

THE EFFECTS OF BLUEBERRY EXTRACT SUPPLEMENTATION ON BASAL
OXIDATIVE STRESS IN YOUNG HEALTHY RATS

by

RACHEL VICTORIA DULEBOHN

(Under the Direction of Joan G. Fischer)

ABSTRACT

Blueberry extracts reduce oxidative stress and increase phase II enzyme activities in vitro. We examined these effects in vivo. Young, non-stressed male Sprague-Dawley rats (n=8) were fed AIN-93 or modified AIN-93 diets for three weeks. Modified diets were 10% crude blueberries substituting sucrose, blueberry polyphenol extract and sugars matching the 10% blueberry diet, sugars equivalent to the 10% blueberry diet, 1% and 0.2% blueberry flavonoids, primarily anthocyanins. Colon mucosa and liver glutathione-S-transferase (GST), quinone reductase and liver UDP-glucuronosyltransferase activities were not significantly increased by crude blueberries or blueberry fractions. However, liver GST activity was approximately 26% higher than controls for the blueberry, polyphenol, and 1% flavonoid groups. Urinary F₂-isoprostanes were unaffected. DNA damage was lowest for liver and lymphocytes in the 1% flavonoid group, significantly in the liver. This study shows in vivo and in vitro findings disagree and higher supplementation or stressed model may be required for significant effects.

INDEX WORDS: Blueberries, Polyphenols, Oxidative stress, Phase II enzymes

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CHAPTER I

INTRODUCTION

Cardiovascular disease and cancer account for approximately 60% of total deaths in the United States (Eyre et al., 2004). Fruits and vegetables, when consumed regularly, are known to reduce the risks of these and other diseases (Hung et al., 2004). Plant foods most likely contain a wide variety of substances that contribute to their health-promoting and disease prevention benefits, including a large class of compounds known as phytochemicals. Polyphenols, one group of phytochemicals, are the most abundant dietary antioxidants and can be found in a wide variety of plant foods. Blueberries are rich in polyphenols which have high antioxidant capacities. Polyphenolic compounds in blueberries that contribute to potential health benefits include phenolic acids and flavonoids such as anthocyanins.

It is widely theorized that oxidative stress contributes to aging and many disease processes. Oxidative stress is the damage caused by free radicals, molecules containing an unpaired electron; these include reactive oxygen and nitrogen species. DNA, lipids and proteins can all be damaged by oxidative stress, and the resulting damage leads to disease and aging.

Polyphenols can reduce oxidative stress through several mechanisms. They can directly scavenge free radical species or chelate transition metals. Polyphenols can also up-regulate detoxification enzymes, which process free radical species and facilitate excretion of foreign compounds that may cause oxidative damage. Additionally, polyphenols can also

protect against radical-induced damage by inducing apoptosis of damaged cells (Yi et al., 2005). Phase II enzymes modify and enhance the excretion of harmful or foreign substances in the body, and are highly expressed in the liver, which is the primary detoxification site (Gandolphi, 1986). Glutathione S-transferase (GST), UDP-glucuronosyltransferase (UGT), and NAD(P)H: quinone reductase (QR) are some of the most commonly studied Phase II enzymes.

There has been great interest in the effects of isolated flavonoid compounds, and more recently, the effects of plant extracts, on phase II enzymes. However, the effects of polyphenol supplementation on phase II enzyme activity have been mixed. Blueberry and other polyphenolic treatments have produced varied effects on enzyme induction in both in vitro and in vivo models. In vitro studies with wild blueberry extracts have shown significantly increased QR activity in one study, but had very little effect at extremely high doses in another study (Bomser et al., 1996; Smith et al., 2000). GST protein expression was increased in humans consuming juice supplements that contained blueberries and other fruits, but response depended on specific genotype (Hofmann et al., 2006). Fischer 344 rats treated with azoxymethane to induce colon cancer had increased hepatic GST activity after supplementation with either blueberries (5% of total diet) or blueberry juice (100 mL per day) for 13 weeks and also had a reduction in aberrant crypt foci (Boateng et al., 2006). Similarly, rats fed a 5% or 10% dried black raspberry diet for three weeks had a 45% and 47% increase in hepatic GST, respectively (Stoner et al., 2006). In contrast, UGT activity was unchanged in rats after a six week 20% fruit and vegetable diet. However catechins, polyphenols found in wine and tea, increased UGT activity in the colons of rats fed at 2% of the diet (Lhoste et al., 2003; Rijnkels and Alink, 1998).

Apoptosis is also affected by polyphenols. Apoptosis is a process of programmed cell death that is controlled by multiple signaling molecules and cascades and is vital for the disposal of damaged or diseased cells (Choucroun et al., 2001). Previous research done in our lab has shown that blueberry fractions reduce proliferation and induce apoptosis of HT-29 and Caco-2 colon cancer cell lines in vitro (Yi et al., 2005). Of four extracts tested, including phenolic acids, tannins, flavonols, and anthocyanins, the anthocyanin extract exerted the greatest effect on growth inhibition and apoptosis. Both cell lines showed at least a 50% growth inhibition with anthocyanin concentrations of 15-50 micrograms/mL. Apoptosis rates were increased 2-7 times at this concentration. This extract concentration has been achieved and measured in vivo in rat plasma (Manach et al., 2005)).

The purpose of this study was to investigate, using an in vivo rat model, the effects of whole blueberries on basal oxidative stress levels by measuring DNA and lipid oxidation levels as well as activities of phase II enzymes, GST, UGT and QR. The blueberry is a good example of a fruit high in anthocyanins. Blueberry fractions consist of mixtures of polyphenols and flavonoids which are representative of a human diet containing multiple fruits and vegetables. The hypothesis of this study was that modest supplementation with whole blueberries and blueberry extracts would reduce oxidative stress in non-stressed rats. The specific aim was to determine if different blueberry extracts reduce markers of oxidative stress and increase detoxification enzyme activity, and if the extent of the protective effect was dependent on the extract.

Young, male, Sprague-Dawley rats were fed one of the following diets for three weeks: control diet, 10% crude blueberry extract, control diet with sugars to match the blueberry diet, control diet with polyphenols and sugars to match blueberry diet, control diet

with 1% flavonoid extract, or control diet with 0.2% flavonoid extract. The flavonoid extract consisted mainly of anthocyanins. The activities of the phase II enzymes GST, QR and UGT were not significantly increased by any of the experimental diets, but some slight increases were seen for GST activity. Compared to controls, the crude blueberry, polyphenol and 1% flavonoid groups had 28%, 27% and 24% higher GST activity in liver tissue, respectively. Oxidative DNA damage was assessed using the comet assay and lipid peroxidation was assessed using the F₂-isoprostane assay. DNA damage was lowest for both liver and colon mucosa in rats fed the 1% flavonoid diet, but results were only significant at p<0.05 for the liver. There were no significant differences in lipid peroxidation levels between supplementation groups.

In summary, although blueberry supplementation did not have a significant effect on phase II enzyme activities and lipid peroxidation and only minor effects on DNA damage for the 1% flavonoid group, there were some trends for improvements in GST activity and DNA damage levels that deserve further exploration. The rats in this study were unstressed, and it is possible that the protective effects of blueberry fractions may only be beneficial in rats subjected to elevated oxidative stress.

CHAPTER II

LITERATURE REVIEW

BACKGROUND

Within the field of nutritional research, there is now a trend to examine nutraceuticals and their potential to promote longevity, health and quality of life. Interest in polyphenols and their effect on human health and disease has increased dramatically within recent years. There is some evidence that polyphenols have the potential to reduce cancer risk, especially colon cancer risk. Cardiovascular disease and cancer are the leading causes of death in the United States, accounting for approximately 60% of total deaths (Eyre et al., 2004). Many of the antioxidant characteristics of polyphenols have been established, but the mechanisms by which they impact health are still being elucidated. Among the fruits, blueberries have one of the highest phytochemical contents, which makes them a desirable and obvious choice for exploring potential health benefits of phytochemicals.

PHYTOCHEMICALS

Fruits and vegetables, when consumed regularly, reduce the risks of many chronic diseases including cardiovascular disease and cancer (Hung et al., 2004). Plant foods contain a wide variety of substances that contribute to their health-promoting and disease prevention benefits, including vitamins and minerals, fiber and a large class of bioactive compounds known as phytochemicals. Phytochemicals are non-nutritive chemical compounds that are formed during normal plant metabolic processes and are thus referred to as secondary metabolites. In plants, these chemicals provide protection from radiation or parasites, but

they also have beneficial effects when consumed by humans and animals. Of the five phytochemical groups, carotenoids, phenolics, alkaloids, nitrogen containing compounds and sulfur containing compounds, the carotenoids and polyphenol classes are the best characterized (Liu, 2004). Polyphenols, the most abundant dietary antioxidants, can be found in a wide variety of plant foods. The general chemical structure for a polyphenol consists of multiple phenol rings with hydroxyl groups attached to aromatic rings. This configuration results in electron rich regions on the hydroxyl groups, which attract and trap electrophilic reactive oxygen species (Issa et al., 2006). Polyphenols are further categorized by structural similarities into phenolic acids, flavonoids, stilbenes, and lignans (Figure 1A). Polyphenols have multiple potential biological activities related to disease prevention. These mechanisms include serving as antioxidants, inhibiting telomerase, controlling signal transduction pathways, inhibiting the inflammatory enzymes COX and LOX and angiotensin-converting enzymes, decreasing matrix metalloproteinase, and altering platelet function (Halliwell et al., 2005).

FLAVONOIDS

Flavonoids are the largest class of polyphenols and include flavonols, flavones, isoflavones, flavanones, flavanols and anthocyanins (Manach et al., 2004). All flavonoids share a basic structure with two interconnected phenol rings and a third peripheral phenol ring (Figure 1B). The subgroups are based on the number and positions of various hydroxyl and other functional groups. Flavonols are the most commonly occurring flavonoids, with quercetin and kaempferol being the most common and present in the largest amount. Quercetin and kaempferol are found at the highest levels in onions, broccoli and blueberries (Manach et al., 2005). The flavanones occur mainly in citrus fruits, include naringenin,

hesperetin, and rutinose and are concentrated in the white, non-juicy sections (Hahn-Obercyger et al., 2005). Flavones are relatively uncommon and appear mainly in celery and parsley (Manach et al., 2004). Isoflavones are one of the more well known groups of flavonoids and are also called phytoestrogens because of their structural similarities to steroid hormones and their estrogenic effects (Cassidy et al., 2006). They occur in legumes, most notably soybeans, and include genistein, daidzen, and glycitein. Flavanols are found at high levels in apricots, chocolate, green tea and red wine (Scalbert et al., 2005). They are referred to as catechins when found as single units and proanthocyanidins when complexed together as polymers (Manach et al., 2005). Because of the many possible forms and combinations of flavonoids, it can be difficult to quantify levels in foods. Flavonols are the only group of flavonoids that do not occur in the glycoside form in plants (Manach et al., 2005).

ANTHOCYANINS

Anthocyanins are pigments of red, blue or purple, depending on the pH, and tend to be concentrated in the skin of the fruit because their synthesis is stimulated by light (Galvano et al., 2004). Anthocyanins are highly unstable in the anthocyanidin, or uncomplexed, form, but are very stable when glycosylated or complexed to other flavonoids (Manach et al., 2004). Thus, they are stable in plants, but become very unstable when subjected to food processing that involves pH changes and high heat (Kalt et al., 1999). Anthocyanins are found primarily in fruits but are also found at lower levels in wine, cereals and some leafy vegetables and root vegetables. Anthocyanin content is proportional to color intensity, with blueberries and blackberries being the richest sources (Prior and Wu, 2006). Cyanidins are the most commonly occurring anythocyanin in plants and average daily human consumption is approximately 180 mg, mainly coming from fruit sources (Galvano et al., 2004).

Cyanidin-3-glucoside is one of the most researched anthocyanins and is found in blueberries ((Wu et al., 2002; Youdim et al., 2000).

ABSORPTION, METABOLISM, BIOAVAILABILITY, AND EXCRETION OF ANTHOCYANINS

It is difficult to determine the quantity of flavonoids or anthocyanins ingested because the amount measured in vivo is not directly proportional to the amount of fruit eaten. The anthocyanin content of fruit varies considerably based on growing conditions (Zheng et al., 2003, Manach et al., 2004). Additionally, after harvesting, anthocyanin content declines with senescence and during storage, and is degraded by heat and pH (Connor et al., 2002). Absorption also seems to vary considerably based on individuals. For human males ingesting about 15 mg/kg, serum anthocyanin levels ranged from 5.43-16.9 ng/mL (Mazza et al., 2002). Average estimates of flavonoid intake for people living in the United States are approximately 65-250 mg/d (Manach et al., 2004).

Once ingested, absorption of anthocyanins begins in the stomach (Passamonti et al., 2003). Only minor absorption occurs here and only native, unmodified forms of anthocyanins are taken up. Talavera et al. (2005) theorizes that the organic anionic carrier, bilitranslocase, is responsible for anthocyanin uptake in the stomach, but the process has not yet been confirmed. Anthocyanins are subjected to modification before, during, and after absorption. It was previously thought that only aglycone forms of anthocyanins could be absorbed, but there is now in vivo evidence that certain glycosidic forms can enter the bloodstream (Galvano et al., 2004). Cyanidin-3-glucoside, cyanidin-3,5-diglucoside, 3-rutinoside and cyanidin-3-rutinoside are absorbed directly and without modification in rat and human intestinal tracts (Matsumoto et al., 2001; Mazza et al., 2002). Mazza et al. (2002)

found that 19 out of 25 blueberry anthocyanins were detected in human serum after ingesting 100 g of dried blueberry powder containing 1.20 g of anthocyanins with a high fat meal. Although the supplementation prevented a mean decrease in serum antioxidant activity, which would be caused by increased lipid peroxidation from the high fat meal, only 0.002-3% of the anthocyanins were absorbed. Peak serum levels of anthocyanins were reached approximately four hours after ingestion and then decreased rapidly.

The major site of absorption for anthocyanins is in the small intestine, particularly the jejunum (Passamonti et al., 2003). Here, some modification occurs as well, including methylation and glucurono-conjugation, but some anthocyanins are left unmodified. It is possible that intestinal beta-glucosidases hydrolyze anthocyanins to remove glucoside groups, but this mechanism has been difficult to determine because aglycones are unstable at basic pH and are hard to measure (Talavera et al., 2005).

Having entered the bloodstream, anthocyanins are quickly metabolized and excreted via the liver and kidneys. The liver is the major site of anthocyanin metabolism, with methylation being the primary modification. Since methylated forms are found at a low concentration in the serum, it is likely that they are excreted quickly after methylation. Anthocyanins can also be glucuronidated in the liver. The kidney modifies anthocyanins as well, by both methylation and mono-glucuronidation, but to a lesser extent than the liver (Talavera et al., 2005). Anthocyanin levels peak in the bloodstream within 0.5 to 2 hours, and excretion begins within 20 minutes, with complete excretion within about 12 hours (Prior and Wu, 2006; Talavera et al., 2005). Anthocyanins circulate to all tissues of the body before being excreted. There are no long term stores of anthocyanins or other polyphenols in the body, so eventually all will be excreted (Erdman et al., 2007). In rats, they have been

detected in brain tissue at 84% higher levels than plasma levels, 0.21 nmol/g tissue versus 0.15 nmol/g serum (Talavera et al., 2005).

Most anthocyanins are excreted in the feces. Up to 85%, depending on the structural forms and glycation varieties, of anthocyanins are not absorbed and pass straight through the digestive tract (Kahle et al., 2006). Anthocyanins are metabolized and degraded within the colon by intestinal bacterial enzymes within several hours (He et al., 2005). The majority of the absorbed anthocyanins are excreted from the body via the biliary route (He et al., 2005). Talavera et al. (2005) found that about 0.19% of ingested anthocyanins were excreted in urine. Another study found that 1.8% of anthocyanins ingested by eating 200 g of strawberries were excreted in urine (Felgines et al., 2003).

BLUEBERRIES

Blueberries are rich in polyphenols and other healthy compounds (Yi et al., 2005). Among fruits and vegetables, berries tend to have one of the highest concentrations of polyphenols due to their high skin to volume ratio (Manach et al., 2004). Blueberries have high hydrophilic oxygen radical absorbance capacity (ORAC), although antioxidant capacity is not directly correlated to polyphenol content (Galvano et al., 2004; Miyazawa et al., 1999; Wu et al., 2004). Blueberries also contain nutrients including vitamin C, minerals, and fiber. Despite the wide variety of healthful blueberry components, the bulk of their protective nature is attributed to the flavonoids (Schmidt et al., 2004). Polyphenols and anthocyanin contents vary among blueberry species, and there is no relationship between berry size and anthocyanin content (Kalt et al., 1999; Yi et al., 2005). Wild, or lowbush blueberries, have higher anthocyanin content than cultivated or highbush blueberries (Smith et al., 2000).

Previous research done in our lab has shown that phenolic compounds extracted from blueberries reduce proliferation and induce apoptosis of HT-29 and Caco-2 colon cancer cell lines in vitro (Yi et al., 2005). Four extracts were tested, including phenolic acids, tannins, flavonols, and anthocyanins. The anthocyanin extract exerted the greatest effect on growth inhibition and apoptosis. Both cell lines showed at least 50% growth inhibition with anthocyanin concentrations of 15-50 micrograms/mL. Apoptosis rates were increased two to seven times at this concentration. This extract concentration has been achieved and measured in vivo in rat plasma (Manach et al., 2005)). The ability of anthocyanins to affect apoptosis appears to depend on the cell type being tested. Yeh et al. (2005) noted that high anthocyanin concentrations did not induce apoptosis in normal liver cells (Chang cells) in vitro.

BERRIES AND DISEASE

Berry Polyphenols and Cancer

Polyphenols can prevent cancer initiation by scavenging reactive oxygen species (ROS) or up-regulating intrinsic antioxidant mechanisms. Anthocyanins and polyphenols in general can negatively affect cancer growth by inhibiting proliferation and by increasing apoptosis. Polyphenols stimulate apoptosis of damaged or cancerous cells via an intrinsic pathway or by acting as a pro-oxidant (Galati and O'Brien, 2004).

Much research has been done to investigate the effects of various purified anthocyanins on various cancer cell types in vitro. For example, reduced proliferation of colon and breast cancer cells occurred following treatment of ten different fruit and berry extracts, including blueberries (Olsson et al., 2004). An analysis of various wild blueberry extracts was used to determine that particular proanthocyanidins reduced proliferation of

mouse hepa 1c1c7 cells and LNCaP human prostate cancer cells whereas other proanthocyanidins had anti-adhesion properties (Schmidt et al., 2004). However, because anthocyanins are absorbed at low concentrations and subsequently undergo metabolic alterations, and most reach the tissues in very low concentrations and as altered forms, it is critical to determine the in vivo effects of anthocyanins (He et al., 2005). Furthermore, anthocyanins tend to be excreted quickly after ingestion and would exert an antioxidant effect for a much shorter amount of time than when incubated for several hours in a flask (Talavera et al., 2005). In addition, because anthocyanins and other polyphenols tend to exert a synergistic effect, it is important that the effects of whole foods be examined (Martin, 2006).

Several animal studies have looked at the cancer prevention potential of anthocyanins. Apc^{Min} mice had fewer and smaller cecal adenomas but not colon adenomas or tumors after consuming a 20% freeze-dried tart cherry diet, anthocyanins or cyanidin in their drinking water when compared to the control diet (Kang et al., 2003). Apc^{Min} mice have a genetic mutation that predisposes them to develop colon adenomas and ultimately colon tumors. It is possible that the colon was less protected due to extensive metabolism of the anthocyanins by colon bacteria (He et al., 2005). In contrast, colon cancer and aberrant crypt foci induced by azoxymethane in male F344 rats were reduced by 29% and 57%, respectively, by a pterstilbene supplementation of 40 ppm for 8 weeks. The supplementation also reduced cellular proliferation and iNOS expression. Pterstilbene is a stilbene, structurally similar to resveratrol, found in blueberries and other berries (Suh et al., 2007). Supplementation for 14 weeks with anthocyanin-rich extracts made from bilberry and grapes reduced cancer markers in the same azoxymethane colon cancer model, in F344 rats. The

rats exhibited reduced aberrant crypt foci, cellular proliferation, and lowered COX gene expression (Lala et al., 2006). Stoner et al. (2006) have investigated black raspberry, blackberry and strawberry supplementation and potential esophageal cancer prevention in NMBA-treated Fischer F344 rats in multiple studies. Supplementation of 5% and 10% of the total diet for 25 weeks reduced esophageal tumors via reduced DNA damage and lowered COX-2 expression. The results are so promising that this group has begun human clinical trials to examine if berry supplementation will have comparable protective effects on the human esophagus.

Berries and Cardiovascular Disease

There is considerably less research on the effects of berries and polyphenols on cardiovascular disease than for cancer. Some epidemiological studies show a correlation between high fruit and vegetable intake and reduced cardiovascular disease. This effect is thought to be due to the antioxidant, anti-thrombotic, and anti-inflammatory nature of the flavonoids found in the fruits and vegetables (Geleijnse et al., 2002). A five year longitudinal study found that dietary flavonoid intake was inversely associated with coronary heart disease, myocardial infarction, and death from coronary heart disease (Hertog et al., 1993). Similarly, a meta-analysis of nine cohort studies showed that fruit and vegetable intake was inversely associated with cardiovascular disease, but noted that the mechanisms are still unknown (Dauchet et al., 2006). Studies attempting to elucidate mechanisms have not been very successful. Subjects who consumed five extra fruit and vegetable servings in the form of soups and juices for four weeks did not show any change in vascular cell adhesion molecule-1 levels or in oxidative stress levels (Paterson et al., 2006). ACE

(angiotensin converting enzyme) activity and blood pressure were unaffected after three weeks of a 250 g/day blueberry supplementation in smokers (McAnulty et al., 2005).

Berries and Neural Disease

Anthocyanins are capable of crossing the blood brain barrier and have even been found in brain tissue at a higher concentration than in the plasma (Talavera et al., 2005). The ability of anthocyanins and their metabolites to enter the brain seems to depend on the lipophilicity of the compounds and the efflux transporters that facilitate transfer (Youdim et al., 2004). Aged rats fed 2% blueberries showed improved but not statistically significant memory improvement; polyphenols were detected in the brain tissue (Andres-Lacueva et al., 2005). Mental problems associated with aging have been attributed primarily by accumulating oxidative damage and increased inflammation (Joseph et al., 2000). The blueberry supplementation appears to have curtailed some of that damage by enhancing antioxidant activity.

Transplant reception is often a major barrier for neural transplant success, and tends to decrease further with age. Older rats supplemented with a conservative 2% blueberry extract diet exhibited significant improvement on hippocampal transplant growth (Willis et al., 2005). Differentiation of transplant cells was also similar to transplant reception in young rats.

Another potential mechanism by which blueberry supplementation may provide neuroprotection is by boosting levels of heat shock protein HSP70. HSP70 protects neurons from oxidative stress and inflammation, but its protective abilities decrease with age, leaving neural tissue vulnerable to damage. Hippocampal sections from old rats that ate a 2% blueberry diet for ten weeks showed significant increases in HSP70 levels in response to an

in vitro inflammatory challenge that were comparable to the response in young rats (Galli et al., 2006).

OXIDATIVE STRESS

Reactive oxygen species, or ROS, can cause oxidative damage. They consist both of molecules containing an unpaired electron and molecules that can easily become radicals. Some examples include superoxide and peroxide molecules. ROS originate from a variety of sources. They are primarily produced endogenously during nutrient metabolism but also come from environmental and dietary sources (Gate et al., 1999). Up to 5% of the oxygen used by cells for aerobic respiration within the mitochondria converts to radical species, with roughly 1% of those radicals escaping deactivation by cell machinery and causing damage to cell components (Montuschi et al., 2004). Cells can also intentionally generate ROS to defend against foreign organisms, such as NADPH oxidase in macrophages, or to serve as short term signaling molecules, as in the vascular system (Collins and Horvathova, 2001). Oxidative stress, resulting in damage, can be measured by determining amounts of the resulting radical-damaged nucleic acids, lipids and proteins (Collins and Horvathova, 2001). Reactive oxygen species are not the only types of radicals that can cause damage. Other radicals include unbound transition metals and reactive nitrogen species. Reactive nitrogen species include nitroso compounds, which form in the intestine after nitrate ingestion and are one of the most carcinogenic compounds known (Gate et al., 1999). Heterocyclic amines, which are formed during the cooking of meat are also reactive nitrogen species (Pool-Zobel et al., 2005).

OXIDATIVE STRESS AND BIOMOLECULAR DAMAGE

Oxidative radicals can damage all biomolecules with oxidation potential, including lipids, proteins and DNA. Because ROS and other radicals are highly reactive and short-lived, they tend to attack whatever molecules are located in the immediate vicinity (Gate et al., 1999). Lipid peroxidation tends to occur preferentially in polyunsaturated fatty acids. This vulnerability of polyunsaturated fat is due to the multiple allylic hydrogens found in polyunsaturated fatty acids, which are extremely susceptible to oxidation, especially by the hydroxyl radical (Gate et al., 1999; Ward et al., 2005). Once an allylic hydrogen is attacked, it becomes a peroxy radical, which can go on to attack allylic hydrogens on other fatty acids, in a chain reaction of lipid peroxidation, which damages the lipids (Ward et al., 2005). Membrane-bound lipids and long-chain free fatty acids in the cytosol can be damaged by ROS, but the polyunsaturated fats attached to lipoproteins seem to be especially open to oxidative attack and to have the greatest biological impact (Bokov et al., 2004). Oxidatively modified LDL can enter the endothelium and recruit cytokines and monocytes, leading to inflammation and ultimately cardiovascular disease (Kaliora et al., 2006).

Radicals can cause different types of oxidative damage to DNA. If the deoxyribose units of the DNA backbone are oxidized, strand breaks or loss of associated bases can occur (de Zwart et al., 1999). Oxidized DNA proteins can crosslink to each other and oxidized bases can cause DNA mutations. DNA is less susceptible to oxidative attack than lipids and has extensive endogenous repair mechanisms (Duthie et al., 2005). Damaged DNA is repaired by glycosylases, which excise damaged bases, and endonucleases, which remove damaged deoxynucleotides (Peoples and Karnes, 2005). Deoxynucleotides are metabolized to deoxynucleosides and are excreted along with bases in the urine (de Zwart et al., 1999).

Of the four bases, guanine is the most susceptible to oxidation due to its low oxidation potential. The most common alteration caused by ROS is 8-hydroxy-2'-deoxyguanosine (8-OH-dG), which can be measured to determine DNA damage levels (Collins et al., 2001).

Virtually all amino acids in proteins can be affected by oxidative radicals, which generally damage the protein backbone chain; this disruption is irreparable. Proteins can also be affected by oxidized carbohydrates and lipids which have been modified into aldehydes (Bokov et al., 2004). The aldehydes are incorporated into the protein to form carbonyl groups. These carbonyl side-chain derivatives tag the protein for proteolytic degradation (Moskovitz et al., 2002). Sulfur-containing amino acids, methionine and cysteine, are extremely sensitive to oxidation (Gate et al., 1999). Proteins containing sulfhydryl groups can be activated or inactivated based on a reversible redox control, but oxidative modification can irreversibly activate or inactivate them (Humphries et al., 2006). Redox-regulated proteins play many roles in managing cell status via signal transduction, transport systems and numerous enzymes. Oxidative damage to these proteins can cause any number of problems for the cell (Gate et al., 1999).

OXIDATIVE STRESS AND THE WESTERN DIET

The Western diet is high in fat, especially saturated and trans fat, high in refined carbohydrate and low in fiber and antioxidants from fruit and vegetables. Western dietary patterns contribute to elevated oxidative stress levels. Postprandial oxidative stress refers to the increased likelihood of oxidative damage occurring during the period following consumption of a carbohydrate and/or lipid rich meal (Sies et al., 2005). Nutrients can be either already oxidized or oxidizable during digestion and absorption. Normally, endogenous antioxidant mechanisms are sufficient to manage the oxidant load. But with low antioxidant

intake and highly oxidized or oxidizable fat intake, the mechanisms are overwhelmed (Natella et al., 2002). For example, glutathione peroxidase in the intestine is usually sufficient to manage lipid hydroperoxide levels (Wingler et al., 2000). However, a high load of hydroperoxide can overwhelm the glutathione pools in intestinal cells, leaving the ROS free to cause damage (Sies et al., 2005). Both animal and human studies show evidence for the link between high fat diet and its consequential oxidative stress causing various diseases. In rats, prolonged high fat diet causes measurable oxidative stress and disease in the pancreas, liver, kidneys and heart (Diniz et al., 2004; Milagro et al., 2006; Scheuer et al., 2000; Yan et al., 2006). These and other diseases have similarly been linked to high fat diet intake in humans. Some types of fat seem to have a greater effect on inflammation than others, especially saturated and polyunsaturated fats. Men aged 30-50 yr. who consume a high fat diet have a greater level of endothelial activation, which is caused by oxidative stress, inflammatory and other factors and indicates cardiovascular disease risk. Saturated fat seems to have the greatest effect on endothelial activation (Couillard et al., 2006). High saturated fat levels affect polyunsaturated fat status on lipoproteins, indirectly increasing oxidative stress of the polyunsaturated fats. Conversely, caloric restriction in rats reduces lipid peroxidation as determined by F₂-isoprostane measurements (Ward et al., 2005).

MEASURING OXIDATIVE DAMAGE

Because ROS can potentially damage DNA, lipids and proteins, it is necessary to measure damage levels in more than just one of these cellular components. DNA damage is the most severe form of oxidative damage because it can cause permanent mutations that are passed on to progeny cells. The two major methods for assessing DNA damage are measuring 8-OH-dG levels and the comet assay. 8-OH-dG is most easily measured in urine

and represents both total body DNA damage as well as DNA repair levels (Collins and Horvathova, 2001). The procedure is time consuming and requires solid phase extraction and other sample preparations followed by HPLC to quantify the 8-OH-dG levels in the samples (Peoples and Karnes, 2005). 8-OH-dG can also be measured in isolated DNA, although it must first be extracted from the DNA and is very difficult to do (de Zwart et al., 1999).

The comet assay, or single cell gel electrophoresis assay, is now perhaps the most commonly used method for assessment of DNA damage. The procedure is based on the fact that damaged DNA is fragmented and no longer tightly bound around histones. Briefly, cells are lysed, embedded in agarose on a slide and subjected to electrophoresis. Cells that have been damaged by oxidation will have fragmented DNA, which will migrate out of the cell and form a comet-like cloud around the cell. The size and shape of the cloud corresponds to the degree of damage, with a large, widespread cloud indicating significant DNA damage (McKelvey-Martin et al., 1993).

Lipid damage is especially detrimental for cells because lipids are concentrated in membranes and therefore lipid damage can compromise the cell membranes. Lipid oxidation can be measured using thiobarbituric-reacting substances (TBARS), conjugated dienes, and F₂-isoprostanes. One problem with TBARS is that it measures malondialdehyde (MDA) production, and MDA is not exclusively a product of oxidation (Montuschi et al., 2004). A newer and superior assay measures F₂-isoprostanes levels. F₂-isoprostanes are prostaglandin-like (isomeric to prostaglandin-F_{2α}) compounds that are produced, not by COX enzymes, but by lipid peroxidation of arachidonic acid residing in phospholipid membranes (Bokov et al., 2004). Phospholipases then release F₂-isoprostanes from the membranes and they are

promptly excreted in the urine (Thompson et al., 2005). F₂-isoprostane levels are known to increase considerably in organisms subjected to high levels of oxidative stress (Basu, 1998). They are regarded as one of the best methods to measure oxidative stress due to their stability and measurement reliability. F₂-isoprostanes can be measured at the picomolar level and are detectable in all normal biological tissues as well as urine (Morrow, 2006). Also, F₂-isoprostane levels are unaffected by the lipid composition of the diet or by F₂-isoprostanes ingested by eating foods such as animal products (Bokov et al., 2004; Gopaul et al., 2000).

Oxidative damage to proteins is most commonly determined by measurement of the carbonyl content of protein, which correlates to the degree of oxidative damage (Moskovitz et al., 2002). Carbonyl levels in tissue are measured based on the affinity of carbonyls for 2,4-dinitrophenylhydrazine. The two compounds complex together to form a stable compound that can be measured by spectrophotometric analysis (Levine et al., 1981). This method can be affected by interference and more recently, antibodies have been used to detect carbonyl levels. Western blots can also be used to determine protein identities after the carbonyl levels have been measured, as some proteins are more susceptible to oxidative damage than others due to their structure and amino acid composition (Bokov et al., 2004).

OXIDATIVE STRESS AND AGING AND DISEASE

Oxidative stress is now thought to be responsible for many elements of aging and also to have role in many diseases. The formal name for this concept is the Oxidative Stress Theory of Aging and it has several components (Humphries et al., 2006). Levels of oxidatively-damaged biomolecules increase with aging and are caused by an increased proportion of ROS to antioxidant levels and reduced repair of damaged molecules. Alterations that reduce oxidative damage by reducing ROS or increasing scavenging or

promoting repair will reduce damage to molecules and retard aging (Bokov et al., 2004). In support of the Oxidative Stress Theory of Aging, many studies have shown increased levels of oxidative stress with aging and disease. Kasapoglu et al. (2001) found that oxidative damage to DNA and protein increase with increasing age in humans aged 20 to 70 years old, as do some antioxidant enzyme activities, superoxide dismutase and catalase. With aging, astrocytes show increased oxidative stress and antioxidant enzyme activity which result in a reduced capacity for neuroprotection in vitro (Pertusa et al., 2007). Cancer, diabetes, cardiovascular disease, liver disease, neurodegenerative diseases and many other disease conditions have been associated with elevated oxidative stress levels (Bhattacharyya et al., 2007; Gate et al., 1999; Klein et al., 2006; Tanikawa and Torimura, 2006; Vecchione et al., 2007; Yao et al., 2004).

POLYPHENOLS AND OXIDATIVE STRESS

Polyphenols can reduce oxidative stress through several mechanisms. They can directly scavenge free radical species and can chelate transition metals. Ability to directly scavenge free radicals is closely related to the chemical structure of the polyphenol. The number and position of hydroxyl groups on the phenol rings determine how electron-rich those regions are and subsequently how well they will attract radicals (Masella et al., 2005). Antioxidants as direct scavengers provide the first line of defense against radicals. However, by direct scavenging, polyphenols form a less reactive compound in the process instead of completely quenching the radical, so there is need for further alteration. This continued metabolism is accomplished by Phase II enzymes (Halliwell et al., 2005). Additionally, polyphenols can trigger cellular antioxidant defenses including up-regulation of some of the Phase II detoxification enzymes and induction of apoptosis of damaged cells (Yi et al.,

2005). Some polyphenols are capable of auto-oxidation and act as pro-oxidants to upregulate cellular defense systems (Halliwell, 2007). For example, activation of polyphenols by phase I enzymes in turn activates genes that are involved in reducing oxidative stress, thus inducing endogenous mechanisms by effectively acting as a prooxidant (van der Logt et al., 2003).

Some polyphenols provide protection specifically against reactive nitrogen species. Epicatechin and one of its dimers inhibited formation of nitroso compounds in vitro under conditions that were comparable to those in the digestive tract. Also, the resulting nitroso-flavanol compounds were not absorbed in rat jejunums, whereas unaltered flavanols were absorbed. However, the nitroso-flavanol compounds are more toxic than flavanol, although less so than nitroso-compounds, so could potentially have a negative effect in the colon (Lee et al., 2006).

Studies with Fruits and Vegetables, Polyphenols and Carotenoids on Oxidative Stress

The antioxidant content of fruits and vegetables has in the past been attributed to vitamins, mainly vitamins C and E as well as carotenoids and other polyphenols. Recent studies, however, have shown that the polyphenols contribute considerably to the total antioxidant ability. Wu et al. (2004) analyzed 100 foods including many fruits and vegetables, to measure their total antioxidant capacity as well as their phenolic content. Total antioxidant capacity was determined by quantifying the antioxidant capacity of both the hydrophilic and hydrophobic food contents. For fruits and vegetables, there tended to be a strong positive relationship between total antioxidant capacity and total phenolic content, although this relationship did not always hold for other types of foods. Similarly, phenolic profiles and antioxidant capacities of blueberries and blackberries were determined by

Sellappan et al. (2002), who also observed a linear relationship between phenolic content and antioxidant capacity. Once this relationship was confirmed, questions concerning the impact of these compounds in the body arose. Subsequent *in vivo* research has attempted to determine if these fruits and vegetables with high phenolic content and antioxidant activity, like blueberries, have potential health benefits. Supplementation studies with various types of polyphenols have looked at oxidative stress alleviation and DNA damage. At least 93 intervention studies have examined the effects of polyphenol supplementation in humans on a wide range of health conditions and biomarkers (Williamson and Manach, 2005). Some of the studies show positive results and others do not. For example, flavonoids from onions and black tea did not change isoprostane levels in healthy human subjects after consumption for 14 days (O'Reilly et al., 2001). Supplementation with 12 mg of mixed carotenoids for 56 days in postmenopausal women reduced endogenous DNA damage by 35.8% (Zhao et al., 2006). Twelve mg is a modest supplementation that can easily be achieved either with supplements, as in this study, or by dietary consumption. For example, one quarter cup spinach contains 4 mg lutein, a third of a carrot contains 4 mg β -carotene and one medium tomato contains 4 mg lycopene. It was noted that the older participants had higher baseline levels of damage and consequently showed greater improvement than younger subjects.

Another study recently showed reduced isoprostane excretion with a diet high in fruits and vegetables (Thompson et al., 2005). After a run-in diet, 208 women, aged 21 and older, consumed either 9.2 fruit or vegetable servings for six weeks or 3.6 servings for four weeks and then 9.2 servings for the remaining two weeks. There was a wide range of baseline isoprostane levels, but the run-in diet reduced variation and decreased excretion levels by an average 33%. Subjects on the high FV diet showed an additional 14% decrease

after two weeks on the experimental diet, with values remaining constant for the rest of the study. Subjects on the low FV diet had steady levels at the baseline values until they switched to the high FV diet for the last two weeks, which caused a significant reduction in isoprostane excretion levels. Subjects on the high FV diet lost weight, an average of 6.5 lb. When weight was included in the analysis, it actually strengthened the effect of the intervention.

OTHER PROPOSED MECHANISMS FOR POLYPHENOLS

Inflammation and Polyphenols

Inflammation is the body's natural mechanism of immune response to infection and injury. Inflammation can occur both as a result of illness or can lead to illness, as in the case of cardiovascular disease. Being closely associated with oxidative stress, inflammation levels tend to increase with aging (Yu and Chung, 2006). Inflammation responses can be categorized into arachidonic acid (AA) dependent and independent pathways (Issa et al., 2006). AA dependent pathways include cyclooxygenase, lipoxygenase, and phospholipase 2, as they involve modification of AA. AA independent pathways include nitric oxide synthase, NF- κ B peroxisome proliferator activated genes and others. Oxidative stress can trigger inflammation by a variety of pathways, including activating NF- κ B, therefore polyphenols that reduce oxidative stress simultaneously reduce inflammation (Rahman et al., 2006). Quercetin and resveratrol suppress components of both the AA dependent and independent pathways (Issa et al., 2006). NF- κ B proteins are activated by free radicals, inflammatory stimuli, cytokines, radiation and others and are thought to play a major role in controlling the overall state of inflammation (Bremner and Heinrich, 2002). When the protein is activated, it induces genes for inflammation, suppression of apoptosis and various cancer

genes. Resveratrol, ellagic acid, and lycopene have been shown to inhibit NF- κ B (Aggarwal and Shishodia, 2006).

Just a single study has specifically examined anthocyanins and mechanistic effects on inflammation. Delphinidin, cyanidin, pelargonidin, peonidin and malvidin all suppressed lipopolysaccharide-induced COX-2 expression in vitro in mouse macrophage cells, with delphinidin being the most potent inhibitor. Transcriptional and translational expression of COX-2 was prevented via downregulation of several promoting factors, NF- κ B, activator protein 1 and CCAAT enhancer binding protein, but not CRE-binding protein (Hou et al., 2005). COX-2 is an inducible form that is expressed in response to inflammation, whereas COX-1 is normally expressed and regulates some cell processes. COX-2 has been shown to affect apoptosis, cell proliferation and tumor promotion (Lee et al., 2004). Another study reported reduced COX-1 and 2 activities using various wild blueberry extracts, which was determined by testing the extracts with a bioassay (Kraft et al., 2005). Twenty-three extracts, including both high and low molecular weight polyphenols were tested for their ability to prevent the conversion of prostaglandin-E₂ production from arachidonic acid by COX 1 and 2 enzymes. The enzymes were each maximally inhibited by one different extract, suggesting that a particular polyphenol or group of polyphenols were responsible for each action.

Biotransformation Enzymes

Biotransformation enzymes are one of the body's mechanisms to manage foreign chemicals. These enzymes modify and tag for excretion substances that are harmful or foreign to the body, such as drugs and toxins and are highly expressed in the liver, which is the primary site for detoxification (Gandolphi, 1986). Biotransformation enzymes are grouped into phase I activation and phase II detoxification enzymes. Phase I enzymes make

molecules more hydrophilic by oxidation, reduction, and hydrolysis reactions, which add or expose functional groups and prepare compounds for detoxification. Phase I enzymes include cytochrome P450s, amine oxidase, esterases and amidases and are located in the endoplasmic reticulum (Lampe and Peterson, 2002). As activators, phase I enzymes can make compounds more carcinogenic. For example, bromobenzene forms epoxides and carbon tetrachloride forms free radicals as a result of phase I alteration (Sturgill and Lambert, 1997). Flavonoids can inhibit phase I enzymes, thereby preventing procarcinogenic compounds from being activated. Capsaicin, ellagic acid, quercetin, genistein and others have all been shown to prevent activation of particular carcinogens (Galati and O'Brien, 2004).

Phase II or detoxification enzymes stabilize reactive compounds by forming a conjugate between the compound and an endogenous molecule, so that the compound can be excreted (Moon et al., 2006). Compounds can be conjugated to glucuronic acid, sulfur hydroxyl group, methyl group, carboxylic acid group and others. Most phase II enzymes are located in the cytosol. GST (glutathione S-transferases), UGT (UDP-glucuronosyltransferases) and QR (NAD(P)H: quinone reductase) are some of the most common detoxification enzymes (Gandolphi, 1986).

Phase II Enzymes

GSTs catalyze the reaction of electrophilic or radical compounds with nucleophilic sulfhydryl on glutathione, which inactivates them, and improves solubility to speed excretion (Moskaug et al., 2005). Glutathione (GSH), a tripeptide composed of glycine, glutamic acid, and cysteine, is thought to be the primary controller of the redox state of the cell. The ratio of the reduced (GSH) and oxidized (GSSH) form of glutathione determines the cell redox

state (Kristal and Lampe, 2002). Glutathione acts as an antioxidant within the cell by scavenging free radicals and is also used by another antioxidant enzyme, glutathione peroxidase. Thus it is critical that levels of GSH can be increased when need is increased, since the GSH pool is used by several cellular compartments (Moon et al., 2006). With increased oxidative stress levels, glutathione and GST synthesis can be up-regulated.

UGTs conjugate glucuronic acid to lipophilic compounds, tagging them for excretion. Glucuronidation effectively adds a carboxyl group, which increases water solubility and aids excretion by both the renal and biliary routes (Gandolphi, 1986). UGTs are one of the few phase II enzymes not located in the cytosol, rather they are found in the microsomes (Martin and Black, 1994). There are two classes of UGTs, UGT1As which mainly glucuronidate exogenous compounds like drugs and chemicals and UGT2Bs which glucuronidate mostly endogenous molecules such as steroids and bile acids (van der Logt et al., 2003). Both groups of UGTs can be upregulated by dietary polyphenols (Kuehl et al., 2005).

QR facilitates the transfer of electron pairs to quinones and some azo dyes, and thus prevents the formation of superoxide radical intermediates (Gandolphi, 1986). GST and QR are the two major cytosolic detoxification enzymes. There are many forms of QR including ubiquinones and vitamin K derivatives (Benson et al., 1980). Quinones are one of the possible products from aromatic hydrocarbon oxidation. Sometimes reduction of quinones can lead to more reactive compounds, which can subsequently be quenched by UGTs (Albert et al., 2000).

Phase II Enzymes and Polyphenols

Polyphenols have been shown to increase activity of phase II enzymes. Some polyphenols can not only up-regulate activity of these enzymes but also increase synthesis

(Gerhauser et al., 2003; Lhoste et al., 2003). The genes for phase II enzymes contain antioxidant or electrophilic response elements (AREs) which control expression. Many flavonoids can activate the AREs and their ability to do so seems related to their individual structure and the presence of Michael reaction centers (Dinkova-Kostova et al., 2001). Flavonoid fractions from blueberries and cranberries induce phase II enzyme expression as do kaempferol and silybin (Bomser et al., 1996; Moon et al., 2006).

GST and UGT activities are strongly associated in the liver but less so in the small intestine and colon. Rats supplemented with angelicalactone, coumarin or curcumin showed related increases in enzyme activity in the liver, but less in the intestines, which might be related to the much lower endogenous levels in the intestines compared to the liver (van der Logt et al., 2003). Brussel sprout polyphenols have been shown to induce GST and UGT *in vitro*, but only at a very high supplementation level (Wallig et al., 1998). Similarly, various catechins have been shown to induce GST and UGT in the liver and intestines of rats (Lhoste et al., 2003).

Blueberry extract has been shown to increase QR activity. QR activity was increased significantly by wild blueberry extract in Hepa 1c1c7 cells after 48 hour exposure to the extract (Smith et al., 2000). The polyphenol concentration and extract dosage were not given, and the exposure level was very long. Most studies now expose *in vitro* cells to extracts for only several hours (Yi et al., 2005). Isolated polyphenols also increase QR activity with quercetin, curcumin and resveratrol, being the most potent inducers; genistein and ellagic acid are less potent inducers (Gerhauser et al., 2003).

One recent study used a non-stressed rat model with citrus supplementation. Citrus is high in the flavanones naringenin, hesperetin and rutinose. Unstressed rats receiving

unlimited grapefruit juice for six weeks had increased phase II liver enzyme production, but the increases were not statistically significant (Hahn-Obercyger et al., 2005). GST activity increased 16.7% and QR activity increased 53.8%. Some of the activity increase was caused by sucrose content. This effect was determined by a control group that received sucrose water instead of fruit juice. The study did not report how much juice the rats consumed on a daily basis, or what the phenolic content of the juice was, so it is difficult to determine dosage amount. Catechins, polyphenols found in wine and tea, were also found to increase UGT and GST activity in the colons of rats when given at 2% of the diet (Lhoste et al., 2003).

Apoptosis

Apoptosis is the process of programmed cell death, in which cells undergo a specific, energy-requiring series of steps to terminate all vital functions. There are several signaling pathways involved in the process. The major physical changes during apoptosis are cell shrinkage resulting from compression of cytoplasm and chromatin, changes in the cell membrane to form blebs, and distinctive nuclear fragmentation. Calcium-dependent endonucleases cleave the DNA into mono- and oligonucleosomal pieces. Oligonucleosomes are strings of repeating units that consist of identical histones, DNA sequence and nucleosome core (Arya and Schlick, 2007). These DNA strand breaks can be measured to detect apoptosis (Gavrieli et al., 1992). Eventually the cell breaks off into small, membrane-packaged globules which are phagocytosed without eliciting an immune response (Martin, 2006). Necrosis, in contrast, does stimulate an inflammatory response in its surroundings (Choucroun et al., 2001). Necrosis is unregulated cell death that occurs passively and generally in response to some significant external disturbance. Damage to the cell membrane

allows extracellular fluid to flow into the cell, resulting in swelling and ultimately bursting of the cell (Qiao et al., 1998). The released cellular contents are detected by immune system components and trigger the inflammatory response.

Apoptosis Regulation

There are two different major pathways for apoptosis, the intrinsic and the extrinsic. The intrinsic pathway is controlled by the integrity of the mitochondrial membranes and the extrinsic pathway is controlled by cell death receptors. Both pathways converge at caspase 3 activation in the caspase cascade, which commits the cell to apoptosis (Martin, 2006). Caspases are proteins involved in the control of apoptosis and consist of activator and executioner caspases, with caspase 3 being the primary executioner caspase (Yeh and Yen, 2005).

When membrane permeability of the mitochondria is increased and the membrane potential is lost, the mitochondrial permeable transition pores open, releasing factors including cytochrome c, which initiates apoptosis by triggering the caspase cascade (Shih et al., 2005). Cytochrome c is contained in the cristae of the mitochondria and is necessary for proper function of the electron transport chain. Upon release from the membrane, cytochrome c binds with apoptotic protease-activating factors, which in turn activate caspase 9, an initiator caspase (Yeh and Yen, 2005). Bcl-2 proteins are a family of 18 or more proteins and include both anti- and pro-apoptotic members. The ratios and dimerizations of these proteins control mitochondrial membrane integrity, by regulating whether or not cytochrome c will be released (Chen and Kong, 2005).

The extrinsic pathway is initiated by tumor necrosis factor receptors on the cell surface. When cytokines bind to their respective receptors, the receptors activate pro-

caspases, notably caspase 8 (Martin, 2006). This pathway can be inhibited by suppressors, as can occur in disease states such as cancer.

Apoptosis and Polyphenols

Apoptosis is an important regulator of tissue maintenance by eliminating unhealthy cells. As such, apoptosis occurs in early response to oxidative damage. Cells that are damaged beyond repair will enter the pathway. Antioxidants can either increase or decrease the apoptotic response (Decoursey and Ligeti, 2005). This regulation is accomplished by controlling various aspects of the related signaling pathways. If a cell is only minimally damaged, antioxidants can sometimes suppress the apoptotic pathway, allowing the cell to repair itself. Conversely, if a cell is severely damaged, antioxidants can induce entrance into the apoptotic pathway.

Apoptosis is an important potential defense against cancerous or cancerous cells, which often have become resistant to apoptosis (Martin, 2006). Some antioxidants promote apoptosis of these damaged cells to the benefit of the organism. Research done in our lab has shown that phenolic compounds extracted from blueberries reduce proliferation and induce apoptosis of HT-29 and Caco-2 colon cancer cell lines in vitro (Yi et al., 2005). Four extracts were tested, phenolic acids, tannins, flavonols, and anthocyanins. The anthocyanin extract exerted the greatest effect on growth inhibition and apoptosis. Both cell lines showed at least 50% growth inhibition with anthocyanin concentrations of 15-50 $\mu\text{g}/\text{mL}$. Apoptosis rates were increased two to seven times at this concentration. This extract concentration has been achieved and measured in vivo in rat plasma (Manach et al., 2005). Polyphenols influence apoptosis via the intrinsic pathway (Chen and Kong, 2005). Malvidin, an anthocyanidin that occurs at a high concentration in blueberries, induces apoptosis in human

gastric adenocarcinoma cells. The mitochondrial membrane potential was greatly reduced, with caspase 3 and Bax/Bcl2 ratios increased, confirming that the malvidin was acting via the intrinsic pathway (Shih et al., 2005). It is important to mention some research suggests that anthocyanins only have a cytotoxic effect on abnormal cells. Yeh et al. (2005) noted that high anthocyanin concentrations did not induce apoptosis in normal liver cells (Chang cells) *in vitro*.

Polyphenolic profile and other fruit components can affect to what extent an extract treatment will affect apoptosis. Methanol extractions of six berry types, bilberry, black currant, cloudberry, lingonberry, raspberry, and strawberry, varied greatly in their apoptosis induction in HT-29 cells. Interestingly, cloudberry had the highest impact on apoptosis despite having undetectable anthocyanin levels, so it likely contains other polyphenolic or nonphenolic compounds with apoptotic-enhancing properties, emphasizing the importance of synergy (Wu et al., 2007).

Polyphenol concentration can also determine whether the polyphenol will have a beneficial or negative effect on apoptosis. Protection against caspase 3 activation was achieved with quercetin and fisetin in rat H411E hepatoma cells at low concentrations, 10-25 $\mu\text{mol/L}$, but activation was upregulated by higher doses, 50-250 $\mu\text{mol/L}$ (Watjen et al., 2005). Others have shown an increase in necrosis rates as the treatment doses increase (Yi et al., 2005). Also, different cell types can respond distinctly to polyphenol treatment. Delphinidin, cyanidin and malvidin strongly induced apoptosis via Bcl-2 proteins in human hepatoma HepG₂ cells but only weakly in Hep3B cells (Yeh and Yen, 2005).

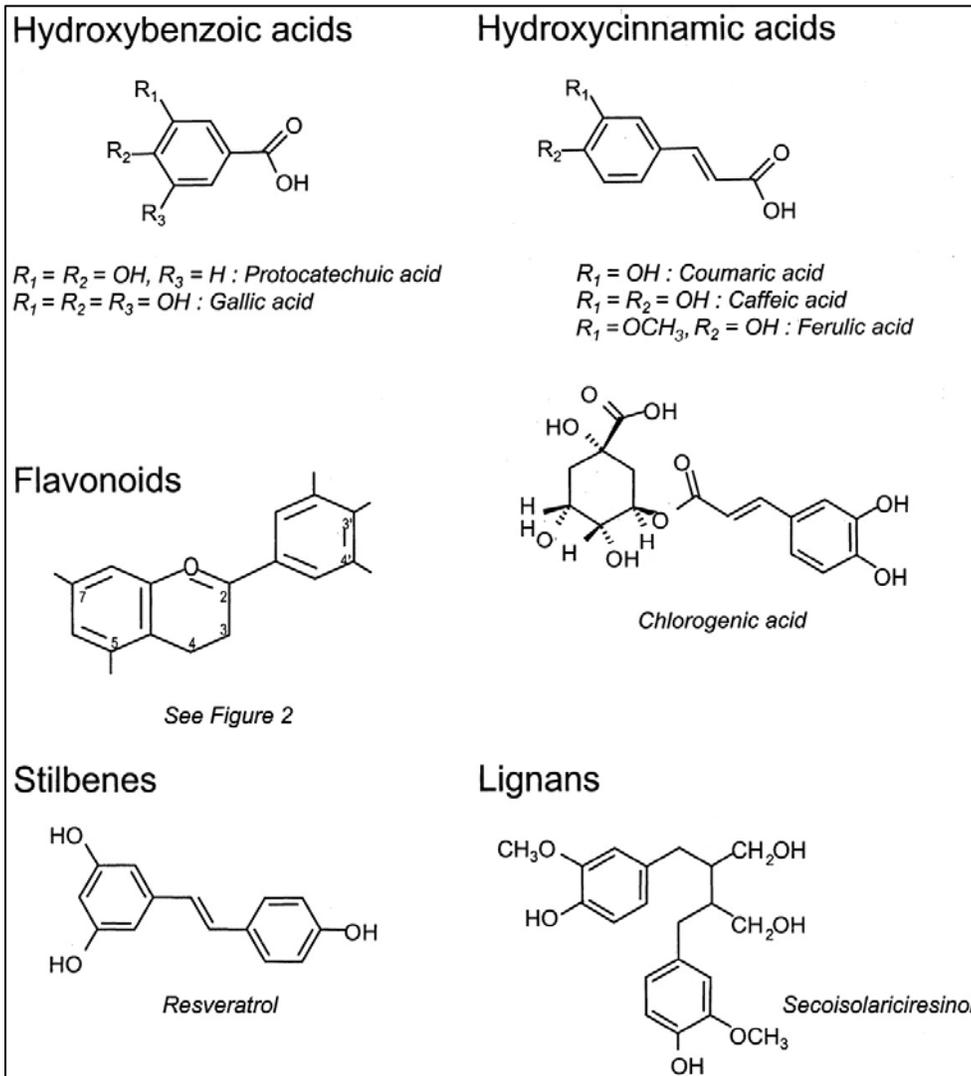


FIGURE 1 Polyphenol Structures.
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 Manach, C (2004) *Am. J. Clin. Nutr.* 79: 727-47.

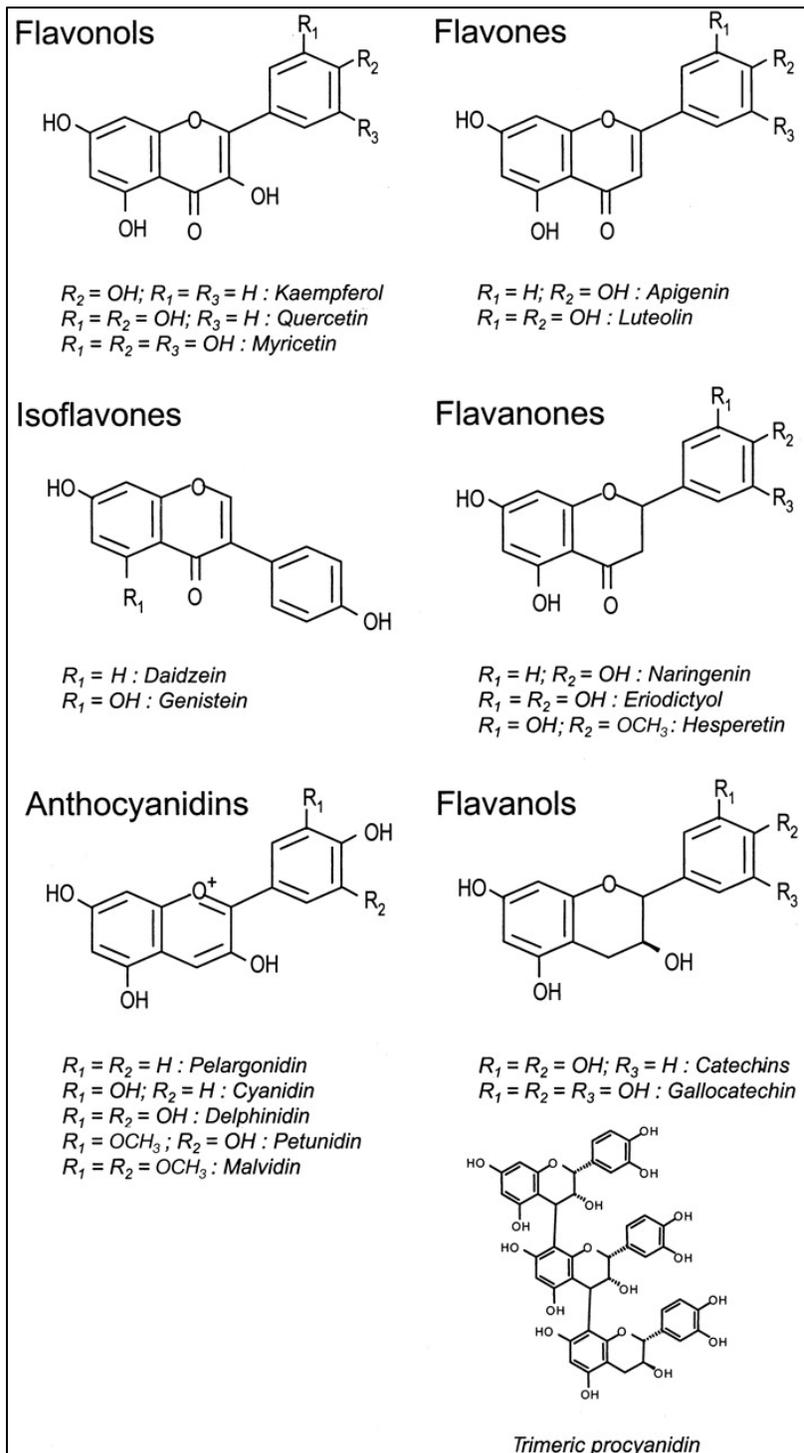


FIGURE 2 Flavonoid Structures.
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CHAPTER 3

EFFECTS OF BLUEBERRY EXTRACTS ON DNA AND LIPID DAMAGE LEVELS AND PHASE II ENZYME ACTIVITIES IN RATS¹

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ABSTRACT

Blueberry extracts have been shown to reduce oxidative stress and increase phase II enzyme activities in vitro. We examined these effects in vivo. Non-stressed male Sprague-Dawley rats (n=8 per group) were fed AIN-93 or modified AIN-93 diets for three weeks. Modified diets were 10% freeze dried crude blueberries substituted for sucrose, blueberry polyphenol extract and sugars to match the 10% blueberry diet, sugars equivalent to the 10% blueberry diet, 1% blueberry flavonoids and 0.2% blueberry flavonoids. The polyphenol extract was obtained using a 40:40:20:0.1 (v:v:v:v) ratio of acetone/methanol/water/formic acid solution. By further purifying the polyphenol extract with an Oasis HLB cartridge then eluting with acidified methanol, total flavonoids were obtained. The flavonoid extract was primarily anthocyanins. Liver and colon mucosa glutathione-S-transferase (GST) and quinone reductase (QR) and liver UDP-glucuronosyltransferase (UGT) activities were measured as were urinary F2-isoprostanes and liver and lymphocyte DNA damage. GST, QR and UGT activities were not significantly increased at $p < 0.05$ by crude blueberries or blueberry fractions, but some slight increases in activity were seen. For example, compared to controls, the blueberry, polyphenol, and 1% flavonoid groups had 28%, 27% and 24% higher GST activity in liver tissue. DNA damage assessed with the comet assay was lowest for both liver and lymphocytes in the 1% flavonoid group, but results were only significant in the liver. This study shows that in vivo results do not always agree with in vitro findings and that extremely high doses or a stressed model may be required to elicit significant effects.

INTRODUCTION

The association between diets rich in fruits and vegetables and reduced chronic disease risk is now well established. Dietary antioxidants, such as polyphenols, found in fruits and vegetables, may contribute to their protective effects. Blueberries are a rich source of polyphenols and have a high antioxidant potential (Manach et al., 2004; Olsson et al., 2004). Sellappan et al. (2002) reported that blueberries contained on average 556.14 mg/100 g fresh weight of total polyphenols and had TEAC (Trolox equivalent antioxidant capacity) values of 27.60 $\mu\text{M/g}$ fresh weight, which is considered high. Much of their protective nature may be attributed to the flavonoids (Yi et al., 2005). Flavonoids comprise the largest class of polyphenols and include anthocyanins, which are pigments of red, blue or purple, depending on the pH. Anthocyanins tend to be concentrated in the skin of the blueberry (Wu et al., 2004).

Recently, interest in polyphenols and their effect on human health and disease has increased dramatically. Previous research done in our lab has shown that phenolic compounds extracted from blueberries reduce proliferation and induce apoptosis of HT-29 and Caco-2 colon cancer cell lines in vitro (Yi et al., 2005). Four extracts were tested, including phenolic acids, tannins, flavonols, and anthocyanins. The anthocyanin extract exerted the greatest effect on growth inhibition and apoptosis. Both cell lines showed at least a 50% growth inhibition with anthocyanin concentrations of 15-50 micrograms/mL. Apoptosis was increased at these concentrations. This extract concentration has been achieved and measured in vivo in rat plasma (Manach et al., 2005). The effects of anthocyanins may dependent on cell type. Yeh et al. (2005) noted that high anthocyanin

concentrations did not induce apoptosis in normal liver cells (Chang cells) in vitro (Yeh and Yen, 2005).

Others have also reported anticancer effects with blueberry fractions. When comparing various wild blueberry extract fractions in in vitro assays, high molecular weight oligomer proanthocyanidins had the highest anti-proliferative and anti-adhesive effect (Schmidt et al., 2004). Proanthocyanidin fractions from both wild and cultivated blueberries had antiproliferative effects on androgen-sensitive prostate cancer cells but not on androgen-insensitive cells (Schmidt et al., 2006).

Many of the antioxidant characteristics of polyphenols have been established, but the mechanisms by which they impact health are still being elucidated. Polyphenols can reduce oxidative stress through several mechanisms. They can directly scavenge free radical species and can chelate transition metals. Polyphenols can also up-regulate detoxification enzymes, which process free radical species and facilitate excretion of foreign compounds that may cause oxidative damage. Additionally, polyphenols can also protect against radical-induced damage by inducing apoptosis of damaged cells (Yi et al., 2005).

A number of studies have suggested that blueberries reduce oxidative stress and the effect is thought to be a result of the high antioxidant potential of blueberries (Manach et al., 2004). Chronic smokers who consumed 250 g of blueberries daily for three weeks had reduced lipid hydroperoxide levels, but not F₂-isoprostanes; plasma antioxidant potential was not increased (McAnulty et al., 2005). In contrast, healthy humans who consumed 330 mL of a fruit juice mixture containing apple, mango, orange, blueberry and boysenberry juices for two weeks showed reduced oxidative damage in lymphocytes, but reduction was dependent on the genotype of GST isoform hGSTP1 (Hofmann et al., 2006).

Blueberries also decrease neuron vulnerability to oxidative stress while at the same time decreasing oxidative stress levels and inflammation (Andres-Lacueva et al., 2005; Joseph et al., 2006). Endothelial cells are less vulnerable to oxidative stress after blueberry extract treatment and have reduced expression of $\text{TNF}\alpha$, an inflammatory marker (Youdim et al., 2002).

Another potential protective mechanisms for blueberry is to increase phase II enzymes involved in detoxification. Detoxification enzymes are one of the body's mechanisms to prevent oxidative damage. These enzymes modify and tag for excretion substances that are harmful or foreign to the body, such as drugs and toxins. Detoxification enzymes are highly expressed in the liver, which is the primary site for detoxification (Gandolphi, 1986). GST (glutathione S-transferases), UGT (UDP-glucuronosyltransferases) and QR (NAD(P)H: quinone reductase) are some of the most common detoxification enzymes. GSTs catalyze the reaction of electrophilic or radical compounds with glutathione, which inactivates the compounds and improves solubility to speed excretion (Moskaug et al., 2005). UGTs conjugate glucuronic acid to lipophilic compounds, tagging them for excretion. Brussel sprout polyphenols have been shown to induce GST and UGT in vitro (Wallig et al., 1998). Similarly, various catechins have been shown to induce GST and UGT in the liver and intestines of rats (Lhoste et al., 2003). QR facilitates the transfer of electron pairs to quinones and some azo dyes, and thus prevents the formation of superoxide radical intermediates (Gandolphi, 1986).

Supplementation with blueberry and other polyphenol treatments have produced mixed results on enzyme induction in both in vitro and in vivo models. Wild blueberry extracts significantly increased QR activity in one study of Hepa 1c1c7 cells but had very

little effect at extremely high doses in another study using the same cell type (Bomser et al., 1996; Smith et al., 2000). Fischer 344 rats treated with azoxymethane to induce colon cancer had increased hepatic GST activity after supplementation with either blueberries (5% of total diet) or blueberry juice (100 mL per day) for 13 weeks and also had fewer precancerous aberrant crypt foci (Boateng et al., 2006). Similarly, rats fed a diet of 5% or 10% dried black raspberries for three weeks had an approximately 45% increase in GST activity above the control (Reen et al., 2006). Catechins, polyphenols found in wine and tea, increased UGT and GST activities in the colons of rats when given at 2% of the diet, but there are no reports of the effect of blueberry on UGT activity (Lhoste et al., 2003). GST protein expression was increased in humans consuming a polyphenol-rich fruit juice blend containing apple, orange, mango, blueberry and boysenberry, in a study by Hoffman et al. (2006), but the effect was also dependent on specific GST genotypes.

The effects of blueberry supplementation oxidative stress and phase II enzymes have not been previously investigated in an unstressed in vivo model. In this study we investigated the effects of whole blueberries and blueberry fractions on basal oxidative stress and activities of phase II enzymes, GST, UGT and QR in a rodent model.

MATERIALS and METHODS

Chemicals and Supplies

Commercial kits were used for the comet assay (Trevigen, Gaithersburg, MD), and urinary 15-F_{2t}-isoprostanes (Oxford Biomedical, Oxford, MI). Diet ingredients were purchased from Harlan (Indianapolis, IN). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Blueberry and Diet Preparation

An AIN-93 diet was used for the studies (Table 1). Blueberries were donated by the Georgia Blueberry Growers Association. Frozen fresh rabbiteye blueberries were freeze-dried. The resulting product was ground but produced crumbles, not a powder, due to the high sugar content of the berries and a small amount of remaining moisture. The polyphenol and flavonoid extracts were obtained using a procedure described by Yi et al. (2005). The polyphenol extract was obtained by homogenization of blueberries with a solvent consisting of a 40:40:20:0.1 proportion by volume of acetone/methanol/water/formic acid solution. The mixture was heated, filtered and finally freeze-dried to remove water. The flavonoid extract was made by further purifying the polyphenol extract using Oasis HLB cartridge and then eluting with acidified methanol. Crude blueberry and flavonoid extracts were analyzed for polyphenol content by HPLC according to Yi et al. (2005). The flavonoid fraction contained mainly anthocyanins, about 90% (Yi et al., 2005).

The blueberry, polyphenol and flavonoid materials were mixed into the respective diets after all other ingredients had been thoroughly blended. The diets were mixed by an institutional Kitchenaid mixer for at least ten minutes to ensure even mixing. The diets were mixed in 15-day batches and stored at -20°C to prevent degradation.

Animal Study

All experimental protocols were approved by the University of Georgia Animal Care and Use Committee. Forty-eight male Sprague-Dawley rats (n=8), 75-99g, were evenly divided into six treatment groups based on weight (Harlan, Indianapolis, IN). The rats were housed individually in wire-bottomed cages and kept at 211°C, constant humidity, and in a 12 hour light/dark cycle. The rats had unlimited access to their assigned diet, which was

provided fresh daily, and distilled water for three weeks. Body weight was recorded weekly and food consumption was recorded daily.

On the day preceding sacrifice, urine was collected overnight and frozen at -80°C until analysis. The rats were sacrificed using CO_2 . Blood was collected via cardiac puncture. Plasma lymphocytes and serum were immediately isolated with Histopaque 1083 and frozen at -80°C until analysis. Livers were removed, sectioned, and frozen at -80°C until analysis. Colons were removed, opened, rinsed with saline and the mucosa was obtained by gentle scraping with a microscope slide. The mucosal samples were then frozen at -80°C until analysis.

Preparation of the Cytosol and Microsome Fractions

Colon mucosal scrapings or 0.5 g liver samples were homogenized with 1:2 parts ice cold Tris HCl buffer (pH=7.4). The homogenate was centrifuged at $9500 \times g$ for 20 min at 4°C . The supernatant was transferred to a thick walled polycarbonate tube and centrifuged at $100,000 \times g$ for 30 minutes. The cytosol supernatant was aliquoted and frozen at -80°C until analysis. The microsomal pellet was washed with Tris HCl buffer, then re-suspended in stabilizing buffer (phosphate buffer with 1 mM EDTA, pH=7.4) and stored at -80°C until analysis.

DNA Damage

DNA damage in lymphocytes and liver cells was assessed using the comet assay kit. Briefly, cells were prepared, embedded in agarose on a slide and lysed in a basic solution. The slides were then electrophoresed and stained with the silver staining kit. Finally, the cells were visually scored according to degree of DNA damage. Visual scoring is consistent with computer image analysis (Zhao et al., 2006). Cells were scored on a scale of 1 to 5,

with 1 representing an undamaged cell with intact DNA and no comet tail and 5 representing a highly damaged cell with a large comet tail. Representative comet images by Tuo et al. 1991 were used as a guide and DNA damage formula by Zhao et al. 2006 was used. Two samples per rat were scored unless otherwise noted. For the lymphocyte scoring, five rats had completely unscorable samples and seven rats had only one scorable sample.

GST Activity

GST activity of liver and colon mucosa samples were measured in duplicate at 25°C using the methods of Habig et al. (1974). The cytosolic activity was measured by conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione. Conjugation results in increased absorbance at 340 nm, with enzyme activity directly proportional to absorbance. The molar extinction coefficient for CDNB at 340 nm is 9.6 mL/mmol. All samples were analyzed in duplicate.

QR Activity

Quinone reductase activity in liver and colon mucosa was measured according to an established protocol established by Kore et al. (1993) using 2,6-dichloroindophenol(DPIP) for the substrate. The ability of the isolated cytosol to reduce DPIP was determined at 600 nm and the dicoumarol-sensitive portion of activity was used to estimate QR activity. Samples were run in triplicate, with and without dicoumarol, an inhibitor of quinone reductase activity. The dicoumarol-containing assays were used as a baseline to determine enzyme activity. The extinction coefficient of 21 mM/cm for DPIP was used. All samples were analyzed in triplicate.

UGT Activity

UGT activity of the liver was determined using the methods of Bock et al. (1983) with modifications by Letelier et al. (2005). Microsomal samples were assayed in duplicate to determine the amount of *p*-nitrophenol that they glucuronidated. Approximately 1 mg microsomal protein was incubated for five min at 37°C with 0.01% w/v Triton X-100, 100 mM Tris-HCl, 5 mM MgCl₂, and 0.5 mM *p*-nitrophenol. Control samples run with Tris buffer (pH=7.4) in lieu of microsomes and used as a standard. The reaction was initiated by adding 3 mM UDP -glucuronic acid. Aliquots of 100 µL were taken at zero and 15 minutes and combined with 2 mL cold 5% TCA to stop the reaction. After centrifugation, 1 mL of supernatant was combined with 250 µL of 2M NaOH. Absorbance was measured at 405 nm, with color being proportional to the remaining, un-glucuronidated *p*-nitrophenol. The extinction coefficient of $18.1 \times 10^3 \text{ cm}^2/\text{mol}$ for *p*-nitrophenol at pH>10 was used to calculate activity. All samples were analyzed in duplicate.

15-F_{2t}-isoprostane Assay

Urinary F₂-isoprostane levels were measured using the enzyme immunoassay for urinary isoprostane kit and glucuronidase. Urine samples were pretreated for two hours at 37°C with glucuronidase to release isoprostanes bound to glucuronic acid. An enhanced dilution buffer was then added to the sample to remove non-specific binding interference. The diluted samples were incubated for two hours with 15-F_{2t}-isoprostane conjugated to horseradish peroxidase in microplate wells with polyclonal antibody to 15-F_{2t}-isoprostane coated in the wells. The plates were washed three times to remove unbound contents. Substrate was then added to the wells for 30 minutes, to activate the horseradish peroxidase, which causes a blue color development. Sulfuric acid was added to the wells, changing the

color to yellow. The plate absorbance was read at 450 nm, with color intensity being indirectly proportional to the amount of 15-F_{2t}-isoprostane in the samples. All samples were analyzed in duplicate. Concentrations were determined from a standard curve prepared at concentrations ranging from 0.05 ng/mL to 100 ng/mL.

Statistics

The Statistical Analysis Systems (SAS, 1996) was used to analyze the data. Numerical data was expressed as mean ± SE. The data were tested for normality using Shapiro-Wilk and Anderson-Darling tests. For normal data sets, differences among means were determined with analysis of variance. For non-normal data sets, differences among means were determined using nonparametric methods. Differences were considered significant at $p < 0.05$.

RESULTS

Body Weight and Food Intake

Final body weight of the rats was measured (Table 2). There was no difference in weight between the treatment groups ($p = 0.88$). Food intake was compared between the treatment groups to confirm that none of the treatments were influencing appetite (Table 3). Overall average food intake was not statistically significant ($p = 0.87$). Average food intake for weeks one and two were also not different. However for week three, the intakes for the carbohydrate control and the 1% flavonoid groups were significantly higher than the polyphenol and 0.2% flavonoid treatment groups, roughly 18 g per day versus 16.5 g per day ($p < 0.05$). Because the intakes were only different for one week and since there was no significant difference in body weight, this intake difference is likely inconsequential.

Treatment Toxicity

Increased liver weight in treatment groups is a frequent symptom of response to a toxic treatment. Liver weight expressed per 100 g of body weight was examined to screen for a toxic effect. There were no statistically significant differences in mean liver weight between treatment groups ($p=0.69$). Therefore, it appears that supplementation with the treatment diets did not enlarge the livers.

GST Activity

Supplementation with blueberry extracts did not significantly increase GST activity in liver ($p=0.514$) or colon mucosa ($p=0.7444$). However, there was a trend for increased GST activity in the liver for the blueberry, polyphenol and 1% flavonoid treatment groups (Table 4). The carbohydrate control and the 0.2% flavonoid treated groups were similar to the control. The blueberry, polyphenol and 1% flavonoid groups had a 28%, 27% and 24% increase in GST activity, respectively, over the control group (Figure 3). GST activity in the colon mucosa was slightly higher in the polyphenol and 1% flavonoid groups. The blueberry treatment group showed no increase in GST activity over the control. The polyphenol group showed a 23% increase over the control and the 1% flavonoid group showed a 22% increase over the control.

QR Activity

The quinone reductase data were not normally distributed, as was determined by the Shapiro-Wilk and Anderson-Darling tests for normality. The Kruskal-Wallis test for non-normal data was used to determine if there were any differences between treatment means. There was no statistically significant difference in QR activity between diet groups for either liver ($p=0.4434$) or colon mucosa ($p=0.6744$) (Table 5). In the liver, the polyphenol

treatment group was the only one that exhibited any increased activity, with a 15.8% increase over the control. All the other groups actually had slight decreases in activity. In the mucosa, the carbohydrate control and the 1% flavonoid groups had a 24% increase in activity.

UGT Activity

UGT activity was not measured in the colon mucosa because preliminary experiments showed that the activity levels were too low to detect. Activities in the liver were not statistically significant and not normally distributed ($p=0.48$). The blueberry treatment group had UGT activity that was 25.6% higher than the control (Table 6). However, this level was only slightly higher than the activity in the carbohydrate control group. The UGT activity in the carbohydrate control group was an unexpected 18% higher than the control group. The remaining groups had UGT activity levels similar to the control group.

DNA Damage

The comet assay was performed to determine DNA damage in liver tissue and lymphocytes. Some of the slides were not scorable. The blueberry treatment had five lymphocyte samples and the carbohydrate group had seven lymphocyte samples included in the statistical analysis. The 1% flavonoid treatment group had the lowest DNA damage levels in both liver tissue and lymphocytes (Table 7). While there were no statistically significant differences in DNA damage levels between diet groups ($p=0.68$ for lymphocytes and $p=0.20$ for liver), analysis of variance suggested that there was a slight trend for a treatment effect in the liver. Post hoc tests showed a significant 22.5% reduction in DNA damage in the liver for the 1% flavonoid treatment group compared to the control ($p<0.05$).

Urinary F₂- isoprostanes

Urinary F₂- isoprostane concentrations were measured to assess lipid peroxidation levels (Table 8). There were no significant differences in lipid peroxidation levels between treatment groups (p=0.95). The 1% flavonoid treatment group had an unexpected slight increase in peroxidation rates, 21.2% over control.

DISCUSSION

Blueberries have been identified as a fruit with high antioxidant capacity that raises in vivo antioxidant status. However few studies have examined whether or not the increased antioxidant capacity provides added protection against oxidative stress in vivo. Further, previous studies have suggested that fractions from blueberries and other high anthocyanin fruits may alter phase II detoxification enzymes, but studies evaluating the effects of blueberry supplementation in vivo are limited. This study examined the effects of blueberry supplementation on oxidative stress. Blueberries and blueberry extracts were fed to rats to determine if lipid and DNA damage levels would be decreased and if phase II enzyme activities would be increased.

Overall, no significant increase in phase II enzymes was seen with blueberry supplementation. GST activities were not significantly increased by supplementation, although there were some slight increases in activity for rats fed crude blueberry and polyphenol fractions as well as 1% blueberry flavonoid diet above control levels. Reen et al. (2006) previously reported increased liver GST activity after three weeks of supplementation with a 5% and 10% black raspberry diet. The control GST activities were comparable to those in our study, and they noted a significant increase of 47% in GST activity for the 10% treatment group compared to an approximately 27% increase with the 10% blueberry and

polyphenol groups ($p=0.25$). Perhaps Reen and collegeaues (2006) were able to achieve a significant increase due to slightly larger sample sizes with nine rats per group. It is also possible that black raspberries have a greater effect on GST activity than blueberries. In addition to differences in intrinsic polyphenol profiles, the dried raspberry extract may have been produced using a process that yielded a more concentrated product than was used for making our dried blueberries. However, further study with direct comparisons of extracts and use of larger sample sizes would be needed to test this conjecture.

Singletary et al. (2001) reported that supplementation of grape seed proanthocyanin for two weeks or longer in rats increased liver GST activity significantly when fed at 1% of the diet, but not less. Similarly, we expected to see a significant increase in our 1% flavonoid treatment group, but perhaps blueberries influence GST activity to a lesser extent than grape seed extract. Ronis et al. (2006) found that when testing grape extracts and blueberry extracts in rats at 10% of the diet, the grape extract was more potent than the blueberry extract in induction of liver GST alpha, one of the GST isoforms.

A high level of un-metabolized anthocyanins is excreted via the intestinal tract, thus the effects of blueberry fraction on colon mucosa were examined. There was a slight and insignificant increase in GST activity in colon mucosa for the polyphenol and 1% flavonoid groups. GST activity responses were different in the liver and colon mucosa, although it is not uncommon to see different tissues respond differently to a treatment. This difference in activities is likely due to tissue specific metabolism of blueberry polyphenols. Thus, metabolites found in liver tissue may be quite different than compounds or metabolites coming into contact with the colon mucosa. These metabolites will most likely have differing effects on enzyme activity.

Similarly, QR and UGT activities were not increased by supplementation. The effects of supplementation on QR activity are mixed in the existing literature. Bomser et al. (1996) reported that crude extracts from blueberry, bilberry, cranberry and lingonberry had little to no effect on QR activity even at high doses in Hepa 1c1c7 cells. However, an ethyl acetate extract did increase QR activity. Similarly, no significant changes occurred in QR activity levels in the kidneys of rats fed black rice, which contains cyanidin 3-O-b-D-glucoside, an anthocyanin also found in blueberries, but the lack of effect could be due to the short supplementation period, of only 6 days (Toyokuni et al., 2002). In contrast, Smith et al. (2000) reported that wild blueberry extracts increased quinone reductase activity in Hepa 1c1c7 cells. It is possible that the difference in results could have been due to different extraction and storage procedures for the cell studies, or an in vitro versus an in vivo effect. The exposure time of 48 hours reported by Smith et al. (2000) was very long and may not represent a condition that would occur in vivo as a result of supplementation.

The effects of supplementation on UGT activity in the literature are also mixed. Hepatic UGT activity was unchanged in rats after receiving a 20% fruit and vegetable diet for six weeks (Rijnkels and Alink, 1998). Catechins, polyphenols found in wine and tea, however, increased UGT and GST activity in the colons of rats when given at 2% of the diet (Lhoste et al., 2003). With all the phase II enzymes examined, the diversity in results could be due to a variety of factors, including supplementation with different polyphenols, different dosages, and varied length of studies.

Although ex vivo studies clearly show antioxidant effects of isolated polyphenols and some plant extracts, it is critical to examine the in vivo effects of plant foods and fractions on oxidative stress. Because ROS can damage DNA, lipids and proteins, it is necessary to

measure damage of more than just one of these cellular components. DNA damage is the most severe form of oxidative damage because it can cause permanent mutations that are passed on to progeny cells. Our results show that there was not a significant reduction in DNA damage from supplementation, except for in the liver of the group receiving a 1% flavonoid diet. DNA damage levels were significantly lowered in the hippocampal regions of rats that had been supplemented with blueberry anthocyanins for 30 days, although DNA damage levels were unchanged in cortex tissue (Barros et al., 2006). The control rats received 0.6–1.0 mg/kg of anthocyanins per day in juice form and the experimental rats received 2.6–3.2 mg/kg per day. Rats fed diets supplemented with 3.85 g/kg monomeric anthocyanins from bilberry, chokeberry and grapes had no change in DNA damage levels after 14 weeks despite lowered expression of COX-2 mRNA (Lala et al., 2006). Human studies with berry and fruit supplementation are even more scarce. Most studies reported no improvements to DNA damage levels from supplementation and the ones that did were not well controlled. Supplementation with blackberry, blood orange and cranberry juices failed to improve DNA damage levels (Duthie et al., 2006; Moller et al., 2004; Riso et al., 2005). It is thought that studies might be more successful if they are more carefully controlled, longer term, and use subjects with elevated oxidative stress levels, such as elderly individuals or those with diabetes (Freese, 2006).

The 1% flavonoid supplementation significantly decreased DNA damage in liver cells ($P < 0.05$). This supplementation level represents a higher polyphenol intake than any of the other treatments, including the 10% crude blueberry diet, which is a likely reason for why it was the only group that showed improvement. However, DNA damage was only decreased in the liver and not in lymphocytes.

The overall small reduction in DNA damage in our study might have been greater if larger sample sizes had been chosen. Larger groups would help to overcome the considerable variation shown in the results. Power analysis had shown that groups of 11 would be needed to demonstrate a protective effect. Another reason for the small effect of flavonoid supplementation on DNA damage levels was the low baseline level of damage. Since the rats were young and healthy, it is likely they had very low DNA damage levels. Supplementation with 12 mg of mixed carotenoids for 56 days in postmenopausal women reduced endogenous DNA damage by 35.8% (Zhao et al., 2006). It was noted that the older participants had higher baseline levels of damage and consequently showed greater improvement than younger subjects. Further studies with larger sample sizes and in models of moderate to high DNA damage may be necessary to fully explain the effect of blueberry function on DNA damage.

Lipid damage is also detrimental for cells because lipids are concentrated in membranes and therefore lipid damage can compromise the cell membranes. Lipid peroxidation can be determined by measuring F₂-isoprostanes. F₂-isoprostanes are prostaglandins that are produced, not by COX enzymes, but by lipid peroxidation of arachidonic acid from oxygen radicals. F₂-isoprostane levels are known to increase considerably in organisms subjected to high levels of oxidative stress. They are regarded as one of the best methods to measure oxidative stress due to their stability and measurement reliability (Morrow, 2006).

Our study showed no effect of blueberry supplementation on F₂-isoprostane levels. Chronic smokers who consumed 250 g of blueberries daily for three weeks had reduced lipid hydroperoxide levels but not F₂-isoprostanes or plasma antioxidant potential (McAnulty et

al., 2005). However, reduction of isoprostanes has been demonstrated in vivo. One human study recently showed reduced isoprostane excretion with a diet high in fruits and vegetables (Thompson et al., 2005). Two hundred and eight women, aged 21 and older, consumed either 9.2 fruit or vegetable servings for six weeks or a 3.6 servings for four weeks and then 9.2 servings for the remaining two weeks. There was a wide range of baseline isoprostane levels, but the run-in diet reduced variation and decreased excretion levels by an average 33%. Subjects on the high FV diet showed an additional 14% decrease after two weeks on the experimental diet, with values remaining constant for the rest of the study. Subjects on the low FV diet had steady levels at the baseline values until they switched to the high FV diet for the last two weeks, which caused a significant reduction in isoprostane excretion levels. In contrast, flavonoids from onions and black tea did not change isoprostane levels in healthy human subjects after consumption for 14 days (O'Reilly et al., 2001).

In conclusion, GST, QR and UGT activities were not significantly increased by blueberries or blueberry fractions. Likewise, lipid peroxidation was also not significantly decreased. There was only a slight decrease in liver DNA damage for the 1% flavonoid group. This study shows that in vivo results are not always in agreement with in vitro findings and that extremely high doses may be required to elicit significant effects. For that reason, more in vivo research needs to be done to confirm results from in vitro studies.

TABLE 1 Composition of diets¹

	diet 1	diet 2	diet 3	diet 4	diet 5	diet 6
	Control	Crude blueberry	Carbohydrate match	Crude polyphenol	1% flavonoid	0.2% flavonoid
Ingredient	g/kg diet					
casein	200.0	200.0	200.0	200.0	198.0	199.6
cornstarch	529.0	529.0	529.0	529.0	524.0	528.4
soybean oil	70.0	70.0	70.0	70.0	69.3	69.7
fiber	50.0	33.0	50.0	50.0	49.5	49.9
mineral mix	35.0	35.0	35.0	35.0	34.7	34.9
vitamin mix	10.0	10.0	10.0	10.0	9.9	10.0
L-cysteine	3.0	3.0	3.0	3.0	3.0	3.0
choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
sucrose	100.0	18.0	26.2	24.3	99.0	99.8
fructose	-	-	37.0	28.5	-	-
glucose	-	-	37.0	28.5	-	-
blueberries	-	100.0	-	-	-	-
polyphenols	-	-	-	20.0	-	-
flavonoids	-	-	-	-	10.0	2.0

¹Diet 1 is an AIN-93M diet, Diet 2 is 10% dried blueberries, which are 17% fiber and 82% sugar. Added fiber and sucrose were adjusted so total values would match control. Diet 3 contains sugars to match the composition in blueberries, 10% sucrose and 45% each of glucose and fructose. Diet 4 contains crude polyphenol extract and sugars to match the 10% blueberry diet. Diet 5 consists of 99% control diet and 1% purified flavonoids consisting mainly of anthocyanins, Diet 6 is 99.8% control diet and 0.2% purified flavonoids consisting mainly of anthocyanins.

TABLE 2 Final body, liver and relative liver weights in rats fed control or blueberry supplemented diets¹

Treatment	Body Weight (g)	Liver Weight (g)	Relative liver weight (g/100 g body weight)
Control	222 ± 7	9.0 ± 0.5	4.05 ± 0.15
Crude blueberry	219 ± 3	9.3 ± 0.3	4.26 ± 0.13
Carbohydrate control	220 ± 4	9.1 ± 0.4	4.11 ± 0.16
Crude polyphenol	214 ± 3	8.6 ± 0.3	4.00 ± 0.10
1% flavonoid ²	219 ± 4	8.8 ± 0.4	4.02 ± 0.12
0.2% flavonoid ²	220 ± 4	8.8 ± 0.2	4.00 ± 0.07

¹ Values are mean ± SE, n=8. No groups were significantly different.

² Flavonoids for the 1% and 0.2% flavonoid diets consist mainly of anthocyanins.

TABLE 3 Weekly and average food intake of rats fed control or blueberry supplemented diets¹

Treatment	Week 1	Week 2	Week 3	Average
Control	13.4 ± 0.3	15.1 ± 0.3	17.1 ± 0.7 ^{a,b}	15.4 ± 0.4
Blueberry	13.2 ± 0.2	15.9 ± 0.3	17.4 ± 0.3 ^{a,b}	15.5 ± 0.4
Carbohydrate control	13.2 ± 0.3	15.4 ± 0.2	18.0 ± 0.3 ^a	15.5 ± 0.4
Crude polyphenol	12.9 ± 0.2	15.3 ± 0.3	16.6 ± 0.3 ^b	15.0 ± 0.4
1% flavonoid ²	13.1 ± 0.2	15.7 ± 0.5	17.9 ± 0.5 ^a	15.6 ± 0.5
0.2% flavonoid ²	13.1 ± 0.3	16.0 ± 0.2	16.5 ± 0.2 ^b	15.2 ± 0.3

¹Intake values are in g/day, mean ± SE, n=8. Means in a row with different superscripts are significantly different, P<0.05.

²Flavonoids for the 1% and 0.2% flavonoid diets consist mainly of anthocyanins.

TABLE 4 GST activity in liver and colon mucosa of rats fed control or blueberry supplemented diets¹

Treatment	Liver	Colon Mucosa
	U/mg protein	
Control	596 ± 94	90 ± 12
Crude blueberry	761 ± 15	89 ± 13
Carbohydrate control	575 ± 47	100 ± 12
Crude polyphenol	759 ± 77	110 ± 18
1% flavonoid ²	736 ± 99	109 ± 12
0.2% flavonoid ²	613 ± 80	96 ± 16

¹ Values are mean ± SE, n=8 for all groups except n=7 for colon mucosa for the polyphenol group. GST activity expressed as U/mg protein; 1 unit of activity is nmol conjugate formed/min. No groups were significantly different.

² Flavonoids for the 1% and 0.2% flavonoid diets consist mainly of anthocyanins.

TABLE 5 QR activity in liver and colon mucosa of rats fed control or blueberry supplemented diets¹

Treatment	Liver		Colon Mucosa	
	U/ mg protein			
	<u>n</u>		<u>n</u>	
Control	8	14.3 ± 3.7	7	75.4 ± 15.4
Crude blueberry	8	11.8 ± 2.8	8	71.7 ± 13.6
Carbohydrate control	8	10.9 ± 3.7	7	93.7 ± 18.2
Crude polyphenol	8	16.4 ± 3.1	6	81.5 ± 14.3
1% flavonoid ²	8	12.5 ± 3.1	8	93.8 ± 26.2
0.2% flavonoid ²	8	10.0 ± 3.8	7	69.3 ± 12.2

¹ Values are mean ± SE. QR activity expressed as U/mg protein; 1 unit of activity is nmol conjugate formed/min. No groups were significantly different.

² Flavonoids for the 1% and 0.2% flavonoid diets consist mainly of anthocyanins.

TABLE 6 UGT activity in liver of rats fed control or blueberry supplemented diets¹

Treatment	Activity in liver cells
	U/ mg protein
Control	16.8 ± 1.5
Crude blueberry	21.1 ± 2.2
Carbohydrate control	19.8 ± 2.1
Crude polyphenol	15.6 ± 2.5
1% flavonoid ²	16.8 ± 1.7
0.2% flavonoid ²	17.5 ± 1.8

¹Values are mean ± SE, n=8. UGT activity expressed as U/mg protein; 1 unit of activity is nmol conjugate formed/min. No groups were significantly different.

²Flavonoids for the 1% and 0.2% flavonoid diets consist mainly of anthocyanins.

TABLE 7 Percent DNA damage in lymphocytes and liver of rats fed control or blueberry supplemented diets^{1,2}

Treatment	Lymphocytes	Liver cells
Control	6.53 ± 0.88	8.35 ± 0.54 ^a
Crude blueberry	7.04 ± 0.90	7.43 ± 0.45 ^{a,b}
Carbohydrate control	6.11 ± 0.48	6.79 ± 0.48 ^{a,b}
Crude polyphenol	6.97 ± 0.38	7.80 ± 0.59 ^{a,b}
1% flavonoid ³	5.68 ± 0.67	6.47 ± 0.82 ^b
0.2% flavonoid ³	6.87 ± 0.79	7.18 ± 0.32 ^{a,b}

¹Values are mean ± SE, n=8 for all groups except n=7 for lymphocyte control and carb control groups and N=5 for lymphocyte blueberry group. Treatment groups with different superscripts are significantly different from each other (P<0.05).

²Cells were scored into 5 categories based on tail size. 100 cells were scored per sample. Percent DNA in the tail was calculated as: (2.5*cells₁ + 12.5*cells₂ + 30*cells₃ + 60*cells₄ + 90*cells₅)/100 according to Zhao et al, 2006. Percent DNA in the tail corresponds to percent DNA damage.

³Flavonoids for the 1% and 0.2% flavonoid diets consist mainly of anthocyanins.

TABLE 8 Urinary F₂-isoprostane levels of rats fed control or blueberry supplemented diets¹

Treatment	n	F ₂ -Isoprostanes Ug/ g creatine
Control	7	0.53 ± 0.14
Crude blueberry	7	0.60 ± 0.10
Carbohydrate control	8	0.59 ± 0.09
Crude polyphenol	8	0.59 ± 0.13
1% flavonoid ²	7	0.65 ± 0.13
0.2% flavonoid ²	7	0.49 ± 0.08

¹Values are mean ± SE, No groups were significantly different.

²Flavonoids for the 1% and 0.2% flavonoid diets consist mainly of anthocyanins.

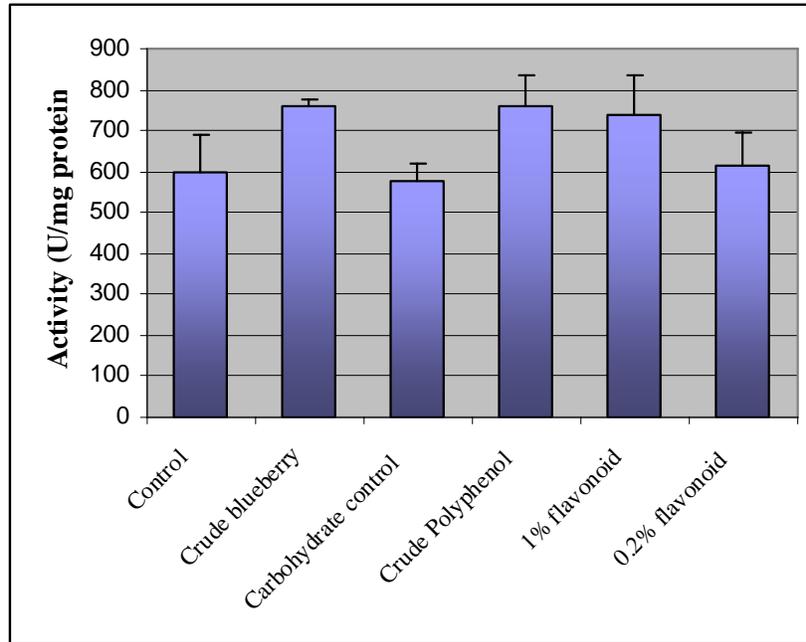


FIGURE 3 GST activity in the liver for rats fed control or blueberry supplemented diets for three weeks. Bars are means \pm SE.

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CHAPTER V

SUMMARY AND CONCLUSIONS

Previous studies have demonstrated that blueberries have high antioxidant capacity. This is typically assessed using *in vitro* studies to determine antioxidant capacity rather than *in vivo* studies to ascertain the effects of high antioxidant capacity. However, some studies in humans and animals have suggested that diets supplemented with plant foods or polyphenols do reduce oxidative stress in some instances. In addition, others have reported that some blueberry extracts increase GST activity *in vitro*. Only a few studies have examined the impact of high anthocyanin fruits such as blueberries on detoxification enzyme activity *in vivo*.

OVERALL PURPOSE

The purpose of this study was to examine the effects of blueberry supplementation on oxidative stress in healthy rats. The specific objectives of this research were to determine if different blueberry extracts reduce markers of oxidative stress and increase detoxification enzyme activity, and if the extent of the protective effect was dependent on the extract.

MAJOR FINDINGS

We hypothesized that modest blueberry supplementation would reduce lipid and DNA oxidative damage and increase activity of the Phase II enzymes GST, QR, and UGT. It was also expected that the whole blueberry supplementation would have a greater protective effect than the polyphenol extract.

For three weeks, rats were fed either a control diet, 10% crude blueberry, control diet with sugars to match blueberry diet, control diet with polyphenols and sugars to match

blueberry diet, control diet with 1% flavonoid extract, or control diet with 0.2% control diet. Results of this study showed that the activities of phase II enzymes GST, QR and UGT were not significantly increased by any of the experimental diets, but some slight increases were seen for GST activity. The blueberry, polyphenol and 1% flavonoid groups had roughly 27% higher GST activity in liver tissue when compared to the control group, which is close to change reported for raspberry supplementation by others (Reen et al., 2006). Oxidative damage levels in DNA and lipids were also not significantly different between groups except for the 1% flavonoid group. DNA damage determined using the Comet assay was lowest for both liver and colon mucosa in the 1% flavonoid group, but results were only significant in the liver. Therefore, this study demonstrated that blueberry supplementation did not have a significant effect on detoxification enzyme levels in rats, although there were some trends for improvements in GST activity. There was only a slight effect on liver DNA damage levels for the rats supplemented with 1% flavonoid extract.

LIMITATIONS

Dietary polyphenols are absorbed at low levels and excreted after several hours. Plasma flavonoid levels were not measured in this study, due to the difficulty of such quantification, but measurements may have been useful in determining the effectiveness of supplementation. A second limitation was that a healthy, unstressed rodent model was used. Oxidative stress levels increase with age and as a result of various stressors. Human studies also tend to use healthy unstressed subjects as well. The rats in the present study were young and had low basal oxidative stress levels, with little to no margin for improvement. Even if the supplementations had the potential to improve oxidative status, the baseline levels may have been so optimal that no improvement was seen. Another limitation of the study was its

small size. The experiment groups only had eight rats per group, and a larger group size would have increased statistical power. Also, the study was short term. Perhaps a time frame longer than three weeks is needed to show significant benefits from supplementation. Finally, the ultimate goal of this research is to determine potential benefits of supplementation for humans. Results from animal studies unfortunately cannot be used to make recommendations for humans, but can be used to test hypotheses concerning mechanisms for disease prevention.

IMPLICATIONS

The results did not support the hypothesis that blueberry supplementation would reduce oxidative stress and increase Phase II enzyme activity. However, there were some trends for reduced DNA damage and increased GST activity in the 1% flavonoid group. Therefore the results suggest that there may be a potential for protection by flavonoid supplementation against oxidative stress that would be more apparent in a longer term study or in stressed organisms.

FUTURE RESEARCH

Future studies should use larger subject numbers to improve the statistical power of the results. Some positive effects were seen in the supplementation groups, but effects were not large enough to produce significance. It would also be very important to use a stressed model to determine if blueberry supplementation would provide a beneficial effect in a situation of elevated oxidative stress. Many studies now report that in order to investigate the effects of a supplement on oxidative damage, it is necessary to use a stressed model to see an effect. Oxidative stress affects many processes in the body, most notably inflammation. It would be valuable to evaluate the effects of blueberry supplementation on inflammation and

other biomarkers associated to oxidative stress to more fully understand the effects of dietary polyphenols on health promotion. And finally, several studies have shown that protective effect is heavily dependent on specific gene polymorphisms. Determining the identity and nature of these polymorphisms would allow for dietary recommendations to be made on an individual basis to promote optimum wellbeing.

APPENDICES

Line up slides at center of tank.

Set voltage at 25 V and adjust buffer level to get as close to 300mA as possible (usually around 295 mA)

(Note: Start with buffer just barely covering slides, and add more to increase ampage, take away to increase voltage.)

Run for 30 minutes.

Tap excess buffer off slides and dip in 70% alcohol for 5 minutes.

Air dry slides over night, then store at RT with desiccant in the dark.

SAMPLE PREP:

For tissue:

Follow a homogenization procedure to isolate cells.

For frozen cells:

Thaw by submerging vial in 37 °C water bath until all ice is gone

(Transfer to ice cold media, centrifuge 10 min at 2000rpm (200xg))

Resuspend in cold PBS.

Cryopreservation:

Harvest cells

Resuspend in medium with 10% DMSO.

Count and pipette aliquots of 2×10^6 cells per eppendorf tube. (Make sure there is room for expansion.)

Put tubes in Styrofoam container and place in -80 °C freezer (to freeze at a rate of -1 °C)

SILVER STAINING PROCEDURE

1) Fixation

Solution prep (mix in this order and only immediately before use)

Per sample, 30 ul DI water

50 ul methanol

10 ul glacial acetic acid

10 ul fixation additive, 10X

Pipette 100ul on each sample and incubate for 20 min at RT.

Get Reagent 4 out of fridge to warm, and mix up 100 ul of 5% acetic acid per sample.

Rinse slides with DI water for 30 min.

2) Staining

Solution prep

Per sample, 35 ul DI water
 5 ul Reagent 1
 5 ul Reagent 2
 5 ul Reagent 3

Mix by tapping tube

50 ul Reagent 4

Quickly pipette to mix and flood each sample with 100 uL of staining solution.

Incubate approximately 11 min. at RT. Monitor staining progress under 10X lens of microscope and allow to develop until the DNA tails are easily visible.

Stop the reaction by pipetting 100 ul of 5% acetic acid on each sample and incubate for 15 minutes.

Rinse slides with DI water, air dry and store in dark with desiccant.

APPENDIX B

UGT ASSAY

Adapted from Bock, K, et al. *Biochem Pharmacol* **32**, 953-5, 1983. and Embola C, et al. *Food Chem Toxicol* **40**, 841-4, 2002.

This assay is to be performed on liver microsomes. Colon mucosa UGT levels are too low to detect.

SOLUTION PREPARATION

(Can make 1-6 in advance, UDPGA and reaction mix should be made directly before assay)

1) 0.25 % Triton X - 50 ul Triton X in 19.95 ml dH₂O

(Triton X is very viscous, cut pipette tip before measuring and be sure to wait long enough to allow the substance to all run out of the tip. Mix triton X and water on the stir plate, at very low heat to aid mixing, which usually takes a while.)

Storage: fridge

Disposal: pour down sink

2) 0.08 M Tris HCl - 2.522 g Tris HCl in 200 mL dH₂O

1.165 g Tris base in 120 mL dH₂O

Slowly add base to acid until pH=7.4.

Storage: fridge for 1 month

Disposal: pour down sink with water.

3) 50mM MgCl₂- 101.6 mg MgCl₂ in 10 ml dH₂O

Storage: fridge

Disposal: pour down sink with water.

4) 5 mM PNP- 3.48 mg per 5 ml 0.08 M Tris HCl

PNP is extremely toxic! Handle with care.

(Note: 1 M PNP standard can be used in place of fresh-made solution, just use half standard half tris buffer when making reaction mixture.)

Storage: fridge

Disposal: Hazardous waste

5) 2M NaOH - 4 g NaOH in 50 ml dH₂O

Storage- RT

Disposal- down the sink with lots of water

6) 5% TCA- 1 g TCA in 20 ml dH₂O

Storage- fridge (since it needs to be chilled for assay)

Disposal- down the sink with lots of water

7) 30mM UDPGA- 24.24 mg in 1250 ml 0.8 M Tris HCl

Note! Only make right before use! This is enough for 2 runs of 12 tubes each (10 samples and 2 blanks) Keep on ice.

Storage- N/A

Disposal- down sink with water.

8) Reaction mix- 500 ul 0.25 % Triton-X

1250 ul 0.8 M Tris HCl

1250 ul 50mM MgCl₂

1250 ul 5mM PNP*

4.5 ml dH₂O

(* Or 625 ul of 1M PNP standard and 625 ul 0.8 M Tris HCl)

Note-Make right before assay! This is enough solution for 2 runs of 12 tubes each (10 samples and 2 blanks) Keep on ice.

Storage- N/A

Disposal – hazardous waste!!

ASSAY PROCEDURE

Run each sample in duplicate, and a duplicate blank with each batch. I recommend doing 5 samples at a time, adding reagents to 3 samples at a time, with a minute between the two groups to ensure even timing.

Beforehand: Label eppendorf microcentrifuge tubes, two per incubation tube, one for T=0, one for T=15. Fill with 1mL cold TCA, keep in fridge or on ice.

1) Dilute liver microsome samples. (Try 1:20 for starters.)

Keep on ice.

2) In a glass test tube, combine 350 uL reaction mixture

100 uL diluted microsomes

Make two tubes per sample. For the blanks, add 100 uL Tris buffer in lieu of microsomes.

Incubate 5 min in 37 °C shaking water bath (Start incubation for 2nd half of tubes 1 minute after 1st batch)

3) Start reaction by adding 50uL UDPGA to each tube.

Immediately vortex and pipette out 100uL into the waiting labeled eppendorf tubes containing 1 mL cold TCA.

(Start rxn for 1st half of samples, take aliquots, then repeat for the 2nd half of samples)

Incubate reaction mixtures for 15 minutes and then again transfer 100uL aliquots to 1mL of TCA, transferring from the 1st 6 tubes, then the 2nd.

4) After taking the aliquots, invert the epp tubes to mix. Centrifuge epp. tubes for 10 min at 13K rpm in the tabletop centrifuge. If the tubes are not centrifuged immediately after being taken, keep on ice until doing so.

5) Pipette out 1 ml supernatant into cuvettes containing 250ul NaOH. Measure absorbance at 405 nm.

CALCULATIONS

Molar extinction coefficient = $18.1 \times 10^3 \text{ cm}^2/\text{mol}$

- 1) Average the readings for a sample at T=0 and subtract the average reading at T=15 to obtain change in absorbance.
- 2) Subtract change in absorbance of the blank from the sample's change in absorbance.
- 3) Divide by 15 to get rate of change per minute.
- 4) multiply by dilution factor and assay volume, divide by extinction coefficient and amount enzyme.

$$\frac{(A1+A2)_{T=0}}{2} - \frac{(A1+A2)_{T=15}}{2} \quad \text{minus} \quad \text{avg delta A for blank}$$

A/15 = rate per min

umol PNP consumed/min/ml enz = (rate per min)(total assay vol)(dilution factor)/(ext. coeff.)(vol enzyme added)

umol PNP consumed/min/ml enz = (sample rate*1.25 *20)/(18.1*0.0181)

Average values are about 10-30 nmol/min/mg prot (~0.01-0.03 umol/min/mg protein)

APPENDIX C

F₂-ISOPROSTANE ASSAY for Oxford Biomedical Kits EA85 and Pretreatment Kit GL85

This assay takes 5-6 hours to complete.

For all microplate incubation steps, be sure to cover the microplate with tin foil.

Wet pipette tips before aliquotting.

Urine collection

For rats, collect overnight urine using metabolic cages. Freeze urine at -80 until analysis. No preservative required.

Pretreatment

- 1) Thaw glucuronidase pretreatment (must be used completely, cannot be refrozen, although it can be kept on ice for 1 hr after thaw).
- 2) Prepare samples: 100 uL urine,
 4 uL glucuronidase
- 3) Incubate 2 hr. at 37 °C.

Assay Prep (start ~20 min before pretreatment incubation is finished)

- 1) Dilute wash 5X wash buffer to 1X.
- 2) Prepare 3N sulfuric acid. 7.36 mL H₂SO₄
 42.6 mL dH₂O
- 3) Prepare standards:

Standard	Ingredients
S7 - 100 ng/mL	50 uL provided std + 450 uL dilution buffer
S6 - 50 ng/mL	200 uL S7 + 200 uL dilution buffer
S5 - 10 ng/mL	100 uL S6 + 400 uL dilution buffer
S4 - 5 ng/mL	200 uL S5 + 200 uL dilution buffer
S3 - 1 ng/mL	200 uL S4 + 400 uL dilution buffer
S2 - 0.1 ng/mL	100 uL S3 + 900 uL dilution buffer
S1 - 0.05 ng/mL	500 uL S2 + 500 uL dilution buffer
B0 - 0 ng/mL	none + 300 uL dilution buffer

- 4) Add 100 uL of the standards to plate in duplicate (standards will occupy 16 wells). Two additional wells will be reagent blanks, and will only contain 200 uL buffer. Do not add conjugate solution to the reagent blanks in step 7!
- 5) Dilute urine samples with enhanced dilution buffer (either 1:4 or 1:8). Add diluted samples to plate in duplicate.

Assay

- 1) Dilute 15-isoprostane F2 Hrp conjugate 1:50. Only prepare amount that is needed. Add 100 uL diluted 15-isoprostane F2 Hrp conjugate to every well except for blank wells, using a multi-pipetter. Add 100 uL buffer to the reagent blanks. Incubate plate for 2 hr. at room temperature.
- 2) Invert plate to drain then tap on a paper towel to remove as much liquid as possible.
- 3) Add 300 uL wash buffer to each well with multipipettor, let sit for 3 min. Remove liquid as in step 2. Repeat washing and emptying twice more. Be sure to remove as much liquid as possible at the end.
- 4) Add 200 uL substrate to every well. Incubate 30 minutes or until the blanks turn a deep blue color.
- 5) To stop reaction, add 50 uL of sulfuric acid to each well, prompting a color change to yellow.
- 6) Wipe bottom of plate with a kimwipe and read plate in a microplate reader at 450 nm.

Calculations

- 1) Average absorbance values for all duplicates so that the samples and standards have one value each. Subtract the average of the reagent blanks from the average of all the other readings.
- 2) Divide standards by B0 and multiply by 100 to get %B0 values. The relationship is logarithmic.
- 3) Use Excel to determine values for logarithmic formula: $y=b*m^x$
 Since y =absorbance and x = concentration, solve for x to get: $x=\frac{\ln(y/b)}{\ln(m)}$

Excel code:

=INDEX(LOGTEST(A1:A7 ,B1:B7 ,1) to determine m and

=INDEX(LOGTEST(A1:A7,B1:B7 ,2) to determine b

Where A1:A7 are concentrations of the standards and B1:B7 are the %B0 values for the standards.

- 4) Now that m and b have been determines, plug in absorbance values (y) for the samples to determine concentrations (x).
- 5) Standardize concentrations with creatinine concentrations determined using the creatinine assay.

APPENDIX D

CREATININE ASSAY for Oxford Biomedical Kit

This assay is used to standardize urinary isoprostane values.

Urine collection

For rats, collect overnight urine using metabolic cages. Freeze urine at -80 until analysis. No preservative required.

Assay

- 1) Add 0.025 mL/well of the provided standards as well as distilled water to the microplate, in duplicate:
 - S1 - 10 mg/dL
 - S2 - 3 mg/dL
 - S3 - 1 mg/dL
 - S4 - distilled water
- 2) Add 0.025 mL/well diluted urine samples to the plate in duplicate (use a 10-20 fold dilution with distilled water).
- 3) Prepare reagent mixture: 1 part Alkali solution to 5 parts yellow picric acid solution (will need 0.18 mL per well).
Add 0.18 mL per well.
Mix plate by gentle shaking (can use a microplate shaker).
- 4) Cover and incubate plate for 10 min at room temperature.
- 5) Read plate at 490 nm.
- 6) Add 0.015 mL/well of acid reagent.
- 7) Mix and let stand for 5 min at RT.
- 8) Read plate again at 490nm.

Calculations

(Delta A is directly proportional to absorbance.)

- 1) Subtract 1st reading taken at time 0 from 2nd reading taken after 5 minutes.

- 2) Average absorbance values for all duplicates so that the samples and standards have one value each.
- 3) Construct a standard curve using the standards with absorbance on y-axis and concentration on x.
- 4) Determine slope and intercept to calculate unknown concentrations.
- 5) To convert creatinine concentrations from mg/dL to $\mu\text{mol/L}$, multiply by 88.4.