Bioactive Properties and Potential Health Benefits of Blueberries and Anthocyanins

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Abstract
Recent interest has focused on the potential of naturally occurring anthocyanins, such as those found in blueberries, to provide an array of health benefits in humans. The purpose of this review was to examine the current literature database and provide an update of the health benefits and potential mechanisms associated with ingestion of anthocyanins in humans. Every effort was made to include relevant research from 2005 to present. The following search parameters were designated for paper selection in this review: 1) Search Terms- anthocyanins, blueberries, antioxidants, polyphenols, vascular performance, immune function, flavanols, berry-sourced, metabolites, oxidative stress, inflammation; 2) Search Engines- Science Direct, PubMed, Google Scholar. A total of 74 scientific papers were found to meet the search parameters and criteria. Critical review of these selected papers indicates that anthocyanins function as antioxidants, anti-inflammatory agents, and modulators of vascular performance and immune function. Consequently, these compounds demonstrate unique abilities to reduce risk of cancer, metabolic syndrome, and cardiovascular disease. In conclusion, increased dietary anthocyanin consumption, especially from blueberries, represents a safe, economical, and important method of reducing incidence of chronic disease in humans and may even serve as non-pharmacologic treatment for such diseases.

Key Words: Blueberries, Anthocyanins, Polyphenols, Disease, Antioxidants, Oxidative Stress, Humans

1. Introduction

The human body functions within a tightly regulated set of balances. One of these critical balances is that of formation of reactive oxygen species (ROS) and the associated antioxidant enzyme systems and low molecular weight antioxidants that neutralize these compounds (Arcaro et al., 2016; Chiurchiu, Orlacchio, & Maccarrone, 2016; Liu et al., 2016; Tsikas & Theodoridis, 2016; Wozniak, Wozniak, Mila-Kierzenkowska, & Kasprzak, 2016). Many different substances act as antioxidants within the human body. Antioxidants are necessary to balance ROS such as superoxide and hydroxyl radicals and to maintain cellular redox. Antioxidants are classified as exogenous (low molecular weight compounds) or endogenous compounds (enzymatics) which are responsible for removal of free radicals, scavenging ROS, inhibiting formation of ROS, and binding metal ions needed for catalysis of ROS generation (Gilgun-Sherki, Melamed, & Offen, 2001). Some of the low molecular weight antioxidants include vitamin E, vitamin C, beta-carotene, and selenium. Low molecular weight antioxidants act by sharing or donating an electron with a radical. This prevents the radical from obtaining electrons from other surrounding sources such as lipids, nucleic acids, or proteins (Godala et al., 2016; Gomes-Rochette et al., 2016; Rezaei et al., 2016). The enzymatic systems include superoxide dismutase, glutathione peroxidase, and catalase. Enzymatic systems neutralize ROS by conversion to less reactive or neutral compounds (Ida et al., 2016).

Oxidative stress is the imbalance between ROS and antioxidants (Son, Park, & Shim, 2016; Stepaniak et al., 2016; Sunnetcioglu, Alp, Sertogullarindan, Bala-haroglu, & Gunbatar, 2016). Recent interest has focused on the potential of naturally occurring anthocyanins found in foods, such as blueberries, to function as antioxidants and anti-inflammatory agents (G. J. Mazza, 2007; G. Mazza, Kay, Cottrell, & Holub, 2002; McAnulty et al., 2014; McAnulty et al., 2011). Blueberries, from the plant genus of *Vaccinium*, have repeatedly been shown to contain the strongest total antioxidant capacity as well as the highest total phenolic content, total flavonoid content, and total anthocyanidin content in antioxidant-rich foods (Huang, Zhang, Liu, & Li, 2012). Polyphenols, such as 3-glucoside/arabinoside/ galactoside-based polymers of delphinidins, petunidins, peonidins, malvidins and antho-cyanins, are types of biological macro-molecules found in large amounts in blueberries (G. J. Mazza, 2007; G. Mazza et al., 2002).

Anthocyanins are responsible for the red, purple, and blue colors of many fruits, vegetables, cereal grains, and flowers. Over 300 structurally distinct anthocyanins have already been identified in nature. Considerable evidence suggests that dietary anthocyanins provide preventive and therapeutic roles in a number of human diseases and contribute an overall anti-obesity effect (Prior et al., 2010). Following blueberry supplementation, a total of 32 polyphenol metabolites are found to be present in circulation. The three major anthocyanin-specific metabolites of polyphenols are hippuric, hydroxyhippuric, and homovanillic acid, all of which were present at 6 hours post-supplementation. Molecularly, these compounds resemble the pharmacological compound apocynin, an NADPH oxidase inhibitor used to promote vasodilation. A maximum benefit and plateau effect was noted at 766 mg of blueberry polyphenols, suggesting an upper limit for future testing in determining dose-dependent effectiveness (Rodriguez-Mateos et al., 2013). Therefore, blueberries, with high concentrations of antioxidants and anti-inflammatory antho-cyanins, are an ideal candidate to test the theory of dietary antioxidant intake and its inverse relation to oxidative damage.

Because of the limited review of the effects of anthocyanins on multiple health parameters, the purpose of this paper is to provide a current and comprehensive review of the multiple health benefits of these compounds. Table 1 indicates the search parameters used in this study. Specifically, this review addresses the benefits of anthocyanins found in blueberries on oxidative stress, inflammation, vascular performance, and immune function.

2. Effects on Oxidative Stress

As stated in the Introduction, oxidative stress is a disturbance in the balance between the production of free radicals, reactive oxygen species (ROS), and antioxidant defenses. Radicals are a common outcome of normal aerobic cellular metabolism. Our bodies produce antioxidant enzymes to prevent complications due to an overproduction of radicals. However, imbalances may occur through over-production of ROS or incorporation of environmental factors. ROS cause alterations to proteins, lipids, and DNA which can potentiate a plethora of human diseases such as cancer, atherosclerosis, ischemic injury, inflammation, and neurodegenerative diseases (Son et al., 2016; Stepaniak et al., 2016; Sunnetcioglu et al., 2016). Humans are constantly exposed to free radicals created by electromagnetic radiation and from the manmade environment with its pollutants. Natural sources such as radon, cosmic radiation, as well as cellular metabolism, also add free radicals to the environment (Uttara, Singh, Zamboni, & Mahajan, 2009).

Recent research has focused on the beneficial effects of edible berries and their link to oxidative stress suppression (Schiffrin, 2010). Blueberries, in particular, have been found to play a positive role in the alleviation of oxidative stress as well as a treatment of oxidative stress in age-related liver injury (Coban et al., 2014). In a recent study performed by the biochemistry department of Turkey's Faculty of Medicine (Bingul et al., 2013), male rats were treated with diethylnitrosamine (DEN), increasing the generation of ROS, apoptosis, necrosis, and proliferation in the liver. Blueberries were fed to the rats on a 5% and 10% blueberry-containing diet for six weeks. Two days before the end of the feeding cycle, 200mg/kg of DEN were given. Oxidative stress was measured in liver samples. Pretreatment with a high dose of blueberry reduced apoptotic, necrotic, and proliferative changes in the liver induced by DEN. Dietary blueberry intake also decreased hepatic lipid peroxidation, protein oxidation and nitrotyrosine levels, and increased GST activity.

In a study from the European Journal of Nutrition, investigators examined the effects of regular consumption of a wild blueberry drink on markers of oxidative stress, inflammation and endothelial function in subjects with risk factors for cardiovascular disease (Riso et al., 2013). Using a crossover study design, 18 male volunteers received 25 grams of freeze-dried wild blueberry powder, providing 375 mg of anthocyanins daily, or a placebo drink, for six weeks separated by a six-week washout period. Oxidative-induced damage in blood mononuclear cells, serum interleukin levels, reactive hyperemia index, nitric oxide, and soluble vascular adhesion molecule concentrations were then analyzed. Wild blueberry drink intake significantly reduced the levels of endogenously oxidized DNA bases and the levels of H_2O_2-induced DNA damage.

With regard to oxidative stress and athletic performance, a study completed in 2011 examined the effect of blueberry consumption on oxidative stress on 25 well-trained athletes (McAnulty et al., 2011). Subjects were given 250 grams of blueberries per day for six weeks, and another 350 grams were given one hour prior to a 90 minute run at 72% maximal oxygen consumption. Blood was examined for F_2-isoprostanes (indicator of oxidative stress), cortisol, cytokines, homocysteine, leukocytes, T-cell function, natural killer and lymphocyte cell counts for inflammation and immune system activation, and ferric reducing ability of plasma (FRAP) for antioxidant capacity. Muscle biopsies were also examined to evaluate stress and inflammation. The treatment group had significantly lower levels of F_2-isoprostanes compared to the control group. This study indicated that daily blueberry consumption for six weeks increased natural killer cell counts, reduced oxidative stress, and increased anti-inflammatory cytokines (McAnulty et al., 2011).

3. Effects on Inflammation

Inflammation is the body's local response to injury or infection. The physiology of various inflammatory diseases is a complex process mediated by various inflammatory and immune cells. Monocytes are circulating blood leukocytes. These cells develop initially in the bone marrow, migrate into peripheral blood, and then differentiate into macrophages (Auffray, Sieweke, & Geissmann, 2009). In response to an infection or damage, macrophages activate a set of early pro-inflammatory genes that up-regulate broad-spectrum cytokine production (IL-1β), prostaglandin synthesis and secretion (Cox-2), and nitric oxide production (NO). Chronic inflammation occurs in many cardiovascular and autoimmune disorders, when the inflammatory response fails to resolve with time. The negative effects of inflammation can be ameliorated through dietary factors, especially through the consumption of antioxidant-rich foods. The antioxidant capacity of blueberries is one of the highest ranking among all fruits and vegetables, with oxygen radical absorbance capacity (ORAC) values ranging between 13.9-45.9 µmol/g depending on the species (Prior et al., 1998).

Two enzymes most involved in activating the inflammatory response are inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).iNOS and COX-2 can catalyze the synthesis of nitric oxide (NO) and prostaglandin E_2 (PGE_2). These in turn cause sepsis, sepsis shock, and systematic inflammatory response syndrome. Therefore, inhibition of the expression of these enzymes would help reduce inflammation and its related conditions. Wild blueberry extracts from Chile and Ecuador were shown to inhibit iNOS up to 61.8%, while PGE_2 expression showed the highest inhibition at 89.1%. In addition, the phenolic extracts from the blueberries inhibited the expression of COX-2 at levels ranging from 16.6-62.0% (Schreckinger, Wang, Yousef, Lila, & Gonzalez de Mejia, 2010). It can be difficult to determine which specific component of the blueberry fruit is responsible for the positive effects. Eighteen major anthocyanins, four major phenolic acids, and multiple monomeric/polymeric proanthocyanidins have been previously reported as components found in blueberries (Kimura, Ogawa, Akihiro, & Yokota, 2011; Yousef et al., 2013).

Macrophages release several inflammatory cytokines. Those that have been found to directly induce tumoricidal or inflammatory activity include interleukin-1 (IL-1), IL-6, IL-8, TNF-α, and NO, all of which directly induce tumoricidal or inflammatory activity. The main causes of inflammation are not well understood, but imbalances in pro-inflammatory TNF-α, interferon-γ (IFN-γ), IL-1, IL-6, and IL-12 and anti-inflammatory cytokines including IL-4, IL-10, and IL-11 are thought to play a direct role. A recent study investigated the anti-inflammatory activity of blueberry polyphenols by using lipopolysaccharide (LPS) induced RAW264.7 macrophages.

Blueberries suppressed the mRNA gene expression of IL-1β (interleukin-1β), IL-6 and IL-12. Blueberries were found to exhibit anti-inflammatory activity by mediating and modulating the balances in pro-inflammatory cytokines of IL-1β, IL-6, and IL-12 (Cheng et al., 2014).

The health benefits of blueberries on reducing inflammation is due to the presence of a wide range of anthocyanins, the two most abundant proving to be malvidin-3-glucoside (Mv-3-glc) and malvidin-3-galactoside (Mv-3-gal) (Huang, Liu, Wang, Wang, & Li, 2014). TNF-α-induced increases of monocyte chemotactic protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and vascular adhesion molecule-1 (VCAM-1) can be inhibited due to the effects of these two malvidin glycosides. Overall, malvidin-3-glucoside has been shown to have better anti-inflammatory effects than malvidin-3-galactoside (Huang et al., 2014).

Increased obesity is often accompanied by inflammation in adipose tissue as well as an increase in inflammatory proteins. Some of these proteins include tumor necrosis factor-α (TNF-α), interleukin-6, monocyte chemoattractant protein-1 (MCP-1), and nitric oxide (NO) (Ferrante, 2007). These proteins may buildup causing inflammatory macrophages to follow and increase in the adipose tissue. Therefore, it is possible blueberries may be of benefit in overweight individuals.

While inflammation is a chief factor behind chronic disease for the overweight and obese, chronic inflammation is a major component of metabolic syndrome (MetS). In the United States, MetS is a public health problem and raises the risk for type II diabetes, coronary heart disease and other health issues. A recent animal study, utilizing a diet supplemented with wild blueberry, reported an overall anti-inflammatory effect on metabolic syndrome. Researchers found that consumption of wild blueberries in overweight rats resulted in decreased plasma concentrations of TNF-α, IL-6, and CRP and increased adiponectin (a protein involved in regulating glucose levels as well as fatty acid breakdown) concentration. The blueberry-enriched diet significantly reversed the increased plasma levels of IL-6 and TNF-α by down regulating the expression of these pro-inflammatory cytokines in both the adipose tissue and the liver. This anti-inflammatory effect may have further implications for MetS in humans and may offer a non-pharmacologic approach to treatment of MetS (Vendrame, Daugherty, Kristo, Riso, & Klimis-Zacas, 2013).

4. Effects on Vascular Performance

Anthocyanins from whole blueberries, through antioxidant and anti-inflammatory action, are associated with decreased cardiovascular disease (Stoclet et al., 2004; Wallace, 2011). However, the direct effects on vascular performance have yet to be fully elucidated (de Ferrars et al., 2014; Del Bo et al., 2013; Stoclet et al., 2004). It is likely that the specific effects of anthocyanins on vascular performance are an indirect result of their anti-inflammatory and antioxidant properties. Vasculature which is spared oxidative damage will inherently have higher-performing functionality. Emerging research involves investigating the relationship of anthocyanins and vascular performance with an aim to determine specific effects and potential mechanisms. A link between a higher intake of anthocyanins from blueberries and strawberries and decreased risk of myocardial infarction was found in a follow-up of 93,600 women of the Nurses' Health Study II (Kruger, Davies, Myburgh, & Lecour, 2014). Thus, ingestion of anthocyanin-rich beverages and food improves antioxidant enzyme activity and plasma antioxidant capacity, protecting the body against oxidative stress and ultimately the inflammatory parameters that play a major role in CVD (Kuntz et al., 2014).

Some studies have shown that blueberries exhibit hypolipidemic actions (Kalt et al., 2008; Kim, Bartley, Rimando, & Yokoyama, 2010). In a recent animal study, plasma lipids in guinea pigs were investigated in relationship to the effect of whole blueberry supplementation. The animals were fed for 75 days on a high cholesterol diet supplemented with fresh blueberries. The blueberry diet was found effective in reducing oxidative stress and cholesterol accumulation in the aorta and liver of the guinea pigs (Coban et al., 2013).

4.1 Biochemical Activity and Physiological Effects of Anthocyanins

Improved vascular performance may be a result of multiple biochemical effects exerted *in vivo* by blueberry anthocyanins, including improved blood lipids, hypotensive effects, decreased capillary permeability and fragility, improved vascular flexibility and elasticity, and clotting and platelet aggregation (de Ferrars et al., 2014; Del Bo et al., 2013; Kong, Chia, Goh, Chia, & Brouillard, 2003; Norton, Kalea, Harris, & Klimis-Zacas, 2005; Rodriguez-Mateos et al., 2013). Animal studies have shown decreased damage by H_2O_2 in the endothelium and red blood cells when supplemented with anthocyanins, which suggests a possibility for a similar effect in humans (Del Bo et al., 2013). In addition, animal studies have shown improvements in vasodilation as a result of blueberry-containing supplements, but human studies have been limited (Del Bo et al., 2013; Kruger et al., 2014).

Vascular performance is affected by vasodilation and vasoconstriction mediators. When these are out of balance, there is a negative impact on vascular performance and the effects of CVD can occur even before other early indicators, such as the onset of atherosclerosis (Norton et al., 2005). Anthocyanins have demonstrated vaso-relaxant effects *in vitro* which improve the balance of dilation and constriction in theory, thereby reducing vascular resistance and improving tone (Kruger et al., 2014). A polyphenol-rich blueberry supplement was found to exert significant endothelium-dependent brachial artery vasodilation as measured by flow-mediated dilation (FMD) at 1, 2, and 6 hours post-administration (Rodriguez-Mateos et al., 2013). The maximally effective dosage producing these results contained 766mg of polyphenols.

A recent study demonstrated beneficial effects of blueberry powder on arterial stiffness in postmenopausal women, reflected in decreases of 5.1% and 6.3% in systolic and diastolic blood pressure, respectively. The resultant decreases in blood pressure were likely attributable to significantly decreased brachial-ankle pulse wave velocity baPWV, the “gold standard” measure of central and peripheral arterial stiffness, in the blueberry powder-receiving group(Johnson et al., 2015). Another study found that sedentary men and women with pre-hypertensive blood pressure experienced significant reductions in diastolic blood pressure following sixweeks of supplementation with 38g blueberry powder (equivalent to 250g fresh blueberries)/day (McAnulty et al., 2014). Findings from this study are in agreement with other research as the vasorelaxant effects of blueberry powder have been shown to decrease blood pressure in obese men and women with metabolic syndrome, subsequently reducing hypertension, and risk of cardiovascular disease (Johnson et al., 2015).

4.2 Biochemical Mechanism of Anthocyanins on Vascular Performance

For the first time, *ex* and *in vivo* animal studies have determined a mechanism by which anthocyanins improve vascular performance (Kalea, Clark, Schuschke, Kristo, & Klimis-Zacas, 2010; Norton et al., 2005). The actual contraction biomechanism of smooth muscle of the vasculature is aided by antioxidant activity and can work more efficiently when subjected to a chemically-induced contraction. Aortic smooth muscle showed lower resistance (maximum force, or F_{max}) in response to L-phenylephrine, an alpha-1 adrenergenic receptor agonist, due to suppression of contraction by anthocyanins (Kalea et al., 2010; Norton et al., 2005). Anthocyanin activity modulates the relaxation of the aorta in response to agonist-induced contraction. This outcome may be related to the ROS-scavenging abilities of blueberry antioxidants. These findings also bring to light one of the obstacles of anthocyanin research which is confirming that adequate amounts of anthocyanin were absorbed to produce an effect. Potential extensions of this research could include the effects of improving vascular performance and response in hypertensive disease states where the blood vessels are experiencing increased contractions and resistance.

4.3 Anthocyanin Metabolites as Initiating Compounds in Improved Vascular Performance

It has been suggested that the actual metabolites of anthocyanins, phenolic acids, may be the responsible compounds for improvements in vascular performance (Rodriguez-Mateos et al., 2013; Wallace, 2011). This observation may address a key problem of this research topic, that is, the fact that anthocyanins are poorly absorbed and remain largely in the intestine, therefore largely unmeasurable in circulation. Anthocyanins are highly unstable and subject to marked biotransformation through the process of metabolism, producing phenolic metabolite compounds that are unique to anthocyanin degradation (de Ferrars et al., 2014). These metabolites are suspected to be the main mechanism of biomarker-indicated physiological effects of anthocyanins. Measurable differences of phenolic metabolites have been noted in the blood following anthocyanin supplementation. Therefore, it may be a matter of shifting focus to these metabolites within a specific window of time, due to their greater accessibility over the parent anthocyanin molecule to detect measurable effects on vascular performance.

Anthocyanin metabolites have been suggested to induce effects on vascular performance by improving the bioavailability of nitric oxide (NO), a primary vasodilator that also has anti-hypertensive, anti-thrombotic, anti-atherogenic, anti-angiogenic, and other properties that exert positive effects on the vasculature (Pergola, Rossi, Dugo, Cuzzocrea, & Sautebin, 2006; Rodriguez-Mateos et al., 2013; Stoclet et al., 2004; Wallace, 2011). Detrimental effects on the vasculature occur from events that initiate inflammation, oxidation, and the enzyme NADPH oxidase. NADPH oxidase produces ROS that lead to inflammation, degradation, and dysfunction of the endothelium. Additionally, ROS bind with NO, reducing NO bioavailability and, consequently, the capacity of processes which depend on NO (Jiang, Liu, Dusting, & Chan, 2014; Kleikers et al., 2012; Takac, Schroder, & Brandes, 2012; Zhang, Perino, Ghigo, Hirsch, & Shah, 2013).

A pro-inflammatory, oxidative environment is a known initiator for arterial stiffness, hypertension, and cardiovascular disease (Johnson et al., 2015). Blueberry anthocyanin metabolites, specifically, have been suggested to decrease the activity of NADPH oxidase, in turn reducing the generation of free radicals and improving both antioxidant capacity and bioavailability of NO, which would have otherwise become oxidized and destroyed (Johnson et al., 2015; Rodriguez-Mateos et al., 2013; Steffen, Gruber, Schewe, & Sies, 2008). The end result of this process is improved NO availability and enhanced endothelial-dependent vasodilation. Because of this chain reaction, NADPH oxidase has been identified as a primary target of polyphenol and cardiovascular performance research (Steffen et al., 2008; Wallace, 2011). In certain states, such as chronic inflammation or carcinoma, excessive production of NO triggered by toxins can lead to tissue damage. A specific anthocyanin, cyanindin-3-O-glucoside, has been identified to provide immune and anti-inflammatory function within the endothelium by regulating NO under these circumstances when excessive concentrations may produce negative effects (Pergola et al., 2006).

Damage to and impairment of the vasculature also occurs as a result of LDL cholesterol oxidation (Stoclet et al., 2004). Through their antioxidant activity and relationship with NO, anthocyanins have been demonstrated *in vivo* to decrease both serum LDL levels and LDL-related oxidative stress, with the ultimate result of promoting effective vasorelaxation. The specific mechanisms through which this may occur have yet to be determined, as results between studies have reached dissimilar conclusions. Regardless, these findings suggest that anthocyanins have the ability to improve vascular function both directly, through antioxidant function, or indirectly, through metabolite action and further biochemical and physiological chain reactions (McAnulty et al., 2011). Additional research is needed in this area to clarify details of mechanism, biochemical interactions, and differences between specific polyphenols.

5. Effects on Immune Function

Anthocyanins are among several bioactive polyphenols that have been suggested to play a role in both innate and acquired immunity. The scientific literature regarding blueberry-sourced anthocyanins and immune function is extremely limited (Fimognari, Berti, Nusse, Cantelli-Fortii, & Hrelia, 2005; Gerhauser, 2008; Hushmendy et al., 2009; Percival, 2009). Although scarce, the research that does exist on this highly specialized topic includes several pioneer animal and human studies that have begun to investigate the specific action of anthocyanins on immunity.

5.1 Immunomodulatory Activity of Anthocyanins

Enhanced immune function was noted in a human study using polyphenol-containing fruit juices as a source of anthocyanins. Immune function biomarkers, including lymphocytes, cytokines, and NK cell activity were improved by supplementation of polyphenols (Bub et al., 2003). Wine and grape pomace (pulp) with high anthocyanin concentrations have been shown to improve immune function in animal studies (Percival, 2009). Blackberry-sourced anthocyanins have been demonstrated to combat endotoxin-triggered inflammation, circulatory failure, and organ dysfunction in rats, likely resulting from antioxidant function (Pergola et al., 2006). In addition, human studies have shown Concord grape juice to effectively increase serum antioxidant status (verified with ORAC analysis), improve lymphocyte activity, and increase γδ T cell counts (Percival, 2009).

A 2011 study supplementing highly-fit athletes with 250g/day of whole blueberries, for six weeks, demonstrated significantly-increased NK cell activity, suggesting a relationship between anthocyanin intake and immune function (McAnulty et al., 2011). It has been suggested that the anti-inflammatory function of antioxidants may also contribute to their immunity benefits by altering the balance of pro-inflammatory and anti-inflammatory cytokines (Wallace, 2011). Endothelial dysfunction and endothelial cell apoptosis have been found to be improved by anthocyanin supplementation in multiple studies, an effect attributed to their inhibition of oxidative damage (Pergola et al., 2006; Rodriguez-Mateos et al., 2013; Stoclet et al., 2004; Wallace, 2011). These preliminary results are a starting point for future research that is necessary to support claims of increased immune function by way of anthocyanin supplementation.

5.2 Immunomodulatory Biomechanism of Anthocyanins

The specific mechanism of the immune-enhancing effect of anthocyanins is thought to occur through their interaction with γδ T cells, a type of immune cell that works with both innate and acquired immunity (Percival, 2009). This specific type of T cell lines the digestive and respiratory tract and interacts with foreign materials that enter the body through either of these routes. γδ T cells have been shown to be responsive to diet, and because they perform both surveillance and pathogen-targeted immune functions, both severity and length of illness, chronic disease, and wound healing could be targeted by supplementation of functional foods.

Anthocyanins work as a type of trigger to immunity cells, similar to how the body's intrinsic trigger system works. Normally, immune cells are triggered by PAMP (pathogen-associated molecular patterns) allowing the immune system to "ramp up" in anticipation of targeting and fighting pathogens. It appears that anthocyanins mimic PAMP and trigger immune function of γδ T cells so that the molecules work proactively and not just when triggered by a pathogen. This improved function also helps to curb the cycle of immunity and inflammation. With ongoing inflammation, the immune system continues to attack damaged tissue (usually the result of ROS activity), causing additional damage and potentially leading to long-term consequences. Anthocyanins prevent this cycle from recurring by reducing the impact of ROS and mediating immune cells (Percival, 2009).

Whole food studies are far less common than those that make use of isolated extracts. In this context,an added bonus of using the whole food source lies in its antioxidant capacity. The activity of immune cells produces ROS to defend the body against pathogens, but ROS can also be damaging to healthy tissues. Because anthocyanins boost immune function and multitask as antioxidants, they are doubly beneficial because they prevent damage to surrounding tissues that would otherwise be caused by ROS (Percival, 2009).

5.3 Preliminary Testing Involving Anthocyanins and Berries

As exciting as these preliminary results are, not all of the studies that have been conducted thus far have produced results that are in agreement with one another. A very recent study examined the antioxidant and immunomodulatory effects of six fruits: rosehip, chokeberry, hawthorn, blackcurrant, blueberry, and rowanberry. Multiple assays of antioxidant function (including ORAC, TRAP, HORAC, and lipid peroxidation) as well as immune function (assessed by lymphocyte proliferation measured using an MTT colorimetric assay and antimicrobial activity) were conducted (Denev et al., 2014). While blackcurrants, chokeberries, rowan-berries, and rosehips all produced measurable effects on lymphocytes, blueberries failed to produce results. Blueberries also failed to exhibit substantial antimicrobial activity, in contrast with the broad-spectrum anti-microbial activity displayed by choke-berries, rowan-berries, and blackcurrants. It appeared in this trial that the most substantial function of blueberries was inhibition of lipid peroxidation, an antioxidant effect (Denev et al., 2014). While blueberries contained the highest concentration of anthocyanins, the fruits that contained the largest amount of total polyphenols and flavanols produced more substantial immune-modulatory effects.

Another study investigating anthocyanins and gut-associated immunity of healthy rats did not find any positive effects upon supplementation of physiological (not pharmaceutical) doses of anthocyanin-rich grape and bilberry juice. Dosages used were 50mg/kg body weight for rats or 15 mg/day (or the equivalent of 8mg/kg body weight for humans), a quantity easily obtained through diet. Anthocyanins are known to have a low bioavailability, with the highest concentrations remaining in the GI tract throughout digestion and absorption (Graf et al., 2013). Even with intestinal concentrations of anthocyanins reaching 570ng/g, over the course of supplementation, there was no influence on T cell and lymphocyte activity or inflammation. This supports other studies that anthocyanins in the intestine do not play an immunomodulatory role and that further investigation into the effects of anthocyanin metabolites is needed (Graf et al., 2013; Wallace, 2011).

6. Future Implications

The future of anthocyanin research is complicated by the nature of human studies research. Specifically, that is using whole foods or nutritional supplements can be difficult to administer in a way that subjects consistently follow procedures. Results are often difficult to directly link to intervention due to the incredibly complicated science of human nutrition. Differences in results of anthocyanin research may also be related to the type of source such as whole blueberries versus isolated supplements. Studies utilizing isolated compounds or nutrients often fail to show significant results compared to studies using the whole food source, and results may be due to synergistic effects of other compounds in blueberries that act in combination with the anthocyanins (Del Bo et al., 2013). In addition, the respective amounts of anthocyanins contained in different supplement types and dietary interventions vary between studies, and this makes meta-analysis and comparison much more difficult (Rodriguez-Mateos et al., 2013).

Another consideration that must be made in regard to research of anthocyanin-induced improvement on inflammation, oxidative stress, vascular function, and immune function lies in the subject pool. Individuals who have compromised or reduced vascular function, such as the elderly or those with specific conditions, may have a lower threshold for improvement due to hypo-functional baseline and therefore produce results of greater significance (Dato, Bellizzi, Rose, & Passarino, 2016; Duggett et al., 2016; Peixoto et al., 2016).

Yet another substantial complicating factor in investigating anthocyanins lies in the active circulation time in the blood. It has been suggested that human studies may fail to show effects of anthocyanins due to the fact that they are absorbed and excreted quickly upon consumption. These types of studies often focus largely on circulating levels (Del Bo et al., 2013). This issue is compounded by the fact that anthocyanins are not highly bioavailable and so exist in the highest concentrations in the intestinal tract (Denev et al., 2014; Graf et al., 2013). Testing shortly after consumption, such as one hour postprandial, has shown significant decreases in oxidative damage as opposed to later measurements of circulating anthocyanins. This poses further implications surrounding the topic, including the comparison of long-term benefits of anthocyanin consumption versus short term benefits.

Because this research is affected by time-response of dosage, study design must be mindful of time lapses in post-supplementation measurement (Bub et al., 2003). It may be the case that anthocyanin metabolites are responsible for immunomodulatory activity rather than the anthocyanins themselves, as is being discovered in the research surrounding vascular performance. Additionally, anthocyanins are absorbed into circulation at a rate of less than 1%. Because of this and the fact that studies achieving high intestinal concentrations have failed to show effects on immune function, future research should shiftto focus on the phenolic acid metabolites of anthocyanins (Graf et al., 2013; Pergola et al., 2006; Stintzing, 2004).

Overall, while animal studies indicate promising results surrounding the relationship of anthocyanins and human biochemistry and physiology, the availability of human studies in this area is limited (Krikorian et al., 2010; Lasekan, 2014; Lau, Shukitt-Hale, & Joseph, 2005; McAnulty et al., 2014; McAnulty et al., 2013). This provides sound rationale for further experiments to be conducted with human subjects to determine if the same results translate from animal models to humans. If this species gap is able to be bridged, an entirely new horizon for application of anthocyanin supplementation in humans could be explored. Anthocyanins and other food-sourced antioxidants may be able to work alongside, and eventually replace, pharmaceutical treatments for chronic lifestyle-related disease. The multitasking and synergistic effects of anthocyanins have the potential to provide compounded benefits to patients, such as functioning as both an immune booster and antioxidant simultaneously, as described previously. Anthocyanins act by reducing the activity of enzymes that perpetuates inflammation. The possibility exists for countless other physiological actions of anthocyanins. This includes, but is not limited to, inhibiting the expression of damaging enzymes as well as further antioxidant activity. Targeting inflammatory responses in this way could tip the balance of oxidative stress by reducing the prevalence of many chronic diseases affecting the population (de Pascual-Teresa, Moreno, & Garcia-Viguera, 2010; Domitrovic, 2011; Faria et al., 2014; Fernandes, Nave, Goncalves, de Freitas, & Mateus, 2012; Singh, Liu, & Ahmad, 2015).

In summary, the bioactive chemicals found in blueberries have been shown to consistently exert multiple beneficial effects in humans. The obvious next series of steps in anthocyanin research shifts to the establishment of recommended intake in humans. Anthocyanin metabolites hold great promise for antioxidant activity and health benefits. The continued testing of blueberry extracts, isolates, and supplements can help to further identify bioactivity of these metabolites and eventually lead to the development of recommended intakes through extensive review of data and consideration of the influence of genomics. Until sufficient data are produced and federal funding is available for an undertaking of this magnitude, anthocyanin intake is encouraged through adhering to the “5-a-day” fruit and vegetable dietary guideline (Williamson & Holst, 2008).

Table 1. Search Methodology

Search Terms	**Search Engines**
Anthocyanins	Science Direct (via ASU library)
Blueberries	PubMed (via ASU library)
Antioxidants	Google Scholar
Polyphenols	
Vascular performance	
Immune function	
Flavanols	
Berry-sourced	
Metabolites	
Oxidative Stress	
Inflammation	

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