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# Cellular uptake and actions of bilberry anthocyanins in retinal pigment epithelial cells

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# CELLULAR UPTAKE AND ACTIONS OF BILBERRY ANTHOCYANINS IN RETINAL PIGMENT EPITHELIAL CELLS

BY

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| | |
|---|---|
| AIF | apoptosis-inducing factor |
| AMD | age-related macular degeneration |
| ANT | adenine nucleotide transporter |
| ARE | antioxidant response elements |
| AREDS | Age-Related Eye Disease Study |
| ATP | adenine tri-phosphate |
| Bax | a pro-apoptotic member of the Bcl-2 family of proteins |
| Bcl-2 | an anti-apoptotic member of the Bcl-2 family of proteins |
| COX | cyclooxygenase |
| DCF | dichlorofluorescein |
| EGCG | epigalocatechin galate |
| EpRE | electrophile response elements |
| FAD | flavin-adenine dinucleotide |
| GSH | glutathione |
| GSPE | grape seed proanthocyanidin extract |
| GST | glutathione-S-transferase |
| GSTP1 | glutathione S-transferase pi class P-1 form |
| $H_2O_2$ | hydrogen peroxide |
| HCL | hydrochloric acid |
| HO-1 | heme oxygenase-1 |
| HPLC | high pressure liquid chromatography |

| | |
|---|---|
| HPLC/ECD | high pressure liquid chromatography with electrochemical detection |
| HPLC/MS/MS | high pressure liquid chromatography with tandem mass spectroscopic detection |
| JNK | c-Jun NH2-terminal kinase |
| Keap1 | Kelch-like-ECH-associated protein 1 |
| LC | liquid chromatography |
| LDL | low density lipoprotein |
| LPH | lactase phloridzin hydrolase |
| LPH | lactase-phlorizin hydrolase |
| MeOH | methanol |
| MPTP | mitochondrial permeability transition pore |
| MTT | a tetrazolium-based colorimetric cell viability assay |
| NF-κB | nuclear transcription factor kappa-B |
| Nrf2 | NF-E2-related transcription factor |
| ORAC | oxygen radical absorbance capacity |
| PBS | phosphate-buffered saline |
| PDA | photo diode array detector |
| PI 3-kinase | phosphoinositide 3-kinase |
| PKC | protein kinase C |
| ROS | reactive oxygen species |
| RPE | retinal pigment epithelium cells |
| RPE-65 | retinal pigment epithelium-specific protein |

| | |
|---|---|
| rt-PCR | quantitative real-time polymerase chain reaction |
| SGULT | sodium-glucose co-transporter |
| tBH | tert-butylhydroperoxide |
| tBH | tert-butyl hydroxide |
| TER | transepithelial electrical resistance |
| TFA | trifluoroacetic acid |
| TFA | trifluoroacetic acid |
| UV | ultraviolet |
| UV-vis | ultraviolet-visible |
| ZO-1 | zonula occludens-1 |

# ABSTRACT

## CELLULAR UPTAKE AND ACTIONS OF BILBERRY ANTHOCYANINS IN RETINAL PIGMENT EPITHELIAL CELLS

by

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Inflammation and oxidative stress play a significant role in the pathogenesis of age-related macular degeneration (AMD). In AMD, retinal pigment epithelium (RPE) cells are damaged by oxidative stress and die via the process of apoptosis. Anthocyanins from fruits and berries, such as bilberry (*Vaccinium myrtillus*), possess significant antioxidant activity *in vitro* and have been used in "traditional medicine" to treat AMD. It is not clear whether intracellular concentrations of anthocyanins are sufficient to quench radical species and mitigate oxidative stress *in vivo*. In this research project, human RPE cells *in vitro* were used to establish an oxidative stress model in which the effects of anthocyanin and phenolics from a bilberry extract could be tested for their antioxidant potential and ability to inhibit hydrogen peroxide–induced apoptosis. High-pressure liquid chromatography with ultraviolet, electrochemical, and mass spectroscopic detection was used to characterize the bilberry extract and to measure uptake, transport, and metabolism in RPE cells. Results suggest that RPE cells internalize and metabolize anthocyanins. Although ineffective in preventing apoptosis, bilberry extract inhibited intracellular radical generation by as much as 60%. Western blot analysis revealed that

physiological concentrations of bilberry anthocyanins up-regulate the oxidative stress protective enzymes heme oxygenase-1 (HO-1) and glutathione S-transferase (GSTP1) proteins in RPE cells by 1.5- to 2-fold over untreated cells in 6 hours and, at pharmacologic doses, up-regulate HO-1 as much as 10-fold over a 24-hour period. Bilberry anthocyanins and phenolics were shown to induced increases in HO-1 and GSTP1 messenger RNA. The observed increases were similar to that observed for protein. Bilberry anthocyanin induction of phase II detoxifying and oxidative stress protective enzymes suggest more significant protective effects than direct radical quenching suggesting these phytochemicals may thus enhance glutathione levels or altered cellular redox states.

# INTRODUCTION

By age 65, approximately one in three elderly persons experiences vision loss due to an age-related eye disease (Ganley and Roberts, 1983). The four most common causes of vision impairment in the elderly include cataracts, age-related macular degeneration (AMD), glaucoma, and diabetic retinopathy (Hyman, 1987; Klaver et al., 1998). While cataract is the most common cause of vision impairment and blindness in the elderly worldwide, AMD is the leading cause of irreversible vision loss in the United States (US).

The inability to defend against oxidative damage is a leading hypothesis as to the possible cause of aging and systemic diseases of aging, including cataract and AMD. Determining whether dietary antioxidants can prevent initiation, progression, or ameliorate the effects of age-related diseases has been an area of extensive research in the last decade. While many epidemiologic, intervention, and mechanistic studies have investigated the effects of antioxidants in eye-related disorders, relatively little attention has been paid to anthocyanins. Anthocyanins are defined as a class of phytochemicals ubiquitous in dietary fruits (especially berries) and vegetables. Bilberry, a member of the *Vaccinium* genus, is rich in anthocyanin and has a long history in traditional folk medicine for the treatment of eye disorders. Reports from Europe suggest improved night vision after use of bilberry supplements (Canter & Ernst, 2004). However, recent eye and nutritional research efforts have overlooked bilberry and other anthocyanins due

to low anthocyanin bioavailability as well as null results from a high-profile study on the visual effects of bilberry conducted by the US Air Force (Muth et al., 2000).

Despite conflicting data from human trials, evidence of the potential health-promoting bioactivity of bilberry continues to mount from animal and *in vitro* mechanistic studies. Mechanistic evidence from *in vitro* studies suggests anthocyanins may protect against age-related disorders including ischemic heart disease, diabetes, inflammation, allergy, angiogenesis, and cancer (Ghosh, 2005). However, the mechanisms by which bilberry may exert protective effects against AMD remain unknown. This research project outlines three hypotheses, both *a priori* and derived:

1) Bilberry anthocyanins can be taken up and transported by retinal pigment epithelium (RPE) cells;
2) Bilberry anthocyanins protect RPE cells from oxidative stress-induced apoptotic cell death;
3) Bilberry anthocyanins induce up-regulation of oxidative stress–protective enzymes, such as heme oxygenase-1 (HO-1) and glutathione S-transferase (GSTP1).

To test these hypotheses, an *in vitro* RPE model was established to study the relationship between dose and time for uptake, transport, and possible metabolism of bilberry anthocyanins. For hypothesis 1, "Transwell" cultures of RPE cells that exhibited complete confluent monolayers with tight junctions were used to determine electrical resistance and for cellular staining and western blot analysis of tight junction proteins

(e.g., zonula occludens-1 (ZO-1)). Transwell cultures not only permitted testing of electrical resistance across an RPE monolayer but also permitted sampling both above and below the monolayer for anthocyanins in media. Chromatographic methodology — high pressure liquid chromatography with electrochemical detection (HPLC/ECD) and high pressure liquid chromatography with tandem mass spectroscopic detection (HPLC/MS/MS) — was developed for application in the task of measuring anthocyanin flavonoids uptake and metabolism.

For hypothesis 2, a stable oxidative stress model of RPE was established. RPE cell cultures were produced in which a reliable apoptotic response could be generated; bilberry extracts and the anthocyanin component of the extract were then tested against the reliable apoptotic responses. It became clear early in the research that establishing this model would not be straightforward. Data suggested that a stable, predictable model of oxidative stress could only be established in non-dividing RPE cells that exhibited markers of RPE differentiation (e.g., expression of retinal pigment epithelium–specific protein (RPE-65)). To monitor oxidative stress–induced apoptosis, mitochondrial function and viability assays, such as MTT (a tetrazolium-based colorimetric cell viability assay) and adenine tri-phosphate (ATP), were selected and validated in RPE. Apoptosis was also monitored using western blot analysis of the mitochondrial apoptosis-related proteins Bcl-2 (an anti-apoptotic member of the Bcl-2 family of proteins) and Bax (a pro-apoptotic member of the Bcl-2 family of proteins). Quantitative real-time polymerase chain reaction (rt-PCR) was used to monitor changes in expression of the genes for these proteins. When it was determined that bilberry extract had no effect on oxidative stress–induced apoptosis in RPE, direct radical quenching properties of bilberry

extract in RPE were examined. Bilberry antioxidant capabilities within RPE cells were monitored using the intracellular dichlorofluorescein assay.

For hypothesis 3, bilberry anthocyanins were separated from other phenolic components within the 25% anthocyanin–enriched bilberry extract used in this project. This separation was accomplished using semi-preparative low-pressure chromatography verified by HPLC\ECD analysis. Here, the goal was to examine the effects of both bilberry extract and its anthocyanin component on up-regulation of the expression of heme oxygenase-1 (HO-1) and glutathione S-transferase (GSTP1). Western blot analysis was again used to monitor changes in protein level, and rt-PCR used to determine up-regulation of the genes.

This research aimed to determine whether bilberry anthocyanins prevent oxidative stress–induced apoptosis in RPE cells. While results indicate that bilberry extract did not prevent apoptosis in this model, bilberry extract diminished intracellular radical levels while increasing levels of HO-1 protein. Here, the third hypothesis was outlined and tested to determine whether bilberry extract and/or the anthocyanin content of this extract up-regulated the HO-1 and GSTP1 genes. If evidence for this relationship existed, bilberry might affect RPE cells in a manner that would influence the etiology or progression of AMD.

# CHAPTER I

# VACCINUM MYRTILLUS: THE BILBERRY

## The Botany of the *Vaccinium* Genus

Bilberry, or European blueberry (*Vaccinium myrtillus L.*), belongs to the family Ericaceae and to the genus *Vaccinium* that comprises some 450 species worldwide. The genus *Vaccinium* includes many economically important cultivated small fruit species, including cranberry (*Vaccinium macrocarpon*) and the domesticated blueberry (*Vaccinium corymbosum*). While bilberry is abundant on alpine heaths and arctic tundra, it is most commonly located in boreal forests and bogs. In these habitats, it is characterized as an ericaceous dwarf shrub in the herbaceous layer of boreal forests (Jäderlund et al., 1998). Bilberry grows over a wide terrain due to insect-pollinated flowers and bird-dispersed seeds. Bilberry, considered a polyploid species, is distributed completely circumpolar and boreal with extension to more southern mountain ranges (Brochmann et al., 2004). *Vaccinium myrtillus L.* grows in acid soils and is nutrient resilient, permitting it to grow in soil conditions ranging from nutrient-sparse mountainous heaths to nutrient-rich soils of forests and ancient peat bogs (Ritchie, 1956). It grows most abundantly in Scandinavia, Eastern Europe, and at higher elevations in Southern Europe. Bilberry and Lingonberry (*Vaccinium vitisidaea L.*), together, represent the most significant number of wild berries in many northern latitudes and alpine areas (Morazzoni & Bombardelli, 1996). Bilberry's habit as a deciduous dwarf

shrub and as a ground cover often permits it to dominate the field layer in boreal coniferous forests, as in Scandinavia (Atlegrim & Sjöberg, 1996; Kardell, 1997).

Bilberry grows to approximately 60 cm and has multiple branched stems, the finer of which are angular and green in color. The leaves alter from oval to elliptical in shape; they are 6–18 mm wide by 10–30 mm long and green with a finely serrulate margin and short petiole. The plant flowers from April to June and produces either white or light green and pink tinted flowers in the leaf axils, which are either solitary or paired. They have short-lobed corolla tubes 3–7 mm in diameter and pink in color, with the ovary located inferior and generally containing 10 stamens. Bilberry fruits are round bluish-black berries measuring 5–10 mm in diameter and containing numerous seeds. Unlike the common blueberry (*Vaccinium corymbosum*), which has a cream-colored berry pulp, the pulp of the Bilberry is purple in color indicating a higher anthocyanin content. Compared to the common blueberry, the bilberry also possesses a generally more pleasing aroma.

## Bilberry as Food and Traditional Medicine

In bilberry habitats, a wide variety of animals — ranging from moose (*Afces alces L.*) and deer (*Capreolus capreolus*) (Cederlund et al., 1980; Morellet & Guibert, 1999) to gray-sided voles (*Clethrionomys rufocanus*) (Hambäck et al., 2002) — feed on bilberry. Bilberry usually produces high seed crops at intervals of 3–4 years. Therefore, vole populations, which feed heavily on bilberry shoots in winter, peak in years following high bilberry seed yielding years. Many birds and insect species populations are also tied to variations in bilberry production. This is because both birds and insect herbivores feed

on bilberry fruits (Atlegrim, 1989; Atlegrim, 1991). The bilberry is, therefore, of central importance in boreal forest ecosystems.

Given its availability, bilberry also serves as an important food source for humans. Bilberries, as with so many other berries (e.g., buckberries, huckleberries, farkleberry, cranberry, whortleberry, and crowberry), have been collected for millennia and either eaten from hand or, in more recent centuries, cooked into tarts, pies, or jams. Evidence of prehistoric human *Vaccinium* berry consumption comes from DNA analysis of the intestinal content of a 5000-year-old Neolithic glacier mummy (Rollo et al., 2002) and from ancient preserved bog bodies (Wood, 2000). Additional evidence exists from prehistoric archaeological sites throughout northern Europe, where carbonized berry seeds have been recovered, as well as in storage pits, where un-carbonized seeds have been sealed from microorganisms and other decay-inducing forces.

Prehistoric evidence cannot determine with certainty whether bilberry was used for the purpose of medicine. Ancient man learned over millennia by trial and error that certain plants were edible and others were poisonous and perhaps lethal. Similarly, early man determined that some plants were useful for treating illness. If these plants served as staple foods prior to their entry into pharmacopeias, they would have continued to serve this purpose even when used medicinally. Perhaps the strongest argument for this belief stems from western medicine's paradigm shift over the past 100 years, in which healthcare has moved toward a pharmacologic view of treating illness. From the time of the writings of Hippocrates (circa 400 BC) to the recent period of "modern medicine", philosophers and physicians made little or no distinction between food and drugs. Indeed, a physician or healer treating a medical condition during this period would often

prescribe not only common herbs but also increased consumption of particular natural foods (Andlauer & Fürst, 2002).

During the Middle Ages in the Roman Empire, the Greek physician/herbalist Dioscorides cataloged the curative properties of plants in a five-book collection entitled *De Materia Medica*. These books on the preparation, properties, and testing of drugs functioned as the preeminent written record of the use of botanicals as medicines and remained the foundation for pharmaceutical and herbal practice until the sixteenth century. However, not until the invention of the printing press did *De Materia Medica* become generally accessible to the European masses. Indeed, the importance of herbs and specific foods in the treatment of illness became apparent by the sheer number of herbals published in the fifteenth century.

Works by the German herbalist Hildegarde von Bingen (1098–1179 C.E.) (Morazzoni & Bombardelli, 1996) reveal that by the twelfth century, bilberry served as a common medicinal. Among the first herbals translated for publication in English was the anonymous *Grete Herbal* (Treveris, 1526). This herbal text prescribed the fruit of the Mirte (bilberry) for the treatment of vomiting, bruising and bleeding, and staunching "flux of the wombe" and "flux of menstrue". Bilberry was also used to treat bad breath stemming from apparent stomach ulcers, and it was commonly used as an aid to combat fever (Tunón et al., 1995). Although its active ingredients were unknown at the time, bilberry's high vitamin C content made it a useful food throughout Europe for the treatment of scurvy. Both the leaves and dried berries were used in a syrupy tea to combat diarrhea and dysentery.

During the 16th century, the German herbalist Hieronymus Bock and others recommended bilberry for the treatment of bladder stones and liver disorders. Bilberries were also used in syrups for treating coughs and lung ailments (Arber, 1938; Harris, 1972). By the 1800s, German physicians and herbalists prescribed bilberry preparations for ailments including intestinal conditions, urinary tract infections, typhoid fever, gout, and rheumatism. Bilberry was also used topically to treat infections of the mouth and skin, and to stop bleeding.

Traditional medicinal use of bilberry was not limited to Europe. Prior to the European influx to the New World, the Native American people of the Kashaya Pomo in Northern California used bilberry as a treatment for diabetes and eye disorders (Goodrish et al., 1980). Centuries of observation by shamans, healers, and priests separated on two continents led to bilberry use in the treatment of surprisingly similar ailments. In those times, medicinal knowledge was transmitted orally and bilberry was considered by native Americans as both food and "healing herb". Its status among the healing herbs implies that prehistoric or "primitive" medical practitioners as well as many physicians in this century considered the plant to have medicinal value.

## Bilberry: the Vision Legend

Modern medical interest in bilberry arose serendipitously after the Second World War. During night bombing missions, British Royal Air Force pilots reportedly experienced an improvement in night vision after eating bilberry jam, according to the legend. However, the British and United States (US) war departments have no records of issuance of bilberry in pilot diets. Undoubtedly, some pilots received jams, a cultural

commodity, from home. Of interest is whether the berries or berry jams provided the benefits that advocates claimed.

In the mid 1960s, bilberry's purported beneficial properties prompted French scientists to conduct the first laboratory and clinical studies on the effects of bilberry fruit extracts on visual function and vascular systems. In recent years, bilberry has been used for the treatment of eye ailments such as cataracts, glaucoma, macular degeneration, poor night vision, and retinopathy. However, the current majority of the medical community considers this dietary/supplement therapy to remain in the realm of alternative medicine.

However, modern claims have been made that bilberry prevents or controls interstitial fluid formation; contributes to controlling blood flow redistribution in the microvascular network; modulates capillary resistance and permeability; improves visual function by promoting dark adaptation after dazzling; promotes wound-healing; and has anti-ulcer and anti-atherosclerotic activity (Morazzoni & Bombardelli, 1996). These claims, and the fact that European doctors continue to prescribe bilberry prompted the European Scientific Cooperative on Phytotherapy to commission a monograph on bilberry for inclusion in the European Scientific Cooperative on Phytotherapy (E/S/C/O/P) Monographs (2003). Bilberry's importance in non-pharmaceutical medicine is highlighted by its inclusion in Pharmacopoeia, including the British Pharmacopoeia, the European Pharmacopoeia, the German Commission E Monographs, the Food Chemical Codex, and the United States Pharmacopeia.

# CHAPTER II

# AGE-RELATED MACULAR DEGENERATION

## Pathophysiology of AMD

Age-related macular degeneration (AMD) is a relatively common degenerative visual disability among the elderly age 50 years and over, affecting over 8 million individuals worldwide (Chopdar et al., 2003). As the name implies, the disease is progressive with age, and it is ultimately diagnosed in one out of every four persons over 80 years. With the present global trend of an aging population, the importance of macular degeneration as a public health problem will undoubtedly increase.

The disorder is diagnosed by loss of vision and the presence of fundal features, the most significant being the presence of drusen (i.e., deposits of extracellular material lying between the retinal pigment epithelium (RPE) and the inner collagenous zone of the Bruch's membrane). Early AMD is characterized by alteration of RPE pigmentation without angiogenesis or visible signs of inflammation. Although drusen are the hallmark of AMD, one or more drusen are found in at least 95% of aged Caucasian populations, with small drusen common in all age groups (McConnell & Silvestri, 2005). Late-stage AMD includes two forms — exudative/neovascular (wet form) or non-exudative/geographic atrophy (dry form) — with an 80:20 ratio observed in the majority of AMD prevalence studies. Exudative AMD is characterized by choroidal neovascularization and the presence of extensive inflammation. At this point in the progression of the disorder, extensive retinal deformation and damage, as well as

extensive drusen, lead to much greater vision loss than experienced in the dry form of AMD.

## The Role of RPE in AMD

Several theories elucidate the role of RPE in AMD. Traditional theories of AMD pathogenesis hold that senescence of the RPE, which metabolically supports and maintains the photoreceptors, leads to AMD (Young, 1987; Eagle, 1984). Some speculate that senescent RPE accumulate metabolic debris as remnants of incomplete degradation of phagocytosed rod and cone membranes; progressive engorgement of these RPE cells leads to drusen formation with subsequent progressive dysfunction of the remaining RPE. This theory explains RPE senescence through the contribution of sunlight-induced photochemical damage, damage by activated forms of oxygen and metabolic radicals, and other potentially damaging mechanisms (e.g., dietary deficiencies). While the "theory of RPE senescence" is attractive, it arose primarily to explain AMD morphology. It thus may be mechanistically close to actuality but does not account for all clinical presentations of AMD. Indeed, other age-related vision disorders present multiple forms of drusen formation, yet all drusen formations do not necessarily lead to AMD.

As noted above, two forms of late-stage AMD exist: There are two forms of AMD; non-exudative AMD or (i.e., "dry" AMD) and exudative AMD or (i.e., "wet" AMD). These pPhysiologic observations of these two forms led to another pathogenesis theory of pathogenesis that, which suggests that primary vascular changes in the choroids are the etiologic events in AMD and that these changes produce secondary effects in RPE

that lead leading to AMD development of AMD. In all likelihood, both theories may apply to AMD, especially if one considers the two AMD forms as representing distinct disorders with as yet to be differentiated pathogenic events. Alternatively, the observable forms of AMD may represent varying degrees of a multi-etiological disorder highly dependent on individual genetic and environmental factors.

It is possible that RPE senescence, choroidal vascular defects, and photoreceptor defects represent primary events in different AMD subsets. In fact, when genetic factors are considered, it is difficult to envision a single etiological explanation for AMD since only 16% of patients with AMD have a known genetic defect in the ABCA4 (ABCR) gene encoding for a retinal rod photoreceptor protein (Allikmets et al., 1997).

In terms of choroidal vascular defects, the choriocapillaris supplies the metabolic needs of the RPE and the outer retina. Perfusion defects in the choriocapillaris serve as the basis for the "vascular pathogenesis theory" of AMD. Studies suggest that delayed choroidal filling may correlate with thickening of Bruch's membrane (Pauleikhoff et al., 1990). Further, delayed choroidal filling and abnormal choroidal blood flow have been reported in both nonexudative and exudative AMD patients. Delayed choroidal filling has also been found to be independently associated with vision loss in 52% of eyes in which it has been detected (Piguet et al., 1992).

Using Doppler imaging, Friedman et al. (1995) measured ocular flow velocities and vessel pulsatilities (a function of the compliance of the vessel wall and the resistance of the capillary bed) in subjects with and without AMD; the investigators found evidence of increased vascular resistance in the choroidal vasculature in AMD patients. As a result of their findings, the authors proposed a hemodynamic AMD pathogenesis, in which lipid

deposition in the Bruch's membrane leads to impaired choroidal perfusion; impaired choroidal perfursion, in turn, adversely affects the metabolic transport function of the RPE. Although choroidal perfusion abnormalities have been associated with AMD, no experimental evidence exists demonstrating that alteration in blood flow causes AMD. While this theory may have relevance to AMD progression, it does not account pathogenically for the initial lipid deposition in sclera and thickening of Bruch's membrane that lead to impaired choroidal perfusion. It is possible that an initial nutrient transport dysfunction in capillary endothelial cells leads to either deficient transport or inappropriate transport of plasma constituents; these defects would then injure RPE resulting in RPE cell dysfunction. Here, events of the senescent RPE theory proceed to produce AMD pathology.

## RPE Death by Apoptosis

Cells eventually die. This is a direct consequence of the way life evolved on this planet. The events and signals triggering cell death, the mechanism(s) by which cell death occurs, and the regulation of cell death constitute one of the fastest growing fields in modern research. To date, we know that cells die by one of two general processes: necrosis or apoptosis. Chaotic and catastrophic, necrosis results from toxic stress or profound cellular damage, and it usually elicits an inflammatory response. Apoptosis, on the other hand, appears orderly and regulated, occurs normally during development, and does not generally elicit inflammation. The latter type of cell death has been observed during retinal development.

The term apoptosis was coined in a now-classic paper by Kerr, Wyllie, and Currie (1972) to describe the form of cell death associated with normal physiological development in which the mitochondria remain relatively unchanged morphologically. Apoptosis, unlike necrosis, involves active participation of the dying cell in its own demise: synthesis of specific messenger RNA (mRNA) encoding "killer proteins", orchestration of an orderly karyorrhexis (nucleus condensation and disassembly), and cytoplasmic fragmentation with relative preservation of organelles. In mammals, phosphatidylserine appears on the outer plasma membrane as cells shrink; the cells then fragment into membrane-bound apoptotic bodies and "label" the dying cell for macrophage-mediated removal. In some cases, apoptotic cells are more easily removed from tissues due to their detachment from surrounding tissue.

In the last half-decade, changes in the physiological patterns of apoptotic cell death have attracted attention as potentially important factors in pathological conditions. Apoptosis has been recognized as an aspect of retinal degeneration and of photoreceptor loss after retinal detachment. It also has been observed after therapeutic irradiation, suggesting that apoptosis may occur after ultraviolet (UV) light–induced damage. Apoptotic death can be triggered by a wide variety of stimuli, and not all cells will necessarily die in response to the same stimulus. Among the most studied is DNA damage induced by irradiation, which in many cells leads to apoptotic death via a pathway dependent on the tumor suppressor protein p53.

Other stimuli — including hormones, caspases, mitochondria, and flavonoids and carotenoids — have also been linked with apoptotic death. Some hormones (e.g., corticosteroids) lead to apoptotic death in particular cells (e.g., thymocytes) while

stimulating necessary physiological functions in other cell types. Some cell types express Fas, a surface protein which initiates an intracellular death signal in response to cross linking. In other cells, a survival factor actively blocks cell surface receptors that appear to be linked to a default death pathway. Here, when the survival factor is removed, the default apoptotic death program is triggered and the cell goes into apoptosis.

Apoptotic death also involves activation of caspases. Caspases are cysteine proteases that have a high degree of homology with ced-3, the product of the so-called "death gene" of the nematode *Caenorhabditis elegans*. In most cells, caspases are expressed in an inactive proenzyme form. They are unusual among proteases in that their proteolytic activity is directed toward aspartic acid residues, with individual specificities determined by the recognition of neighboring amino acids. Upon activation, many caspases can activate other pro-caspases, resulting in a protease cascade. The importance of proteases to apoptosis and normal development is reinforced by the fact that knockout mice lacking caspase-3, -8, or -9 fail to complete normal embryonic development.

Activation of caspases appears to trigger a cell's apoptotic death. Aggregation of some pro-caspases (those with large pro-domains) allows them to "auto activate". Much evidence suggests that mitochondria are involved in one major pathway involving activation of pro-caspase-9 (Hengartner, 2000). Other research shows ligands that cross-link death receptors, such as Fas, trigger formation of a cytoplasmic complex in which pro-caspase-8 is aggregated and activated (Budihardjo et al., 1999). In both cases, these initiator caspases activate the previously mentioned cascade of other pro-caspases, leading eventually to apoptotic cell death. As the study of apoptosis progresses, it is

becoming clear that the molecular pathways leading to apoptotic cell death are indeed complex.

Mitochondria also play critical roles in apoptosis. This is not surprising since apoptosis is a eukaryotic process and is regulated by highly conserved pathways involving ancient cell death activators and inhibitors, namely EGL-1 and BH3-domain only proteins; CED-9 and Bcl-2; CED-4 and Apaf-1; and CED-3 and caspases, in nematodes and mammals, respectively. Apoptotic degradation of chromosomal DNA involves a mitochondrial endonuclease (endonuclease G (EndoG)) in mammals. EndoG is a mitochondrial pro-apoptotic factor, as are cytochrome c, apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI (Smac/DIABLO), and Omi/HtrA2 (Gulbins et al., 2003). These pro-apoptotic factors are liberated to the cytosol during mammalian apoptotic progression. These factors are either caspase-dependent (cytochrome c and Smac/DIABLO) or caspase-independent (AIF and EndoG).

Abundant evidence supports a role for mitochondria in regulating apoptosis. Specifically, a number of death triggers may target these organelles and stimulate, by an unknown mechanism, the release of several proteins, including cytochrome c. Once released from mitochondria into the cytosol, cytochrome c binds to its adaptor molecule, apoptotic protease activating factor-1, which oligomerizes and then activates pro-caspase-9. Caspase-9, in turn, signals downstream and activates pro-caspase-3 and -7. The release of cytochrome c can be influenced by different Bcl-2 family member proteins, including Bax, Bid, Bcl-2, and Bcl-X(L). Bax and Bid potentiate cytochrome c release, whereas Bcl-2 and Bcl-X(L) antagonize this event.

AIF may be a particularly important mitochondrial contribution to apoptosis. When released from mitochondria, it is translocated to both the cytosol and the cell nucleus. Within the mitochondria, AIF is a flavin-adenine dinucleotide (FAD)–binding oxidoreductase essential to operation of the electron transport chain and reduction of cytochrome c. When released, however, neither AIF's FAD-binding ability nor its oxidoreductase activity is required for apoptotic activity. In the nucleus, AIF induces condensation and fragmentation of chromatin. Exactly how AIF induces DNA fragmentation or apoptosis is unclear and is an area of current study. However, current evidence suggests that AIF translocation to the nucleus remains a general feature of apoptosis in mammalian cells. Once in proximity to DNA, AIF binds to DNA and induces chromatin condensation; however, the complete process of DNA fragmentation is still not clear. It has been postulated that AIF may have some nuclease activity, that AIF binding to DNA renders DNA more susceptible to latent nucleases, or that, once AIF binds to DNA, nucleases are recruited to induce partial chromatinolysis.

AIF also can act as a caspase-independent death effector, driving a cell to apoptosis. However, the process appears to be complex since crosstalk takes place between AIF and the caspase cascade on multiple levels. Studies have shown apoptosis initiation by activation of caspase-8 or caspase-2, where release of AIF occurs as a subsequent event. In other cases, AIF release precedes caspase initiation and triggers the release of cytochrome c from mitochondria for cytochrome c–dependent caspase activation cascade. Conversely, studies have revealed that mitochondrial release of AIF can occur well after cytochrome c (Arnoult et al., 2002). These findings support the

concept that different modes of mitochondrial membrane permeability may control apoptosis in different cells or in response to different stimuli.

Finally, flavonoids and carotenoids affect apoptosis (Watson et al., 2000). As research progresses, data support the notion that apoptosis functions as a normal part of natural biological processes as well as a part of disease pathology, including cancer, heart disease, and AMD. However, little is known regarding an association between dietary flavonoids or carotenoids and apoptosis. Evidence does exist, though, that links diet and tumor development in cancer initiation and progression. Indeed, lycopene, lutein, and β-carotene (but not α-carotene) have been shown to inhibit the development of aberrant colonic crypt foci induced by N-methylnitrosourea in Sprague-Dawley rats. This implies involvement of anticancer mechanisms apart from the transactivation of the retinoic acid responsive promoter of retinoic acid receptor beta2 (RAR-beta2). Furthermore, since β-carotene oxidizes much more readily than other carotenoids, simple free radical scavenging appears to be less important.

Since the realization that flavonoid-rich foods (e.g., tea) are associated with decreased incidence of cancer risk (Le Marchand, 2002), more research has been devoted to these phytochemicals. In the last two decades, *in vitro* biological effects have been observed in flavonoids, including free-radical scavenging; modulation of enzymatic activity; inhibition of cellular proliferation; and antibiotic, antiallergic, antidiarrheal, antiulcer, and anti-inflammatory properties (Middleton et al., 1982; Moon et al., 2006). Among the flavonoid classes, flavones, flavonols, flavanones, and isoflavonones have demonstrated anti-proliferative activity in the absence of cell cytotoxicity; however, anthocyanins have not been tested *per se*, and no notable structure-activity relationships

have been noted on the basis of subclass. Yang et al. (1997) have shown that tea polyphenols inhibit growth and induce apoptosis in human cancer cell lines. Wenzel et al. (2005) evaluated the effect of flavones on the expression of cell cycle and apoptosis-related genes in a human colon cancer cell line and reported dramatic changes in mRNA levels of cyclo-oxygenase-2, nuclear transcription factor kappa B (NF-κB), and Bcl-X. These effects were, however, highly selective for apoptosis in the transformed cells.

This evidence implies a potential mechanism for the anticancer effects of flavonoids; however, it also suggests flavonoids may impact genomic events very differently in healthy cells compared to transformed cells. Indeed, one of the few studies testing the effects of anthocyanins on apoptosis found that *Ginkgo biloba*, a complex proanthocyanidin mixture, inhibits apoptosis (Ni et al., 1996). While the mechanism was undetermined, the authors attributed the results to the antioxidant properties of the mixture. Regardless of the mechanistic explanation, it is evident that speculation concerning the possible anti-apoptotic roles of the active constituents of *Ginkgo biloba* requires confirmation.

Since apoptosis is intimately linked to oxidative stress, and since oxidative stress appears to be a major process involved in AMD pathology, the marketers of EGb 761 (a commercial extract of *Ginkgo biloba*) are funding research into its possible beneficial effects in treating diseases whose pathogenesis may involve oxidative stress and apoptosis (Maclennan et al., 2002). Additional studies have determined that the mechanism of action of EGb 761 on cell survival may be due to the prevention of mitochondrial damage, attenuating release of cytochrome c and DNA fragmentation (Eckertet al., 2003). Further, DNA microarray assay results indicate that transcription of

multiple apoptosis-related genes is either up- or down-regulated in cells treated with EGb 761; this suggests that inhibition of apoptotic machinery by this complex mixture is mediated via several apoptotic pathways (Smith et al., 2002). Still more recent research indicates that *Ginkgo biloba* effectively inhibits chemically induced apoptosis, but it does not modulate the activities of endogenous antioxidant enzymes. While anthocyanins are present in this mixture, these effects are more likely due to the ginkolides that represent a much higher proportion of the mixture (Altiok et al. 2006).

Other flavonoids, specifically those in tea, inhibit NF-κB, a complex of proteins that binds to DNA and activates gene transcription. Here, the flavonoids prevent phosphorylation of the inhibitory protein, thereby inhibiting release of NF-κB from its bound and inactive form in the cytoplasm. Consequently, NF-κB fails to translocate to the nucleus and bind to DNA. This "blockade" activates the transcription of multiple inflammatory genes and is the putative mechanism whereby flavonoids from tea play important roles in the etiology of diseases with inflammation components, including cardiovascular disease and cancer. However, NF-κB inhibition could block the downstream expression of survival factors and this could be a downside of flavonoid action in preventing apoptosis.

Flavonoids also exhibit more or less potent and selective effects on some signaling enzymes. Among the identified signal transducers, both phosphoinositide 3-kinase (PI 3-kinase) and protein kinase C (PKC) are considered components in many cellular responses, including cell multiplication, apoptosis, and transformation (Gamet-Payrastre et al., 1999). Despite their lack of specificity, some flavonoids provide

valuable bases for design of analogues intended to block particular isoforms of PI 3-kinase or PKC and their downstream-dependent cellular responses (Stoclet et al., 2004).

Finally, some evidence suggests that flavonoids may play a role in inducing apoptosis in cancer cells. Unlike normal cells, cancer cells lose the ability to undergo programmed cell death (i.e., apoptosis). Flavonoids have been found to induce apoptosis in cancer cells *in vivo*; however, it is unclear whether they induce apoptosis in pre-cancerous cells or cancer cells in living organisms. For most cells, once they divide, they pass through the cell cycle before they divide again. However, cells *in vivo* have a limited number of cell cycles before they no longer divide and some cells (e.g., nerve cells) having undergone cell cycle arrest will never divide again. In a seminal paper in this field, Bodnar et al. (1998) linked the inability to continue to divide with cell senescence and demonstrated that telomere loss in the absence of telomerase is the intrinsic timing mechanism that controls the number of cell divisions prior to senescence. Here, exogenous stimulation of the induction of telomerase expression contributed to telomere maintenance and cellular lifespan. This prevented cellular senescence and some age-related cellular decline, which contributed to AMD (caused by accumulation of lipofuscin and down-regulation of a neuronal survival factor in RPE) as well as atherosclerosis (caused by loss of proliferative capacity and over expression of hypertensive and thrombotic factors in endothelial cells).

Unlike normal cells, cancer cells divide and grow rapidly. However, flavonoids, such as the catechins, appear to induce cell cycle arrest, thereby preventing cancer cells from continuing to divide and proliferate in a manner akin to certain tumor suppressor proteins (Manson, 2005; Sah et al., 2004). Of note, flavonoids do not appear to have

lethal or even deleterious effects on normal cells during development or differentiation; this suggests fundamental differences may exist between cancer and normal, non-cancer cells with respect to flavonoid influence on apoptosis and cell signaling. These differences have yet to be fully explored.

## Anthocyanins and Apoptosis

Acetaminophen (AAP) induces liver injury leading to apoptosis. In one of the few polyphenolic-related *in vivo* apoptosis studies conducted, mice were placed on short- or long-term grape seed proanthocyanidin extract (GSPE) feeding prior to induction of liver apoptosis (Bagchi et al., 1998). Studies have demonstrated that during digestion proanthocyanidins are converted to anthocyanins and absorbed (Deprez et al., 2000). Since it was demonstrated that hepatocytes do not express Bcl-2, other apoptosis-regulating genes, such as the anti-apoptotic gene, Bcl-xL, were investigated. AAP metabolism triggers production of reactive oxygen species (ROS), which when coupled with disturbances in cellular calcium homeostasis, leads to oxidative stress and ultimately to down-regulation and modification of Bcl-xL expression that results in apoptotic death. Western blot analysis of the level of expression of Bcl-xL revealed that GSPE alone significantly enhanced Bcl-xL expression compared to control and completely prevented the effects of AAP, while AAP-treated mice poorly expressed Bcl-xL, indicating diminished anti-apoptotic power. Although experiments have not been performed to test the hypothesis, the authors felt that the pattern of subunit expression and Bcl-xL reflects the inactivation of Bcl-xL by phosphorylation. This assumption is entirely plausible, given that flavonoids inhibit some kinases (Bagchi et al., 2002, Ray et al., 2004).

In contrast, in a recent study on the effects of ethanol extracts of 10 edible berries, bilberry extract induced apoptotic cell bodies and nucleosomal DNA fragmentation in HL60 human leukemia cells (Katsube et al., 2003). Pure delphinidin and malvidin, like the glycosides isolated from the bilberry extract, also induced apoptosis in HL60 cells. These findings indicate that anthocyanins can play a protective role against apoptosis in initially healthy cells and a deleterious role in cancer cells. However, major gaps in our understanding of apoptosis regulation — including the chain of events connecting intracellular calcium dysregulation, ROS production, oxidative stress, perturbation in cellular energy status, DNA fragmentation, poly(ADP-ribose)polymerase activation, and select expression of death retarding genes (e.g., Bcl-2 or Bcl-xL) — limit identification of the mechanisms by and extent to which anthocyanins regulate apoptosis. Indeed, anthocyanins may play a role in all of these elements; however, studies to evaluate these possible interactions are only just beginning. A cause-and-effect relationship between oxidative stress, apoptosis, and expression of Bcl-xL appears likely.

Induction of apoptosis in endothelial cells has been shown to occur by at least two pathways exhibiting differential sensitivity to the anti-apoptotic protein Bcl-2. The first pathway involves activation by transduction signals originating from the tumor necrosis factor (TNF) receptor family; this activates upstream caspases (-6, -8, -9), which in turn activate pro-caspase-3 and which have been shown to be Bcl-2 insensitive. The second pathway involves signals originating from numerous other stimuli (including ultraviolet-B (UVB) irradiation), which are potently inhibited by Bcl-2. Endothelial cells are relatively apoptotic resistant in another manner that separates them from other cells. They are protected by anti-apoptotic proteins, such as the family of viral inhibitors of

death receptor-mediated cell death (specifically, FADD-like IL1ß–converting enzymes (FLICE) and caspase-8 inhibitory proteins, which interfere with the Fas-induced apoptotic pathways) (Sata & Walsh, 1998). No studies have investigated the role of Bcl-2, Bcl-xL, or FLICE in RPE. The cellular and molecular mechanisms underlying retinal cell death in AMD remain poorly understood other than the recognition that apoptosis plays a significant role.

Anthocyanins have the potential to positively influence expression of the anti-apoptotic genes, particularly Bcl-2 and Bcl-xL, which can have significant downstream effects in preventing mitochondrial release of cytochrome c and AIF. Thus, anthocyanins may have effects on kinases, including isoforms of PI 3-kinase or PKC, that could have beneficial effects in preventing transglutamase activation and activation of the caspase-activated DNase (CAD) pathway or in preventing endonuclease activation, which affect DNA fragmentation. It is yet unclear what effect NF-κB activation will have on healthy RPE. It may turn out that anthocyanins will in effect "push" compromised RPE cells toward apoptosis.

While the preceding discussion suggests that flavonoids, including anthocyanins, may possess specific properties that could benefit human health, the experimental *in vivo* and *in vitro* data have produced conflicting results. Data from epidemiological studies regarding flavonoids in human health are also far from convincing. More studies at all levels are needed to characterize both the potential health benefits and potential harmful attributes of individual flavonoids. It is possible that the sum of the parts (e.g., total fruit and vegetable intake) is more important in providing a health benefit to humans than any particular plant phytochemical.

## Oxidative Stress, Redox Biology, Aging and AMD

Evolution has produced a fascinating paradox that exists in the biochemistry of all existing aerobic organisms. The emergence of photosynthesis three and a half billion years ago, along with cyanobacterial dominance over the preceding billion years, fundamentally changed the earth's atmosphere from a reducing environment to an oxidizing environment through the atomospheric introduction of oxygen. Beginning in the middle proterozoic period, oxygen build-up in the atmosphere led to widespread bacterial extinction while driving evolution of antioxidant defensive biochemical pathways in the bacterial survivors and eukaryotic life forms, including multi-cellular algae (Kaufman et al., 1997; Knoll, 1991; Rye et al., 1995). By the late proterozoic period, the first animals evolved. Rapid evolution was possible by the availability of cellular energy in excess of fundamental needs, a condition set in motion by the symbiotic relationship that lead to mitochondria and oxidative phosphorylation in eukaryotes.

This biochemical paradox in aerobic organism biochemistry represents a complex series of tradeoffs that leave an organism with survival advantages and disadvantages. The net result provides organisms with a survival advantage in their *in situ* environment (Lang et al., 1999; Searcy, 2003; Vellai & Vida, 1999). However, the tradeoff in producing cellular energy (ATP) by mitochondria via oxidative phosphorylation is the production of toxic ROS (Barja, 1998; 1999; Lennaz et al., 1999). ROS are defined as free radical species that, if not well controlled by cellular processes, can react with and/or modify or damage other critical cellular molecules (Cutler & Rodriguez, 2003). On the

other hand ROS can be useful to organisms as, later in evolutionary history, animals evolved strategies to incorporate ROS in immune defense mechanisms, as secondary messengers, and in redox signaling. Thus, we see the paradoxical result that animals cannot live without oxygen, yet oxygen leads to eventual demise.

For the past decade, one theory of aging has suggested that aging ensues from the damaging effects of life's normal, essential processes (Rose & Finch, 1994). This proposal — that free radical–induced damage leads to the aging process — implies the process is not evolutionarily driven but instead is a process and consequence of the natural laws of increasing entropy and chemical stability. The "theory of longevity" (defined as duration of life), on the other hand, maintains that a species survival advantage for longer life drives the evolution of anti-aging or longevity processes. These processes may include increased resistance to or protection from biomolecular radical damage. Alternative longevity processes include enhanced repair processes. Indeed, when comparing human centenarians with other age classes, studies show an association between longevity and a phenotype characterized by enhanced redox defense status, including high glutathione reductase activity and higher levels of reduced glutathione (Andersen et al., 1998; Lang, 2001; Marcotte & Wang, 2001).

The major source of ROS in eukaryotic cells is the mitochondria, where ROS are the byproducts of oxidative phosphorylation reactions (Sohal, Sohal & Orr, 1995). In more highly evolved animals, another important ROS source is the production of byproducts of the "oxidative burst" process performed by macrophage cells in the course of their destruction of foreign organisms (Libby & Ridker, 2004). Perhaps more significant, cellular damage pertinent to the aging process originates from radicals

produced by chronic, low-grade inflammation. This appears to be an exacerbating component of many age-related related diseases (Finch & Crimmins, 2004).

ROS are, in most cases, tightly controlled within living systems; however, this control is not perfect and the occasional stray radicals cause inevitable "collateral" damage to critical cellular components. To prevent organism dysfunction, endogenous defense and repair systems evolved, particularly for nuclear DNA and mitochondrial DNA. With time, however, un-repaired damage accumulates throughout the entire cell, with the damage build-up resulting in loss of functional capacity of most physiological processes. This gradual loss of functionality has been referred to as the "wear-and-tear oxidative stress hypothesis of aging" or the "free radical theory of aging". Theories postulate that loss of functionality leads to increasing cellular/organism dysfunction on many levels and eventual system failure (Beckman & Ames, 1998; Harman, 1998).

The concept that free radical–mediated damage accumulates in older organisms and leads to dysfunction (Finkel & Holbrook, 2000) has given rise to the idea that removal of damaged components might restore function (Gray et al., 2004). Another concept of free radical–induced aging includes the dysdifferentiation hypothesis of aging (Cutler, 1991). This theory postulates that low levels of free radicals exert their most devastating effects on functionality and progression of the aging process by damaging sensitive and critical processes within a cell, thereby controlling and maintaining the proper state of cell differentiation. Free radicals cause changes in regulatory proteins, signal transduction pathways, transcription factors, and even the structure of chromatin protein, resulting in altered transcription profiles. The changes can produce stable but less efficient or perhaps improperly differentiated cellular states (Sohal & Allen, 1985).

Further, recent evidence supports the important role of these epigenetic processes, in addition to or in place of mutational events, in the etiology of aberrant disease states, including cancer (Sutherland & Costa, 2003). In this light, one could conceive of cancer increasing in prevalence with increasing age; increased cancer prevalence would arise from the general aging process, where dysdifferentiation would involve genes (particularly oncogenes) controlling cellular proliferation and apoptotic ability.

Oxidative stress, or the imbalance between free radical production and adequate protective control leading to radical-induced damage, perturbs the cell's differentiation state through epigenetic mechanisms. Researchers already know that a cell's oxidative stress state or oxidative/reductive status plays a role in those processes that control cell growth, development, signaling, and state of differentiation (Schafer & Buettner, 2001). Stem cell research has demonstrated that general tissue maintenance depends on the existence of particular stem cells specific for each tissue type (Reya et al., 2001). Over time, due to epigenetic drift, stem cells gradually lose their special state of differentiation, and the tissues dependent on their renewal become increasingly defective (Tzukerman et al., 2002). Epigenetic alterations in stem cells also occur by free radical mechanisms. Thus, oxidative stress may impact an organism's aging process by affecting the stem cell tissue renewal system in a manner that results in altered or "aged" tissue.

Longevity can be interpreted as a measure of the ability of an individual or a species to stave off the debilitating effects of age-related disorders, which lead to organ or tissue dysfunction and death. While it is not true of all species, notably nematodes and drosophila, the longevity of mammalian species is dependent upon stem cell renewal maintenance. Therefore, it might be expected that the ability of a species to protect stem

cells from free radical–mediated dysdifferentiation would correlate with longevity. This line of reasoning led to the concept of longevity determinant genes (Calabrese et al., 2006; Cutler, 1991; Vijg & Suh, 2005). A positive correlation exists between the tissue concentration of specific antioxidants and mammalian lifespan (Borrás et al., 2003; Sohal, Sohal & Brunk, 1990). Furthermore, markers of oxidative damage, such as oxidative damage to DNA and tissue resistance to spontaneous autoxidation, are inversely correlated with lifespan in mammals. Compelling data also indicate that disease (more generally) and age-related disease (in particular), as well as the aging rate, are all associated with the general intensity of oxidative stress occurring within an organism (Cutter, 1992). These data suggest that radical damage may play a causative role in aging and that the antioxidant status, whether determined by endogenous or exogenous factors, could be important in determining the risk of age-dependent diseases.

During the conversion of earth's atmosphere from a reducing environment to an oxidizing one, and during the evolution of oxidative phosphorylation, organism survival depended on the co-evolution of processes to reduce the destructive effects of ROS. A number of such protective/defense processes have been identified (Fridovich, 1989; Sohal & Weindruch, 1996; Sundquist & Fahey, 1989). Phylogenic evidence indicates that organisms with more efficient mitochondria and reduced metabolic rates enjoy a longer lifespan (Criscuolo et al., 2005; Papa, 1996). However, mammals, such as humans, that fall outside the correlative curve between size and lifespan appear to have higher tissue levels of antioxidant protection (Barja, 1998).

Many studies show that increases in tissue antioxidants — through dietary supplementation, antioxidant induction, or gene transfection — can increase survival or

mean lifespan (Cutler, 1991b) but not maximum potential lifespan. Recently, both caloric restriction and genetic manipulations in the nematode *C. elegans* resulted in lifespan extension up to 500% (Houthoofd et al., 2004; Vanfleteren & Braeckman, 1999). As organisms evolved, increased resistance to membrane peroxidizability and to structural oxidative damage afforded increased lifespans. For example, this phenomenon is most evident in the evolutionary trend toward decreases in peroxidizable fatty acid molecules, such as the unsaturated fatty acids, in cellular membranes. Once radical production decreases and molecular targets "harden", further protection occurs by "quenching" (i.e., removing) radicals using antioxidants (Sundquist & Fahey, 1989). The classic definition of antioxidants defines them as molecules that react more readily with free radicals than with critical cellular components. Such classic antioxidant molecules (e.g., α-tocopherol, ascorbate, urate, and glutathione ) are thus "sacrificed" in the act of removing radicals and in so doing, provide the cell with less reactive and less lethal alternative radicals. These antioxidants are either synthesized endogenously or taken in as dietary components.

If these "quenching and removal" mechanisms fail and damage is inflicted on critical cellular components, elaborate repair systems have evolved to repair cellular nucleic acid, protein, and lipid components. Repair mechanisms, such as DNA excision repair, are highly specific in recognizing oxidative damage and in repairing damage, thereby restoring molecular function (Lombard et al., 2005). In phylogeny, lifespans are longer where these repair systems are more active (Promislow, 1994). As a last resort, cells damaged by oxidative mechanisms are removed, and normal function is restored via general tissue renewal and remodeling processes (i.e., cell division).

Several definitions of oxidative stress exist. Sies (1997) described oxidative stress as a level of oxidatively mediated damage that is above a cell's or an organism's normal level. This definition implies that oxidative damage to cellular components occurs at a given rate appropriate for a particular organ or organism and that repair and removal mechanisms within that organ or organism cope with the damage to maintain normal function. When those mechanisms fail to cope adequately, a state of oxidative stress exists. Others have defined oxidative stress as an absolute level of oxidative stress rather than as a measure of the flux of damage; here, oxidative stress occurs when any damage is done (Cutler, 1992). The debate over these two definitions may be academic, as the crux of the matter lies perhaps more in the fate of the damaged molecules rather than in whether the damaging radicals originate under a state of redox balance or redox imbalance. If a damaged molecule is repaired or removed and replaced adequately, normal function is restored. If a damaged molecule cannot be repaired or removed, then dysfunction is likely and the processes of aging and disease ensues.

While oxidative stress is *associated* with most human diseases (Halliwell & Gutteridge, 1999), evidence does not suggest that oxidative stress *causes* most diseases. In many cases, increased free radical levels and/or increased oxidative stress are secondary to the disease process. Increased free radicals and oxidative stress exist in many age-related diseases, including diabetes, cancer, cardiovascular, pulmonary, and neurological diseases, such as Alzheimer's and Parkinson's disease. Additionally, free radicals are associated with inflammatory processes (e.g., arthritis). Reduction in both cardiovascular disease and cancer risk are associated with diets rich in fruits and vegetables (McDermott, 2000). While antioxidant phytochemicals present in these foods

may be responsible for this protective effect, proving the underlying mechanisms of these protective effects as antioxidant effects has been difficult. This lack of convincing and overwhelming evidence has led to a controversy about the use of antioxidant supplementation as therapy for age-related disorders (Heinecke, 2001; Witzum, 2000).

Increased oxidative stress likely plays an important role in AMD pathogenesis (Beatty et al., 2000). The retina is particularly susceptible to oxidative stress for several reasons. Retinal oxygen consumption is high compared to oxygen consumption in other tissue (Yu & Cringle, 2005). The retina is exposed to high levels of cumulative irradiation (Glickman, 2002; Godley et al., 2005; Young, 1988), and photoreceptor outer segment membranes in the retina are high in polyunsaturated fatty acid content. When oxidation events occurs, these lipids are easily oxidized leading to initiation of lipid peroxidation chain-reactions (SanGiovanni & Chew, 2005). Furthermore, as neurosensory retinal tissues and the RPE age, lipofuscin is deposited even in normally functioning eyes. These compounds exhibit significant photoreactivity, and in high oxygen partial pressure environments form potentially cytotoxic end products (Rozanowska et al., 1995). Among the more overlooked ROS-generating mechanisms in the eye is the process of phagocytosis. Within the retina, the RPE removes debris and spent metabolites from photoreceptor outer segments (Miceli et al., 1994; Tate et al., 1995).

Moreover, the RPE is exposed to high levels of radicals produced by the cones and rods as byproducts of the visual process. For example, studies in canine eyes showed remarkable compartmentalization of enzymes involved in the production or degradation of peroxides in the eye. Whereas the retina seems well-protected against superoxide free

radicals and hydrogen peroxide by virtue of the presence of superoxide dismutase, peroxidases, and catalases, the rod outer segments are only protected by superoxide dismutase (Armstrong et al., 1981). This lack of protection implies that, after phagocytosis of rod outer segments, any peroxidized lipids, organic peroxides, or unconverted superoxide radicals contained within the rod outer segments must be detoxified by a specific peroxidase in the RPE.

Evidence from trials and meta-analyses suggests that the antioxidant vitamin E, used alone, does not have a protective effect against AMD; however, *in vitro* and animal studies provide evidence that a combination of vitamin E and vitamin C can protect the retina against photochemical damage. Two xanthophylls with antioxidant properties, lutein and zeaxanthin, are concentrated as macular pigment in the fovea of the retina and are thought to protect the retina from oxidative damage by filtering out short wavelength light. As in the case of cardiovascular disease, results from AMD risk–related observational studies of antioxidant intake or antioxidant blood levels have been inconsistent (Delcourt et al., 1999; Seddon et al.,1995; Smith et al., 1999; VandenLangenberg et al., 1998; West et al.,1994). Over the past decade, several randomized controlled trials have been conducted to explore uncertainty about the role of antioxidants.

## Epidemiology and Intervention Studies in AMD

**Antioxidant Effects on AMD.** Epidemiologic evidence suggests a genetic basis for AMD (Heiba et al., 1994). People of young age with macular diseases have mutations also found in AMD-susceptible populations (Allikmets et al., 1997); however, it is

unknown whether these mutations cause AMD or merely contribute to AMD susceptibility. Several studies of risk factors associated with cardiovascular disease and AMD found common associations but these findings are inconsistent (Hyman et al., 1983; Vingerling et al., 1995). As with cardiovascular disease, cigarette smoking is associated with higher risk of AMD development and progression (Smith et al., 2001), which suggests that both oxidative stress and inflammation play a significant role in the disease. Other epidemiological evidence reveals an association between sunlight exposure and AMD development (Taylor et al., 1992), but not all studies have found this relationship (Mitchell et al., 1998). These findings combined with pathophysiologic changes in the RPE have led investigators to conclude that RPE failure to repair oxidative damage may play a role in AMD etiology.

In the past decade, mounting evidence suggests that diet and/or dietary supplements may prevent the onset and progression of both AMD and cataracts. To investigate this possibility, case-control and cross-sectional studies have been undertaken to determine associations between AMD and antioxidant vitamin intake or plasma concentrations (Delcourt et al., 1999; West et al., 1994). Results have been inconclusive and contradictory. These inconsistent and disconcordant conclusions have been attributed to methodological issues, inadequacies in dietary ascertainment, and/or biochemical measurements of antioxidants.

Two nutritional studies, a population-based case-control study of serum antioxidant levels and AMD (Mares et al., 1995) and the multi-center Eye Disease Case-Control Study (Seddon et al., 1995) support the theory that increased intake of dietary antioxidants, specifically carotenoids, reduces advanced AMD risk. However, findings

from these studies regarding vitamin intake other than carotenoids were less consistent. Data suggest an inverse association between vitamin A intake and AMD risk. However, this association appears to result from the carotenoid component, since a beneficial effect for high vitamin C intake, particularly through foods, cannot be ruled out. Interestingly, study data offer no evidence of a vitamin E protective effect; in fact, overall results are in the opposite direction. Nevertheless, these epidemiologic observations indicate that nutrition may play a role in macular degeneration.

Despite this evidence, speculation persists that the antioxidants vitamin C and vitamin E as well as the carotenoids, especially lutein and zeaxanthin, may have a protective effect against AMD by limiting oxidative damage. These speculations motivated design of the Age-Related Eye Disease Study (AREDS), in which the value of long-term dietary antioxidant supplementation for severe AMD development was investigated. AREDS results provide some evidence that antioxidants, vitamins, and minerals may prevent or treat AMD. Results also indicate a protective effect of antioxidant supplements; here, antioxidant supplements prevented either progression of moderately advanced dry macular degeneration cases or vision loss in individuals with unilateral wet macular degeneration (Age-Related Eye Disease Study Research Group, 2001).

As previously mentioned, prior epidemiologic studies suggest antioxidants reduce the risk of eye diseases (Taylor et al., 1992); however, results from a small clinical trial indicate that zinc may delay AMD development and progression (Newsome et al., 1988). Evidence from these two studies, combined with lack of AMD treatments as well as with enthusiastic commercial supplements marketing, led to increased use of high-dose

antioxidant vitamins and zinc for self-medicated AMD prevention/treatment. The efficacy and safety of such a "nutritional intervention" was unknown; therefore, AREDS offered an opportunity to assess these factors in a controlled clinical trial.

Because AMD and cataract are chronic aging–related diseases with slow progression rates, AREDS was designed as a large and lengthy study in order to test the effect of nutritional supplementation on these conditions and other eye disorders. However, because lutein and zeaxanthin were unavailable commercially, AREDS made a controversial decision by using the antioxidant carotenoid β-carotene in the study. While seemingly not an optimal choice, it was a rational one in that pharmaceutical companies were already marketing β-carotene supplements, it was already used in heart disease and cancer clinical trials, and it was commercially available. Vitamins C and E were also included in the study as known antioxidants.

While the literature revealed zinc's protective effect against AMD, no evidence existed regarding its usefulness with regard to cataracts. Therefore, researchers tested zinc only with regard to AMD. Further, zinc supplementation was tested only in those study subjects who manifested risk of AMD-related vision loss as determined by evidence of drusen or RPE anomalies.

Eligible participants included individuals aged 55- to 80-years-old and free of illness, such as cancer or cardiovascular disease (CVD), at enrollment. Participants were enrolled in one of five AMD categories based on fundus photograph grading (i.e., the central reading center) of drusen size, number, and area on corrected visual acuity (defined as less than 20/32 and either due to AMD or not); participants were also categorized based on ophthalmologic evaluations. Categories are defined in Table 1 of

the AREDS report no. 8 (Age-Related Eye Disease Study Research Group, 2001a). Other health measures also were collected on the state of eye health.

AREDS study outcome variables included (1) change in visual acuity and (2) change in AMD status or lens opacities. The relationship between antioxidant and no antioxidant groups, as well as between zinc and no zinc groups for visual acuity or AMD progression was tested. For cataracts, the comparison was between antioxidant and no antioxidant groups for any progression of lens opacity or for cataract surgery.

Descriptive results of the study follow. A total of 3,640 participants enrolled in this 2 × 2 factorial design of antioxidants and zinc. At entry, 57% of participants used vitamins, zinc, or multivitamin supplements and half of patients took the recommended daily allowance dosages below the pharmacologic dosages of the study medication. A standardized multivitamin (Centrum) was provided to those subjects (95%) who chose to continue using multivitamins. Subjects in the placebo or active groups who chose to use Centrum had intakes of vitamins C, E, beta-carotene, and zinc, from both dietary sources and Centrum, as well as, in the case of active treatment, the pharmaceutical dose provided. This decision weakened the study's statistical power in testing its primary hypothesis on pharmacologic doses; however, it was thought that inclusion of RDA level supplementation on top of the study doses would better reflect the supplementation habits of the population 55 to 80 years.

AREDS results confirmed prior findings regarding smoking, hypertension, hyperopia, lens opacities, education level, gender, increased body mass index, and white race. New findings included a risk of developing geographic atrophy associated with use of thyroid hormones and antacids, as well as an increased AMD risk for persons with

extensive intermediate or large drusen who have arthritis or who use hydrochlorothiazide (Age-Related Eye Disease Study Research Group, 2000).

Regarding antioxidant supplementation, AREDS found that people at high risk of developing advanced stages of AMD lowered their risk by approximately 25% when treated with a high-dose combination of vitamin C, vitamin E, beta-carotene, and zinc. In the same high-risk group, which included people with intermediate AMD or advanced AMD in one eye but not the other eye, the nutrients reduced the risk of vision loss caused by advanced AMD by about 19%. For those study participants who had either no AMD or early AMD, the supplements did not provide an apparent benefit (Age-Related Eye Disease Study Research Group, 2001a). In the cataract portion of the study, the same nutrients had no statistically significant effect on age-related cataract development or progression (Age-Related Eye Disease Study Research Group, 2001b).

Data from AREDS suggest that once AMD pathogenesis has begun (as evidenced by the presence of intermediate drusen, large drusen, or noncentral geographic atrophy), zinc alone or in combination with antioxidants can reduce advanced AMD progression. Too few individuals with early AMD (i.e., milder drusen and retinal pigment epithelial abnormalities) advanced sufficiently within the study's timeframe to assess whether any treatment tested could slow progression to advanced AMD. Of note, AREDS results did not validate results from a prior (small) randomized trial that suggested a benefit of large doses of zinc on visual acuity in persons with AMD (Newsome et al., 1988).

In contrast to the AREDS findings, another randomized trial of 1,193 subjects reported that after 4 years of supplementation, 500 IU per day of vitamin E had little benefit in reducing the risk of AMD development or progression (Taylor et al., 2001).

The suggestion has been offered by AREDS investigators that, as in the AREDS study, too few of the subjects in this study progressed to advanced AMD. Indeed, at present this study may validate the AREDS finding that antioxidant vitamins at the tested dose appear to be of little benefit in the early stages of AMD.

AREDS could not prove the safety of high dose antioxidant supplementation; however, AREDS reported mortality of half that observed in the general population. Decreased mortality may be due to several factors: First, the typical volunteer for this study was healthy, mobile, and motivated. Second, the vitamin C dose (500 mg) used in formulation was approximately 5 times what the general population receives from diet alone. Third, the 400-IU vitamin E dosage was approximately 13 times the recommended daily allowance (RDA) and the dose of zinc as zinc oxide was approximately 5 times the RDA. These levels of zinc and vitamins C and E can generally be obtained only by supplementation.

AREDS lacked the power to address whether either zinc or antioxidants held differing abilities to reduce the risk of developing advanced AMD. Practically, however, evidence indicated that individuals older than age 70 who risk developing advanced AMD should take zinc/copper and multivitamin supplements. Further, the study's findings led to two major recommendations: (1) persons >55 years should undergo dilated eye examinations to determine risk of developing advanced AMD and (2) those with extensive intermediate size drusen, $\geq$1 large drusen, or noncentral geographic atrophy in one or both eyes or those with advanced AMD, should take a supplement of antioxidants plus zinc.

The AREDS study found no benefit on cataract progression from supplementation with antioxidants plus zinc. In contrast to the AREDS study are results of a double-blind, placebo-controlled pilot study testing the effect of long-term antioxidant lutein and α-tocopherol supplementation on serum levels and visual performance in cataract patients (Olmedilla et al., 2003). The study involved dietary supplementation of subjects with lutein (15 mg; n = 5), alpha-tocopherol (100 mg; n = 6), or placebo (n = 6), three times a week for up to 2 years. Serum carotenoid and tocopherol concentrations were determined, and visual performance (i.e., visual acuity and glare sensitivity), biochemical, and hematologic indexes were monitored. Findings indicated that serum concentrations of lutein and alpha-tocopherol increased with supplementation, although statistical significance was reached only in the lutein group; visual performance (visual acuity and glare sensitivity) improved only in the lutein group. There was a trend toward the maintenance of and decrease in visual acuity with alpha-tocopherol and placebo supplementation, respectively. This study suggests that a higher lutein intake, through lutein-rich fruits and vegetables or through supplements, may improve visual performance in individuals with age-related cataracts.

With regard to cataracts, results reported by both the AREDS and Olmedilla et al. indicate fundamental differences between the carotenoids β-carotene and lutein in preventing ocular oxidative stress. Indeed, lutein as well as zeaxanthin, also a carotenoid xanthophyll, are thought to have two AMD-related beneficial effects. Lutein and zeaxanthin, both yellow in color, filter short wavelength light; this is considered a primary protection mechanism from free radical damage to the photoreceptor cells (rods and cones). Photooxidative damage is induced by the formation of reactive oxygen

species such as singlet molecular oxygen ($^1O_2$), superoxide radical anion ($O_2^{\bullet -}$), and peroxyl radicals (Darr & Fridovich, 1994). Lutein and zeaxanthin can also act as antioxidants in the photooxidation process by physically quenching radicals, especially singlet oxygen (Foote & Denny, 1968). Lutein is 10 times more effective than Vitamin E in quenching photo-induced radicals. It is probable that lutein and zeaxanthin have other significant cellular effects not yet substantiated.

A study out of Johns Hopkins University (Dagnelie et al., 2000) examined the effects of lutein supplementation on visual acuity, central visual-field area, and subjective visual disturbances in retinitis pigmentosa and related retinal degenerations. Participants tested their own visual acuity via a computer screen test and their central visual-field extent via a wall chart. These remote monitoring techniques of 23 subjects demonstrated that short-term (26-weeks) lutein supplementation (40 mg/day for 9 weeks, 20 mg/day thereafter) produced vision improvements. These results imply that if lutein had been used in the AREDS study, AMD progression may have slowed to an extent capable of impacting the study's primary outcome measures .

Another 12-month randomized, doublemasked, placebo-controlled clinical trial was conducted in 90 subjects to determine whether supplementation with lutein or lutein in combination with other antioxidants, vitamins, and minerals improved vision and decreased AMD symptoms (Richer et al., 2004). On average, study subjects were older and had more severe disease than subjects enrolled in AREDS. Results showed that visual function, including Snellen equivalent visual acuity and contrast sensitivity, improved with lutein alone or with other antioxidants. While the data were promising regarding visual function improvements using both lutein and other antioxidants on

visual function, a larger and longer study is needed to determine the long-term effect of these compounds on AMD prevention and pathogenesis.

Wolfberry, *Lycium barbarum L.*, is rich in zeaxanthin dipalmitate, and is valued in Chinese culture for providing "visual benefits". In a single-blinded, placebo-controlled human intervention trial of parallel design, consumption of whole 15 g/d wolfberries (containing an estimated 3 mg zeaxanthin) for 28 days increased plasma zeaxanthin concentration in healthy subjects (Cheng et al., 2005). This human supplementation trial showed that zeaxanthin in whole wolfberries was bioavailable and that intake of a modest daily amount markedly increased fasting plasma zeaxanthin levels.

While this particular wolfberry study was a bioavailability study and did not address the question of efficacy of zeaxanthin, a recent animal model study investigated the efficacy of another antioxidant containing berry, bilberry, on AMD and cataracts. To determine the effects of antioxidant flavonoids on senile cataract and macular generation, Fursova et al. (2005) undertook one of the first *in vivo* bilberry intervention studies in animals. Senescence-accelerated OXYS rats exhibit early senile cataract and macular degeneration. The diet of OXYS rats was supplemented with 25% bilberry extract. By age 3 months, 70% of the control OXYS rats had cataract and macular degeneration while those OXYS rats whose diets were supplemented with bilberry extract suffered no impairments in the lenses and retina. Vitamin E supplementation had no statistically significant protection against impairments in the lenses and retina. However, both bilberry extract and vitamin E decreased lipid peroxides in the retina and serum of OXYS

rats. The authors offered no hypotheses to explain the mechanisms of dietary antioxidants in preventing AMD in OXYS rats.

**Bilberry Studies.** Few *in vivo* studies exist on the effects of bilberry flavonoids. Searches of document databases internal to the National Institutes of Health (NIH) reveal the Institutes' consensus of mixed scientific evidence for bilberry use as an herbal medicine. To date, bilberry extract has been evaluated for efficacy as an antioxidant, anti-inflammatory, "vasoprotectant", mucostimulant, hypoglycemic, and lipid-lowering agent. Pre-clinical studies show promise, but data from human studies remain sparse and of poor quality. Existing data neither prove nor disprove the case for bilberry efficacy.

While the mechanisms of action behind bilberry flavonoid's beneficial effects on the eye are not completely understood, traditional uses of bilberry suggest its ability to improve oxygen and blood delivery to the eye, scavenge free radicals, and stabilize collagen structures, thus contributing to cataract and macular degeneration prevention. Anthocyanidins also appear to have effects related to light and dark visual adjustments (Caselli, 1985; Wegmann et al., 1969). Such findings have prompted approximately 30 trials of bilberry effects on night vision in Europe and the US since the early 1960s. In a systematic review of 30 studies by Canter & Ernst (2004), only 12 studies were placebo-controlled, and 4 randomized controlled trials yielded null outcomes. Improvements in night vision were found in eight controlled trials, including one randomized controlled trial. Study investigators concluded that the data do not support the theory that bilberry flavonoids improve night vision in subjects with normal healthy eyes (Canter & Ernst, 2004).

While studies have been conducted on the association between bilberry flavonoids and night vision in healthy subjects, no trials exist regarding the effect of bilberry extract on vision in subjects with impaired night vision or pathological eye conditions. In contrast to studies in healthy eyes, Fursova et al. (2005) investigated long-term bilberry extract supplementation in OXYS rats and found decreases in oxidative stress markers, cataract formation, and AMD development. If one extends the search for the effects of anthocyanins to include those of commercially produced *Vaccinium* species, studies in rats with blueberry feeding show reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits (Joseph et al., 1999).

Evidence suggests that bilberry flavonoids offer some relief from glaucoma, although few studies have investigated facilitating aqueous outflow in response to bilberry intake (Caselli, 1985). More convincing data show the effect of bilberry extracts on delaying cataract development in both animals (Hess et al., 1985; Pautler & Ennis, 1984) and humans (Bravetti, 1989). An Italian study showed that 260 mg/day of a 25% anthocyanin extract in combination with vitamin E arrests cataract formation patients with senile cortical cataracts. In addition, several European clinical studies showed bilberry anthocyanin efficacy against diabetic retinopathy (Chaundry et al., 1983; Perossini et al., 1987; Scharrer & Ober, 1981; Varma et al., 1977). Indeed, one-month bilberry extract supplementation significantly improved ophthalmoscopic parameters in patients with diabetic and/or hypertensive retinopathy.

Traditional uses of bilberry also include treatment of vascular fragility and microcirculation. Animal studies (Colantuoni et al., 1991; Lietti et al., 1976) and clinical trials in humans (Ghiringhelli et al., 1978) have shown bilberry extracts to decrease

vascular permeability and improve vascular tone and blood flow. Many uncontrolled trials of patients with venous insufficiency indicate bilberry extract effectiveness in improving symptoms; however, controlled studies are required before non-European medical practice will adopt bilberry use for treating microcirculation disorders.

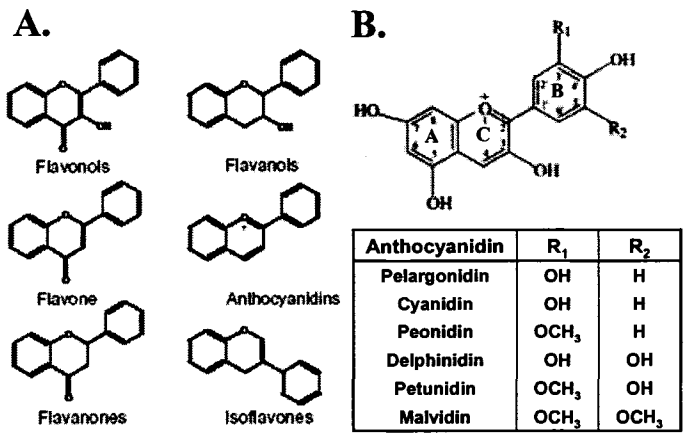
Bilberry extracts have been shown to possess anti-inflammatory properties in animals (Rao et al., 1981) and to have strong anti-platelet aggregating activity in humans when supplemented for a month at 480 mg/day (Puilleiro et al., 1989). Despite traditional medicine's centuries of bilberry use for eye-related disorders, few well-controlled bilberry trials of sufficient duration have been conducted in populations with eye disease risk.

# CHAPTER III

# BILBERRY ANTHOCYANINS

## Anthocyanins as Antioxidants

Anthocyanins exist as glycosides of polyhydroxy and polymethoxy derivatives of the 2-phenylbenzopyrylium cation. They are distinct from other flavonoid classes due to an electron deficiency in their ring structure. Structural differences among flavonoid classes can account for differences in their bioavailability and bioactivity (Figure 1). In addition, variations in the substitution patterns of hydroxyl and methoxy groups as well as differences in sugars attached at the 3 position of the B ring can confer significantly different bioavailability and bioactivity. Anthocyanins and their aglycones, the




| Anthocyanidin | $R_1$ | $R_2$ |
|---|---|---|
| Pelargonidin | OH | H |
| Cyanidin | OH | H |
| Peonidin | $OCH_3$ | H |
| Delphinidin | OH | OH |
| Petunidin | $OCH_3$ | OH |
| Malvidin | $OCH_3$ | $OCH_3$ |

**Figure 1.** Basic flavonoid and anthocyanin structure. Panel A shows the basic structures of the classes of flavonoids. Flavonoids contain two aromatic rings (A and B) linked by an oxygenated heterocycle (ring C). R substitutions include hydroxy, methyl, and mono- or disaccharide groups. Panel B shows the structure of the basic anthocyanidin flavylium cation and the substitution patterns of bilberry's major anthocyanidins.

anthocyanidins, may differ in bioavailability and bioactivity from other flavonoids, since they contain a flavylium nucleus with a positively charged oxygen atom as well as appear to be more amphipathic than other flavonoids.

In 1936, Szent-Gyorgyi and colleagues found lemon juice extract to decrease capillary wall permeability and called the active ingredient "vitamin P" (later identified as the flavonoids hesperidin and eriodictiol glycoside). Though flavonoids were subsequently found to be nonessential and, therefore, not vitamin candidates, this early work did note a synergy between flavonoids and vitamin C and suggested their activity as antioxidants. *In vitro*, flavonoid aglycones, including the anthocyanidins, are potent antioxidants due to their degree of hydroxylation and the presence of a B-ring catechol group. However, the B-ring catechol is metabolized *in vivo*, principally by O-glucuronidation and formation of sulfate esters (Bors et al., 2001). The literature contains little information on antioxidant capacity of flavonoid conjugates *in vitro*, although these metabolites predominate *in vivo* and have different properties than their parent compounds.

The non-catechol hydroxyl groups on flavonoids can chelate transition minerals such as copper and iron; this inhibits Fenton-Weiss-Haber reactions and the generation of reactive oxygen species. Some dietary flavonoids may serve as sufficiently effective chelators of non-heme iron in the gut to aggravate or precipitate iron-deficiency anemia (Zijp et al., 2000).

Interventions with some flavonoid-rich foods have revealed an increase in plasma measures of "total antioxidant capacity" (e.g., by using the ferric reducing antioxidant power and oxygen radical absorbance activity assays) and a reduction in biomarkers of

oxidative stress (e.g., phospholipid peroxides, malondialdehyde, and $F_2$-isoprostanes in plasma and 8-OH-deoxyguanosine in leukocytes). However, other studies have found no statistically significant antioxidant action by these compounds *in vivo* (Lean et al., 1999; Widlansky et al., 2005; Wiswedel et al., 2004). Such contrasting reports may reflect differences in the specific flavonoids tested, as well as differences in patient-level health or oxidative stress status, dosing, and treatment duration. Nonetheless, the direct stoichiometric contribution of intracellular flavonoids to quenching reactive species *in vivo* appears small in relation to contributions from other dietary antioxidants whose *in vivo* concentrations are significantly higher. For example, supplementation with 50 mg of epigallocatechin gallate results in peak plasma concentrations of approximately 0.15 μmol/L, while the usual status of ascorbate is 3–7 mmol/L. Anthocyanins are among the lowest of flavonoids with regard to their absorption (Milbury et al., 2002; Wu et al., 2005). However, a marked synergy between flavonoids and other components of the antioxidant defense network, including vitamins C and E via mutual recycling, sparing, or other mechanisms, may result in a significant impact on the quenching of reactive oxygen and nitrogen species (Chen et al., 2005).

Flavonoids may act indirectly to increase antioxidant defenses and redox status by inducing phase II enzymes, including those regulating glutathione synthetase, peroxidase, and S-transferase (Kong et al., 2001). For example, using transgenic mice, berry fruit flavonoids have been found to increase the activity of the heavy subunit promoter (GCSh) of γ-glutamylcysteine synthetase, the rate limiting step in GSH synthesis (Carlsen et al., 2003). Flavonoids may act as pro-oxidants by reducing $Fe^{+3}$ to $Fe^{+2}$ to yield hydroxyl radicals, although no such effects have been demonstrated *in vivo*. While

flavonoid catechols can be oxidized to quinones, which may generate reactive species through redox cycling, enzymes such as quinone reductase and catechol-O-methyltransferase limit their formation in tissue.

Some theorize that anthocyanins and their aglycones, anthocyanidins, contribute to the prevention of age-related diseases via antioxidant mechanisms (Renaud & DeLorgeril, 1992). However, no epidemiological evidence proves this mechanistic connection. As mentioned for other flavonoids, anthocyanidins are potent antioxidants due to their high degree of hydroxylation and conjugation. This property has been demonstrated *in vitro* by oxygen radical scavenging assays (Tsuda et al., 1996; Yamasaki et al., 1996) and their metal chelation properties (Amorini et al., 2001). Several studies also have shown that anthocyanins inhibit lipoprotein oxidation *in vitro* (Kerry & Abbey, 1997; Ghiselli et al., 1998; Tsuda et al., 1996a). Further, studies have investigated foods, especially fruits and berries, for their anthocyanin content and antioxidant activity *in vitro* using radical interception assays such as the oxygen radical absorbance capacity (ORAC) or ferric ion reducing antioxidant power (FRAP) assays (Gabrielska & Oszmianski, 2005; Fukomoto & Mazza, 2000; Kähkönen et al., 1999; Moyer et al., 2002; Prior et al., 1998; Wang et al., 1996).

In the most general terms, these *in vitro* assays determine an antioxidant's ability to protect a target molecule from oxidation by quenching radicals generated within the assay. The studies suggest that, as with other flavonoids, anthocyanins or anthocyanidins entering the vascular circulatory system exhibit an antioxidant effect by quenching radicals. Indeed, investigators theorized that lowering radical load would decrease the

risk of developing age-related diseases associated with oxidative stress. This hypothesis has proven difficult to substantiate.

In comparison to the numerous studies on flavonoids, such as catechins and quercetin, relatively few studies have addressed anthocyanidin antioxidant activity *in vivo* or in isolated cells. Duthie et al. (1997) demonstrated that myrecetin acts as a pro-oxidant by increasing DNA damage and inhibiting proliferation in some human cells exposed to $\geq$100 μM concentrations. These effects were not observed at lower concentrations; therefore, the relevance of these studies to the *in vivo* conditions are unknown, especially considering that anthocyanidins may not circulate freely *in vivo*.

Even fewer publications focus on the antioxidant activity of anthocyanins, which represent the form found in plants and in human diets. The literature has revealed red wine's ability to alter *ex vivo* antioxidant capacity of human serum; however, wine is also a complex mixture of which anthocyanins are only one component class showing antioxidant properties *in vitro* (Whitehead et al., 1995). To determine intracellular antioxidative capacity of anthocyanin, Pool-Zobel et al. (1999) assessed the ability of anthocyanin-enriched fruit juice concentrates from fresh berries — i.e., aronia (*Aroniamelanocarpa*), elderberry (*Sambucus nigra),* macqui *(Aristotelia chilensis*), and the tintorera grape — to prevent *in vitro* oxidative damage to DNA in HT29 primary human colon cells. In non-cellular assessment, results showed that pure anthocyanidins and anthocyanins were 2- to 5-fold more potent antioxidants than equimolar concentrations of ascorbic acid or the water-soluble vitamin E analogue Trolox. Antioxidant activity of aglycon cyanidin, measured using the FRAP assay, was less than that of the glycosides, cyanin, and idaein. In this study, however, the mechanism for

preventing DNA damage was not determined. Intracellular oxidative stress was only weakly affected by the anthocyanin/anthocyanidin concentrates. Interestingly, this study demonstrated that glycosides are as effective or even more effective than the aglycons in the FRAP assay. This suggests that, while direct radical quenching capabilities of anthocyanins are less than that of their aglycones, their iron chelation capability is not impaired by the presence of a sugar moiety.

Several studies have used a highly purified extract of *Vaccinium myrtillus L.*, labeled Myrtocyan®, which contains 36% anthocyanosides of which cyanidin 3-glucoside predominates (Morazzoni & Bombardelli, 1996). This study found that cyanidin 3-glucoside inhibited tetrachloride-induced lipoperoxidation (Morazzoni & Bombardelli, 1996). *In vivo*, Myrtocyan® has been shown to promote wound healing, inhibit ulcer formation, and display anti-atherosclerotic activity. Myrtocyan® has displayed effects on microvascular vascular control and capillary resistance and permeability, improving dark adaptation after dazzling. This and other studies have not determined mechanisms; however, Wang et al. (1997) showed that Myrtocyan® displayed high radical quenching capability via the ORAC test.

Berry phenolic inhibition of both protein and lipid oxidation in liposomes *in vitro* was measured by determining the loss of tryptophan fluorescence, formation of protein carbonyl compounds, and by conjugated diene hydroperoxides and hexanal analysis (Viljanen et al., 2004). This study and others like it, though, did not distinguish between proanthocyanidins, anthocyanins, and other phenolics. A recent study demonstrated the ability of bilberry anthocyanins to inhibit oxidation of the pyridinium bisretinoid, A2E, *in vitro*. A2E, an autofluorescent pigment that accumulates in retinal pigment epithelial

cells with age and in some retinal disorders, can mediate perturbation of cell membranes and light-induced damage to the cell. The authors suggest that cells taking up anthocyanins also exhibited a resistance to the membrane permeabilization occurring from the detergent-like action of A2E.

Anthocyanins from bilberry extracts also show *in vitro* antioxidant activity toward nitric oxide (NO) and peroxynitrite (ONOO-) (Ichiyanagi et al., 2004). With the exception of delphinidin glycosides, anthocyanins exhibit slightly less antioxidant activity toward these radicals than does the flavonoid catechin. These effects have yet to be verified *in vivo.*

## Enzyme Activity and Cell Signaling Effects

In addition to their role as antioxidants, flavonoid, and likely anthocyanin, bioactivity modulate enzyme activity and affect cell signaling events (Williams et al., 2004). Many activities shown for other flavonoids have not been tested for anthocyanins; however, some flavonoid functionalities appear to be shared across classes and may well extend to anthocyanins and anthocyanidins.

Flavonoids have been shown to interact with all major enzyme classes, including hydrolases, isomerases, ligases, lyases, oxidoreductases, and transferases, although the majority of investigations on which these findings are based have been conducted *in vitro* (Middleton et al., 2000). Flavonoids selectively inhibit kinases by binding directly to enzymes or associated membrane receptors; in so doing, they influence signal transduction pathways (Hollosy & Keri, 2004). By activating the antioxidant response element (ARE), flavonoids may induce phase II detoxification enzymes such as

glutathione S-transferase, UDP-glucuronosyltransferase, NAD(P)H:quinone oxidoreductase 1, and epoxide hydrolase (Song et al., 1999; Zhang & Gordon, 2004).

The gene promoter regions for these enzymes are transcriptionally regulated by several xenobiotic response elements, including the antioxidant and electrophile response elements (ARE and EpRE, respectively) (Nguyen et al., 2003). Upstream regulation of ARE and EpRE is partly coordinated through binding of NF-E2–related transcription factor (Nrf2). Nrf2 translocation from the cytosol to the nucleus is inhibited by the cytoskeleton-associated protein Keap1. Nrf2 binding depends on thiols in Keap1 and, thus, on feedback control of ARE- and EpRE-regulated enzyme systems. Flavonoid oxidation products (e.g., quinones and quinone methides) not recycled by ascorbate or other antioxidants may arylate protein thiols and thereby affect enzyme expression.

In addition to their potential for quenching reactive oxygen species, flavonoids can affect cellular redox status via other mechanisms. For example, flavonoids with a B-ring catechol moiety can inhibit succinoxidase and promote a mitochondrial respiratory burst of hydrogen peroxide and superoxide anion. Flavonoids with a 2,3 double bond/3-OH, in conjugation with the 4-oxo function on the C-ring, can reduce mitochondrial membrane fluidity and cause uncoupling or, particularly in compounds with a B-ring o-di-OH, inhibit the respiratory chain (Dorta et al., 2005). Other flavonoids may induce mitochondrial permeability transition and release of $Ca^{2+}$. Anthocyanins likely share many of these activities.

Anthocyanin-rich extracts of grape, bilberry, and chokecherry have been investigated for their chemopreventive activity against colon cancer cells (Zhao et al., 2004). Extracts inhibited the growth of colon cancer, as compared to non-tumorigenic

colon cells. The data suggested varying compositions and degrees of growth inhibition were dependent on anthocyanin chemical structure. Using more refined materials, five anthocyanidins and four anthocyanins were tested for cell proliferation inhibitory activity against six human cancer cell lines representing stomach, colon, breast, lung, and central nervous system (Zhang et al., 2005). Cell viability after exposure to anthocyanins and anthocyanidins was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric methods. Anthocyanidins, but not anthocyanins, showed cell proliferation inhibitory activity.

Likewise, anthocyanidin delphinidin exerts anti-proliferative effects on basal and VEGF-induced proliferation of aortic endothelial cells in culture (Martin et al., 2003). Here, the data suggest that the mechanism involved in cell cycle progression arrest for $G_0/G_1$ phase occurs via ERK-1 /-2 pathways. Delphinidin was shown to inhibit angiogenesis via alteration of the expression of proteins key to cell migration and proliferation (Favot et al., 2003).

Cyanidin and delphinidin are both potent inhibitors of the tyrosine kinase activity of the epidermal growth-factor receptor in carcinoma cells (Meiers et al., 2001). Shutting off downstream signaling from growth factor receptors might contribute substantially to the growth-inhibitory properties of anthocyanidins. These signal transduction effects that involve Elk-1 phosphorylation and MAP kinase pathway activity resemble those of chemopreventive flavonoid Epigalocatechin galate (EGCG). The extent to which the anthocyanins share the effects of anthocyanidins on these systems is relatively unknown. One report on anti-tumor effects *in vitro* and *in vivo* of red soybean extracts attributed the effect to cyanin conjugated with glucose and rhamnose, since these were the major

cyanin forms within the extract (Koide et al., 1997). The anti-proliferative effects of anthocyanins in neoplastic and non-neoplastic cells warrant further investigation.

Evidence reveals anthocyanins to be both pro- and anti-apoptotic. For example, hibiscus anthocyanins induced apoptosis in human promyelocytic leukemia cells (Chang et al., 2005). The authors attributed this pro-apoptotic mechanism to activation of p38 MAP kinase, which resulted in protein c-jun phosphorylation and signal transduction to further activate the apoptotic protein cascades containing Fas-mediated signaling. The resulting mitochondrial cytochrome c release led to cleavage of caspase-3 and apoptosis. The literature also suggests anthocyanins are pro-apoptotic in human gastric adenocarcinoma cells (Shih et al., 2005).

In contrast to evidence on anthocyanin pro-apoptotic activities, data indicate that the phenolic extract of strawberries is neuroprotective by preventing apoptosis *in vitro* on PC12 cells treated with hydrogen peroxide ($H_2O_2$) (Heo & Lee, 2005). Anthocyanin effects on differing cell types and apoptotic conditions require further investigation to resolve mechanistic or experimental differences that account for variations in apoptotic modulation.

Those flavonoids exhibiting anti-inflammatory properties act via interactions with tyrosine and serine-threonine protein kinases, as well as via other elements of signal transduction pathways (Middleton et al., 2000). *In vitro*, flavanols and procyanidins modulate the interleukin transcription in activated peripheral blood mononuclear cells and inhibit mitogen-induced T-cell proliferation and polyclonal Ig B-cell production (Sanbongi et al., 1997). Bioavailability studies suggest that procyanidins are broken down to monomers prior to absorption. Some of the metabolites are anthocyanidins and

anthocyanins that may contribute to the anti-inflammatory effects. The putative antioxidant and anti-inflammatory effects of blueberry and cranberry anthocyanins and hydroxycinnamic acids against $H_2O_2$ and tumor necrosis factor-α (TNF-α)–induced damage to human microvascular endothelial cells have been investigated (Youdim et al., 2002). These berry polyphenols reduced TNF-α–induced up-regulation of various inflammatory mediators (i.e., IL-8, MCP-1 and ICAM-1). While these actions suggest health benefits against vascular disease initiation and development as well as age-related related deficits in neurological impairments, they also suggest signal pathway regulation that may prevent other diseases with inflammation components, such as wet AMD.

Red wine flavonoids, polyphenols, and possibly anthocyanins inhibit platelet-derived growth factor–induced vascular smooth muscle cell migration through inhibition of signaling cascades of phosphatidylinositol-3 kinase and p38 mitogen-activated protein kinase (Iijima et al., 2002). Modulation of these same kinases may also affect inflammatory responses. The dietary anthocyanin, cyanidin 3-glucoside, naturally activates endothelial nitric oxide synthase (eNOS) in endothelial cells by promoting its phosphorylation at Ser1179 and dephosphorylation at Ser116 (Xu et al., 2004). Ameliorating endothelial dysfunction may explain, in part, the cardiovascular protective effects of anthocyanin-rich foods like berries and wine.

While the literature lacks a large body of research on anthocyanin biochemical and molecular effects on mammalian cells, research to date suggests that anthocyanins have many potential mechanistic actions. Data exist showing physiological activities of flavonoids on mammalian cells. Common effects have been observed between anthocyanidins and other flavonoids, although these may not translate to the glycosylated

anthocyanins. Evidence also exists suggesting that the presence of sugar moieties significantly affect activities. Nevertheless, as the review above indicates, semi-purified anthocyanin mixtures affect signal transduction pathways and enzyme activities in manners that can significantly affect cellular states. Much research remains regarding the nature and extent of anthocyanin bioactivities.

# CHAPTER IV

# METHODS AND MATERIALS

## Materials and Chemicals

The bilberry extract material used in these studies was a commercial extract of bilberry (*Vaccinium myrtillus*). The extract was enriched in anthocyanin content to approximately 25% of the final product weight and was obtained as a gift from Artemis International, Inc. (Fort Wayne, IN, USA). This product is currently the same product incorporated into bilberry dietary supplements alleged to promote eye health. All chemicals not otherwise specified in the sections below were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All reagents were of the highest purity available.

## Chromatography, Polyphenolic, and Glutathione Assays

**Sepak Separation of Anthocyanins**. To determine whether effects resulted from anthocyanins or other polyphenolic compounds present in the bilberry extract, anthocyanins were recovered by solid phase extraction using an octadecylsilane (ODS) solid phase extraction cartridge (Sep-Pak C18), as previously described (Cao & Prior, 1999). Briefly, anthocyanins in the bilberry extract were extracted using an ODS solid-phase extraction cartridge (Sep-Pak C18). The cartridge was washed with 10 mL of methanol and equilibrated with 10 mL of 0.44 mol/L trifluoroacetic acid (TFA) before use. TFA was obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA). One mg of bilberry extract was dissolved in 1 mL of 0.44 mol/L TFA, then centrifuged, and the

supernatant applied to the cartridge. Water-soluble compounds, polar lipids, and neutral lipids were eluted from samples with 10 mL of 0.44 mol/L TFA, 10 mL of dichloromethane, and 10 mL of benzene, respectively. Finally, samples were eluted with 5 mL of 0.44 mol/L TFA in methanol. The methanol phase, collected and dried under nitrogen, contained anthocyanins. Samples were redissolved in buffers or mobile phase for anthocyanin analysis and used either the total anthocyanin method or high pressure liquid chromatography (HPLC) methods, as described below.

**HPLC with ECD and UV Detection.** Chromatographic separation and compound identification by HPLC with electrochemical detection (ECD) was accomplished using a coulometric array system and modifications of methods previously described (Milbury, 2001). Anthocyanin separation was achieved on a Zorbox SB-C18, 4.6 × 250 mm– column with a complex mobile phase gradient. The gradient ran at a 1.0 mL/min flow rate over 130 minutes from 25 mM sodium acetate (pH 1.5) to 25 mM sodium acetate in methanol carrying equivalent trichloroacetic acid.

**Liquid Chromatographic Mass Spectrometry (LC/MS/MS).** To verify compound identities, dried samples were reconstituted in 200 μL 1% Formic acid/$H_2O$ and separated on an Agilent 1100 HPLC. A dual column system was utilized, with a switching valve between the columns to segregate the anthocyanin "region" eluting from the first column for passage to the second analytical column. Chromatography was conducted at a 0.3 mL/minute flow rate using a complex gradient from acidified $H_2O$ (9% Formic acid) to 100% acidified acetonitrile (9% Formic acid) over a 120-minute analytical run. Anthocyanin elution was monitored on an Agilent G1315A diode array detector at A520. Mass fragmentation and detection were accomplished using a Brucker Esquire ion trap

LC/MS/MS fitted with an electrospray interface; the instrument operated in positive ion mode using selected reaction monitoring. Based on elution times established in each analytical run, selected reaction monitoring windows were determined. Anthocyanins were quantified by calculating the area under the curve in the extracted ion chromatogram of fragmentation ions.

**Total Phenolics Assay.** Total phenolics were assayed colorimetrically via the Folin-Ciocalteu method, as modified by Singleton & Rossi (1965) and Singleton et al. (1999). Two and a half mL of ten-fold diluted Folin-Ciocalteu reagent, 2 mL of 7.5% sodium carbonate, and 0.5 mL of phenolic extract were mixed. Absorbance was measured at 765 nm after 15 minutes of heating at 45°C. A mixture of water and reagents was used as a blank. The phenolics content was expressed as g/kg powder using catechin as a standard phenolic material.

**Total Anthocyanins Assay.** Total anthocyanins were estimated by a pH differential method (Cheng & Breen, 1991). Absorbance was measured in a Shimadsu UV-1601 spectrophotometer at 510 nm and at 700 nm in buffers at pH 1.0 and 4.5, using [(A510–A700)pH1.0–(A510–A700)pH4.5] with a molar extinction coefficient of cyanidin 3-glucoside of 29,600. Results were expressed as grams of cyanidin 3-glucoside equivalent per 100 g of dry weight (i.e., percent dry weight).

**Glutathione Analysis.** For glutathione (GSH) studies, media were removed from the cultures by aspiration and culture plates were washed with phosphate-buffered saline (PBS) (pH 7.4) before addition of 1 mL perchloric acid (5% vol/vol) containing 0.2 M boric acid. Contents were scraped and stored in 1.5-mL microcentrifuge tubes at -80°C until analysis. GSH was derivatized by mixing 300 μL sample supernatant with 60 μL

iodoacetic acid (7.4 mg/mL $H_2O$) and adjusted to pH 9.0 with a KOH/tetraborate solution (1 M KOH in saturated $K_2B_4O_7$). The samples were incubated for 20 minutes at room temperature before addition of 300 µL dansyl chloride solution (20 mg/mL acetone) and mixed thoroughly on a votex. The mixture was stored at room temperature in the dark for 24 hours before addition of 500 µL chloroform. These samples were then stored at 0°C without further processing until analysis via HPLC.

After centrifugation, aliquots of the aqueous layer were injected onto a 3-aminopropyl column (Custom LC, Houston, TX, USA) for separation. Initial mobile phase conditions consisted of a mixture of 80% methanol (solvent A) and 20% 4 M sodium acetate (pH 4.6) containing 64% methanol (solvent B) delivered at a flow rate of 1 mL/min. After 10 minutes, a linear gradient was established to achieve 20% solvent A and 80% solvent B by a 30-minute chromatogaphic time point. At this point, the 20%:80% A:B mixture was run isocratically to 46 minutes before returning to 80% A and 20% B in 2 minutes for equilibration prior to the next analysis. The GSH derivative was detected by fluorescence (305–395 nm excitation and 510–650 nm emission; Gilson Medical Electronics, Middleton, WI, USA). GSH quantitation was obtained by integration relative to a derivatized GSH standard curve.

## RPE Cell Culture Model Characterization

**APRE-19 Cell Culture.** ARPE-19 cells were obtained from the American Type Culture Collection (ATCC) (ATCC, Manassas, VA, USA), grown, and routinely passaged under conditions previously described (Dunn et al., 1996). Passage 15 ARPE-19 cells were seeded at 20,000 cells/cm$^2$ (sub-confluent) and grown 10 days to confluence (80,000

cells/cm$^2$). Cells were plated in a growth medium comprised of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/Hams F12) medium, which contained 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate, and 10% fetal bovine serum. Cells were cultured at 37°C under 5% carbon dioxide ($CO_2$). After three days in culture, the medium's fetal bovine serum concentration was decreased to 5% in order to slow division and encourage differentiation. At 10 days in culture, the medium was removed and the cells washed twice with Hanks balanced salt solution (HBSS) (Gibco BRL, Bethesda, MD, USA). Chemical treatments were performed in 1:1 DMEM/Hams F12 medium without phenol red, and cells were harvested after 4 hours. Hydrogen peroxide ($H_2O_2$) (30%; Fisher Scientific Company LLC, Pittsburgh, PA, USA) was diluted in water; the dilution was then placed into a final treatment concentration of 1:1 DMEM/Hams F12 medium without phenol red. Tert-butyl hydroxide (TBH) (70% aqueous solution; Sigma Inc., St. Louis, MO, USA), bilberry extracts, and bilberry anthocyanins were diluted in 1:1 DMEM/Hams F12 medium without phenol red immediately prior to addition to cells.

**Microscopy.** Retinal pigment epithelium (RPE) cells grown on Transwell membranes (described above) were fixed and stained using a Diff-Quik Stain Set (Dade Behring AG, Dudingen, Switzerland). The membranes were then rinsed with water, cut from the Transwells, and mounted onto glass slides for photomicroscopy. The cultures were photographed using an Olympus IX70 inverted microscope, with 40x and 100x high n.a. oil-immersion objectives. The microscope was fitted with a QImaging Retiga 1300 CCD digital camera and images captured using QCapture Software for Windows.

**Uptake and Transepithelial Electrical Resistance.** To determine transepithelial resistance and bilberry anthocyanin uptake and transport, ARPE-19 cells were plated at a density of $2 \times 10^5$ cells/cm$^2$ on a 24 mm permeable membrane insert (12 mm diameter, 0.4 mm pore size, Transwell; Costar, Cambridge, MA, USA). The insert was coated with laminin (5 mg/cm$^2$; Becton Dickinson Laboratory, Franklin Lakes, NJ, USA) in DMEM/Hams F12 medium with 20% fetal bovine serum (FBS). After the RPE cells became confluent, the medium was changed to DMEM/Hams F12 medium with 5% FBS until the transepithelial electrical resistance (TER) stabilized. At this stage of culture, the RPE was tested for expression of the tight junction proteins zonula occludens-1 (ZO-1) and retinal pigment epithelium-specific protein (RPE-65). TER was measured with an epithelial voltohmmeter (World Precision Instruments, New Haven, CT, USA) at regular intervals and was corrected for background resistance contributed by the blank filter and culture medium. Three independent experiments were performed. Results were expressed as mean ohms $\times$ cm$^2$ $\pm$ SD and compared by Student's t-test. Statistical significance was set at $p \leq 0.05$.

## Redox Status and Apoptosis Assay

**Dichlorofluorescein Assay.** Intracellular oxidative stress was determined using a dichlorofluorescein (DCF) assay similar to the method described by Rota et al. (1999) and Marchesi et al. (1999). The fluorescent probe, 2′,7′,-dichlorofluorescin (DCFH)-diacetate (DA) (Molecular Probes, Inc., Eugene, OR, USA) diffuses freely into cells where it becomes trapped after deacylation as the non-fluorescent DCFH. During oxidative stress, DCFH is oxidized to fluorescent dichlorofluorescein (DCF) (Wan et al.,

2003). DCFHDA, aliquotted in dimethyl sulfoxide (DMSO) at a stock solution of 5 M and stored in a desiccator in the dark at -20°C, was diluted in PBS immediately before the experiment. Sterile, 96-well tissue culture plates (black plates with clear bottoms) (Costar, El Sobrante, CA, USA) were seeded with ARPE-19 cells and grown as described above. After culture for 10 days, the cells were washed twice with DMEM/Hams F12 medium without phenol red. ARPE-19 cells were exposed to media with treatment chemicals for 4 hours followed by exposure to 500 μM $H_2O_2$ for 2 hours. After rinsing with PBS, cells were incubated with DCFH-DA (5 mM) at 37°C for 30 minutes. Fluorescence was measured using a FLUOstar Optima multifunctional plate reader (BMG Labtech GmbH, Offenburg, Germany) set to excitation and emission wavelengths of 485 and 528 nm. After measurements were obtained, an excess of $H_2O_2$ (500 μM) was added to each well to determine maximum fluorescence and assure equivalent DCF loading.

**MTT Cell Viability Assay.** Following treatment with bilberry anthocyanins or $H_2O_2$, cell viability was determined using the MTT assay, as previously described (Ballinger et al., 1999; Hansen et al., 1989; Hussain et al., 1993). The assay cleaves tetrazolium salt MTT to formazan by mitochondrial dehydrogenases in viable cells. The MTT assay was purchased from Molecular Probes (Eugene, OR, USA). Cells, grown in 96-well plates as described above, were treated with serum-free medium containing doses of bilberry extracts or $H_2O_2$ for 2 or 4 hours; they were then allowed to recover in conditioned medium for either 1 hour with MTT or for 24 hours where MTT was incorporated in the last hour. The cells were lysed and absorbance measured at 570 nm using a FLUOstar Optima multifunctional plate reader (BMG Labtech GmbH, Offenburg, Germany).

Absorbance values were converted to MTT reduction using a standard curve generated with known numbers of viable cells. MTT reduction for treated samples was then normalized to non-treated control samples and was reported as a control fraction.

**JC-1 Assay.** The JC-1 assay is an apoptotic cell death assay capable of indicating early changes in mitochondrial membrane permeability. The cationic carbocyanin dye, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide), obtained from Molecular Probes, Inc. (Eugene, OR, USA) has been used to determine apoptosis and changes in mitochondrial trans-membrane potential, an index of mitochondrial integrity (Woollacott & Simpson, 2001). JC-1 is present as a green-fluorescent monomer at low concentrations, and on excitation at 490 nm, it emits light at 527 nm. At high concentrations, i.e., >0.1 mM, and in a reducing environment "J-aggregates" form; these aggregates can be excited at 490 nm to emit light at 590 nm resulting in red fluorescence (Cossarizza et al., 1993; Reers et al., 1995). Typical healthy cells possessing high mitochondrial transmembrane potential are capable of concentrating JC-1 dye and thus generating a red-fluorescent signal. Assessing the ratio of green to red fluorescence in a plate reader assay provides a measure of a cell population's relative health. Those cells having lost capacity to maintain high membrane potentials cannot concentrate the JC-1 dye. After treatment with bilberry and $H_2O_2$, adherent cells were incubated in 1 mL phenol- and serum-free DMEM/Hams F12 medium containing JC-1 (1 μg/ml) for 30 minutes at 37°C. The cells were then rinsed with PBS and fluorescence read in a FLUOstar Optima multifunctional plate reader (BMG Labtech GmbH, Offenburg, Germany).

**ATP/ADP assay.** The adenine tri-phosphate (ATP) and adenosine di-phosphate (ADP) assay measures the relative levels of adenylate nucleotides and allows calculation of an ATP/ADP ratio reflective of the cell state. Cells were grown in 96-well microplates for 10 days, as described previously. The cells were rinsed twice with PBS and then incubated with the test compounds in serum- and phenol red–free medium prior to assaying for ATP and ADP. Utilizing a luciferase reaction that consumes ATP, the intensity of light emission (luminescence) was monitored on a FLUOstar Optima multifunctional plate reader (BMG Labtech GmbH, Offenburg, Germany ). ADP was then measured in the same well after its conversion to ATP. The lucerin-luciferase method was conducted with a validated, commercially available kit (ApoGlow™, Adenylate nucleotide ratio assay kit, Cambrex; Alexis Biochemicals, San Diego, CA, USA) per manufacturer instructions.

In brief, the culture plate was removed from the incubator and cooled to room temperature for at least 5 minutes. The assay was conducted at ambient temperature (18–22°C), the optimal temperature for the luciferase enzyme. One hundred μL of Nucleotide Releasing Reagent (NRR) were added to each plate well, and the plate was allowed to stand at room temperature for 5 minutes. A baseline 1-second integrated luminescence reading was made at an emission of 565 nm for each well prior to addition of 20 μL Nucleotide Monitoring Reagent (NMR) per well; this was followed by an immediate 1-second integrated reading (Reading A). After 10 minutes, another 1-second integrated reading was made (Reading B). At this point, 20 μL ADP-Converting Reagent was added to each well and allowed to stand for 5 minutes; the final 1-second integrated luminescence reading was then made (Reading C).

For viability assay purposes, ATP values were sufficient. Comparing ATP concentration and ATP/ADP ratio to appropriate controls allowed for differentiation between apoptosis, cell growth arrest, cell proliferation, and necrosis in the same assay (Bradbury et al., 2000). The ADP/ATP ratio was calculated from measurements A, B, and C as follows: (C – B)/A. Thus, not only were potentially beneficial actions of the test botanical products examined, but their potentially toxic actions were examined as well.

## Protein and mRNA Analysis

**Western Blot Analysis.** Test culture medium was removed from the RPE cells, and cells were washed twice with PBS and then aspirated dry. ZO-1, RPE-65, Bcl-2, Bax, and pro-caspase-3, heme oxygenase-1 (HO-1), glutathione S-transferase pi class P-1 form (GSTP1), and β-actin protein levels were determined by western blot analysis; this was done by homogenizing cells with CytoBuster Protein Extraction Reagent (Novagen via CALBIOCHEM, A Brand of EMD Bioscience, Inc., La Jolla, CA, USA), which contained protease inhibitors (Protease Inhibitor Cocktail Set III, CALBIOCHEM, A Brand of EMD Bioscience, Inc., La Jolla, CA, USA) at a dilution of 1:100 (10 μL protease inhibitor cocktail in 1 mL Cytobuster). Cytobuster volumes were adjusted to yield a protein concentration of about 20 ng protein in 15 μL solution, and the cellular protein mixture was centrifuged in eppendorf tubes for 5 minutes at 10,000 revolutions per minute. The retained supernatant was stored at -80°C until electrophoresis.

Protein concentration was determined using the Pierce BCA Protein Assay (Pierce Biotechnology Inc., Rockford, IL, USA) per company instructions. Samples were boiled

in loading buffer for 5 minutes prior to gel loading. Loading buffer — comprised of Tris HCl 0.05 M (pH 6.8), glycerol, 1 % sodium dodecyl sulfate (SDS), phenol blue (0.01 g/mL) and beta-mercaptoethanol (50 μL/mL) — was added to 20% (v/v). Samples containing 30 μg protein were loaded for electrophoretic resolution on a 12% SDS–PAGE gel and separated under reducing conditions at constant 200 V, 80 mA, 16 W for approximately 40 minutes. Separated protein was electro-transferred onto polyvinylidene difluoride (PVDF) membrane (Invitrogen Corporation, Carlsbad, CA, USA) at 4°C and 30 V overnight. The membranes were blocked with 5% nonfat dry milk powder in PBS containing 0.05% Tween 20 for 60 minutes at room temperature or incubated overnight at 4°C. Primary antibodies, anti human ZO-1, Bcl-2, Bax, pro-caspase-3, HO-1 and GSTP1, were IgG1 isotype from mouse and were obtained from BD Biosciences (San Jose, CA, USA). These antibodies were diluted 1:1000 from the supplied concentration for use on the western blot membranes.

To control for protein loading, the membranes were probed with β-actin mouse monoclonal antibody obtained from Novus Biologicals, Inc. (Littleton, CO, USA), used at 1:50,000 dilution. After rinsing, blots were incubated for 60 minutes at room temperature, with a horseradish peroxidase–conjugated secondary antibody. Secondary antibody was sheep anti-mouse IgG and was obtained from GE Healthcare Biosciences Corp. (formerly Amersham Biosciences; Piscataway, NJ, USA), and used at 1:5000 dilution. The primary antibody against human RPE-65 was a polyclonal rabbit serum and was obtained as a gift from Dr. Rosalie Crouch (Medical University of South Carolina, Charleston, SC, USA) and used at either 1:250 or 1:1000 dilutions. The secondary antibody used to visualize the RPE-65 antibody was a stabilized goat anti-rabbit HRP-

conjugated antibody obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA), and used at a 1:1000 dilution. The secondary antibody substrate was dura, femto, or pico SuperSignal West Chemiluminescent Substrates from Pierce Biotechnology, Inc. (Rockford, IL, USA), and was matched to the primary signal empirically. Exposed x-ray film was quantified by densitometry analysis using the Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Quantitative Real-Time Polymerase Chain Reaction.** Total RNA was extracted from RPE cell cultures using the RNeasy Kits (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's protocol. Briefly, cell-culture medium was completely aspirated and cells were washed with PBS; 0.10–0.25% trypsin in PBS was added. After cells detached from the dish or flask, medium containing serum was added to inactivate the trypsin and cells were transferred to a centrifuge tube and pelletted by centrifugation at 300 × g for 5 minutes. Supernatant was completely aspirated. Cells were lysed and cellular RNA was stabilized by addition of RNeasy guanine thiocyanate buffer. RNA was isolated from the samples by binding to a silica gel based membrane in an ethanolic mixture and then eluted with water. Collected RNA was stored at -80°C until used for quantitative real-time polymerase chain reaction (rt-PCR) determinations.

First-Strand cDNA synthesis from 500 ng RNA from RPE cells was accomplished using SuperScript™ III Reverse Transcriptase (RT) (Invitrogen Corporation, Carlsbad, CA, USA) in a 20-μL reaction volume. Briefly, 250 ng of random primers, 500 ng total RPE RNA, 1 μL 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH), and 13 μL sterile, distilled diethylpyrocarbonate-treated water were added to a nuclease-free microcentrifuge tube. The reaction mixture

was heated to 65°C for 5 minutes and then placed on ice for at least 1 minute. After a brief centrifugation, 4 μL 5X First-Strand Buffer, 1 μL 0.1 M DTT, 1 μL RNaseOUT (Recombinant RNase Inhibitor (40 units/μL, Invitrogen Corporation, Carlsbad, CA, USA), and 1 μL of SuperScript III RT (200 units/μL) were added to the reaction; this was followed by further incubation at 25°C for 5 minutes before a temperature increase to 42°C for 50 minutes. The enzyme was inactivated by heating at 70°C for 15 minutes. To remove RNA complementary to the cDNA, 1 μL (2 units) of *E. coli* RNase H was added and the mixture incubated at 37°C for 20 minutes. The resulting cDNA was used as a template for amplification in rt-PCR.

An ABI PRISM® 7000 instrument was used to conduct rt-PCR using LUX™ Fluorogenic Primers. Both certified LUX housekeeping gene primers and custom LUX primers were obtained from Invitogen Corporation (Carlsbad, CA, USA) for use in rt-PCR reactions. GAPDH and β-actin were Invitrogen proprietary 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein (JOE) dye–labeled housekeepng primers. HO-1 and GSTP1 primers were sequence designed and displayed in Table 1. These primers were 6-carboxyfluorescein (FAM)–labeled to permit concurrent use with the JOE-labeled housekeeping primers.

**Table 1.** Heme oxygenase and glutathione S-transferase Lux Primer definitions and sequences

| Gene | Primer | Strand | 3' Loc | Sequence |
|---|---|---|---|---|
| HO-1 | X06985.1_633RL | Reverse | 633 | cgcatATCTCCAGGGAGTTCATGcG |
| HO-1 | X06985.1_633RL/611FU | Forward | 611 | ACATTGCCAGTGCCACCAAG |
| GST-pi | NM_000852.2_135FL | Forward | 135 | cggtcGAAGGAGGAGGTGGTGACcG |
| GST-pi | NM_000852.2_135FL/155RU | Reverse | 155 | TAGGCAGGAGGCTTTGAGTGAG |
| GAPDH | NM_002046 | | | Invitrogen Certified LUX™ Primer Set |
| β-Actin | NM_001101 | | | Invitrogen Certified LUX™ Primer Set |

A Master Mix was prepared (reaction volume of 50 μL) containg Platinum® qPCR SuperMix-UDG 25 μl (1X), LUX labeled #1 primer 100 nM, Unlabeled #1 primer 100 nM, LUX™ labeled #2 (housekeeping) primer 100 nM, Unlabeled #2 (housekeeping) primer 100 nM, ROX Reference Dye 500 nM., MgCl2 3 mM, and autoclaved distilled water to 40 μL. Ten μL of cDNA generated from 500 ng of total RPE mRNA was added to the reaction and the mixture placed in the ABI PRISM® 7000 programmed to cycle as follows: UDG reaction 50°C for 2 minutes; UDG inactivation/template denaturation 95°C for 2 minutes; 45 cycles of denaturation 95°C for 15 seconds, hybridization at 55°C for 30 seconds, elongation at 72°C for 30 seconds; a hold at 60°C for 30 second; and a final melting curve analysis. Rt-PCR analysis of gene expression data was accomplished using the 2-ΔΔCT method as described by Livak & Schmittgen (2001). The HO-1 mRNA levels were normalized to that of GAPDH mRNA and expressed relative to control using the ΔΔCt method.

**Data Analysis and Statistics.** Results are expressed as means ± standard deviation (S.D.). Where appropriate, data were expressed as fold increases over values obtained under control conditions. Baseline characteristics of the treatment groups were compared using independent paired t-tests. Treatment effects as well as interactions were determined by repeated measures analysis of variance (ANOVA) using the Statistical Analysis Systems statistical software (SAS Institute, Inc., Cary, NC, USA). Linear regression analysis was also conducted using SAS. Minimum established criterion for data inclusion involved at least three independent experiments using independent cell cultures, with each condition within an experiment performed in triplicate. Differences of $p \leq 0.05$ were considered statistically significant.

# CHAPTER V

# RESULTS

## Bilberry Extract

**Bilberry Anthocyanin Material.** Using a spectrum pH differetial method, total anthocyanins in the bilberry extract material was calculated as 26.1%. When determined by high pressure liquid chromatography/ultraviolet (HPLC/UV) and expressed as cyanin 3-glucoside equivalents, anthocyanin content was 27.7% of dry weight. Total polyphenols, as determined with the Folin-Ciocalteu assay and using catechin as the reference phenol, showed 620 g/kg phenolics content. This suggests that the anthocyanins represented approximately 45% of the extract's phenolic content.

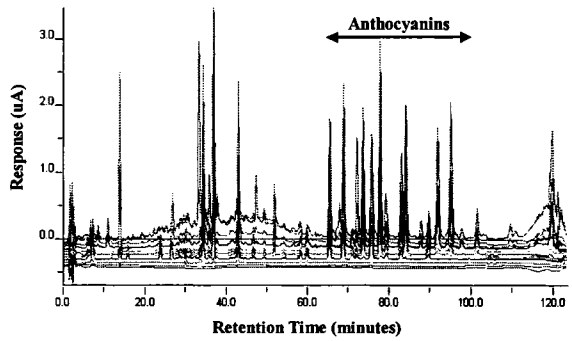



**Figure 2.** HPLC/ECD analysis of bilberry anthocyanin–rich extract.

Analysis using high-pressure liquid chromatography/electrochemical detection (HPLC/ECD) (Figure 2) shows a typical anthocyanin pattern with resolution in the 60–110 minute retention time region for the 15 bilberry anthocyanins previously reported. Using authentic standards (obtained from Polyphenols Laboratories AS, Sandnes, Norway), the following anthocyanins, in order of elution, were identified in the bilberry extract: delphinidin 3-galactoside, delphinidin 3-glucoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, cyanidin 3-glucoside,

petunidin 3-galactoside, petunidin 3-glucoside, cyanidin 3-arabinoside, peonidin 3-galactoside, malvidin 3-galactoside, peonidin 3-glucoside, malvidin 3-glucoside, and malvidin 3-arabinoside.

Anthocyanin confirmation of the bilberry extract's compound identities was accomplished using liquid chromatographic mass spectrometry (LC/MS/MS) with selected ion monitoring. Figure 3 shows a typical elution pattern of bilberry anthocyanin glycosides from HPLC using a UV detector monitoring at 520 nm. Chromatographic methods were synchronized by monitoring retention time patterns of eluting compounds; montoring was performed using photodiode array detection on both the HPLC/ECD and LC/MS/MS systems. This enabled determination of four chemical properties (i.e., retention time, UV absorption, oxidation potential, and mass fragmentation patterns) for identification of compounds in the bilberry extract or experimental samples.

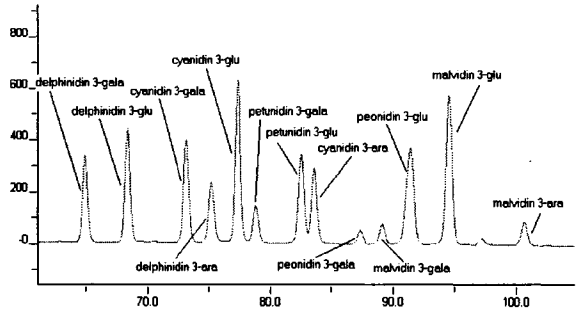



**Figure 3.** UV 520 nm spectogram of anthocyanins from bilberry extract.

Portions of the study required securing an anthocyanin-only fraction. Here, the original extract material was dissolved and applied to a semi-preparative low-pressure chromatography column packed with C18 solid phase. Eluted fractions were analyzed using HPLC/ECD and appropriate fractions combined to yield two pools: an anthocyanin pool and a pool containing the extract's other phenolic components.

Figure 4A (upper panel) shows the HPLC/ECD analysis of fraction 72, which represents compounds isolated in fractions 70–75. These compounds were collected,

combined into an anthocyanin pool, dried down, and reconstituted in the original bilberry extract concentration for cell treatment. Figure 4B (middle panel) shows fraction 58, which represents fractions 55–70 containing compounds other than anthocyanins (e.g., phenolic acids) in the bilberry extract material. Figure 4C (lower panel) shows five authentic purified anthocyanin standards. The standards were, in order of elution, cyanidin 3-galactoside, cyanidin 3-glucoside, petunidin 3-glucoside, peonidin 3-glucoside, and malvidin 3-glucoside.

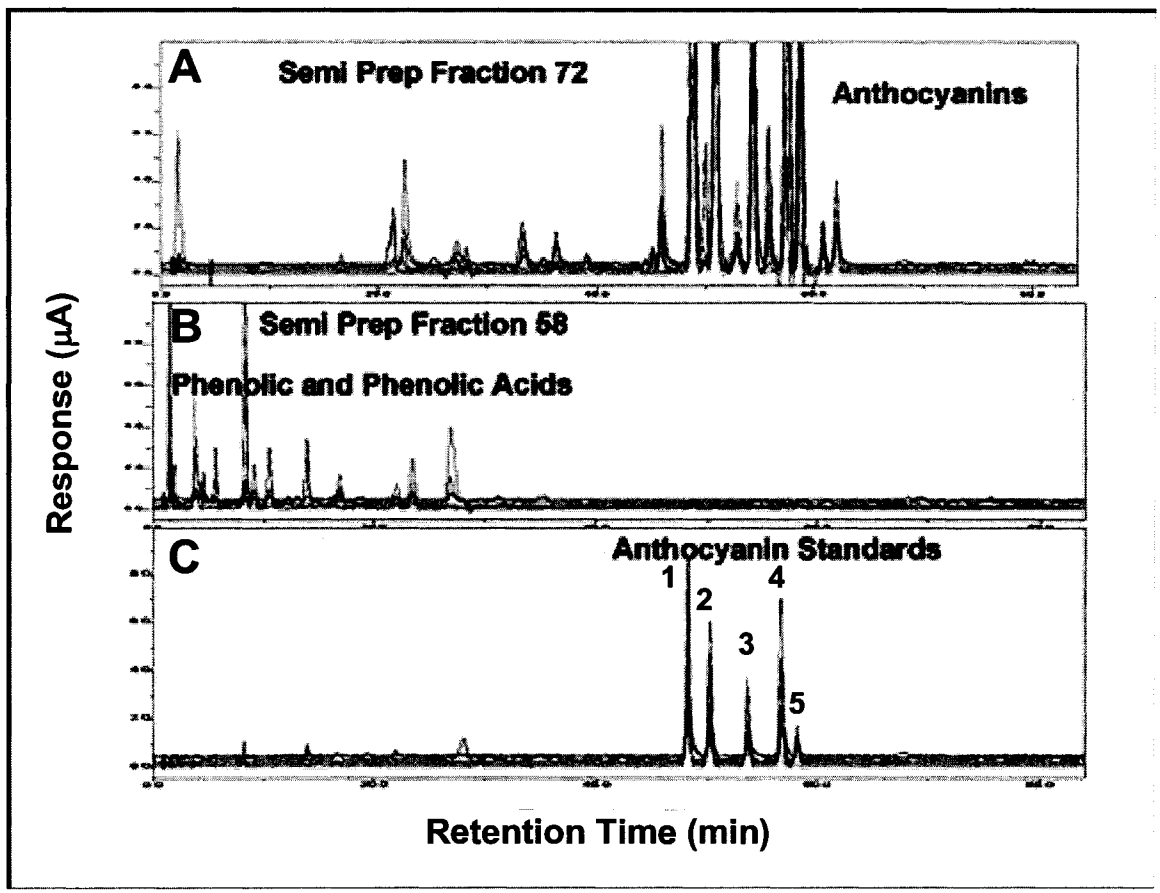



**Figure 4.** Fractionation of bilberry extract. Panel A represents HPLC/ECD pattern from a n anthocyanin-containing fraction. Panel B represents an analysis of a phenolic acid–containing fraction. Panel C is an injection containing five authentic purified anthocyanins: (1) cyanidin 3-galactoside, (2) cyanidin 3-glucoside, (3) petunidin 3-glucoside, (4) peonidin 3-glucoside, and (5) malvidin 3-glucoside..

## RPE *in Vitro* Model and Anthocyanin Cellular Uptake

**RPE Cell Culture.** Initial experiments established a stable or reproducible hydrogen peroxide ($H_2O_2$) lethal dose 50% ($LD_{50}$) within the retinal pigment epithelium (RPE) cell culture model and measured resulting changes in glutatione (GSH) levels. Inducing a reproducible level of $H_2O_2$-induced oxidative stress proved difficult as time changes in culture of as little as half a day resulted in dramatic changes in $H_2O_2$ $LD_{50}$. ARPE cells were grown as described by Dunn et al. (1996) until sufficient cell numbers were produced to permit plating for experimentation. A review of the literature regarding $H_2O_2$ treatment of RPE cell cultures revealed a wide range of culture times, which varied from undefined to sub-confluent overnight cultures to several months in culture (Choudhary et al., 2005; Garg & Chang, 2003; Geiger et al., 2005; Godley et al., 2002; Jarrett & Boulton, 2005; Kasahara et al., 2005; King et al., 2005; Lu et al., 2006; Marin-Castano et al., 2005; Sreekumar et al., 2005; Tate et al., 1999; Tsao et al., 2006; Yu et al., 2005; Zareba et al., 2006). Likewise, data reported from RPE experiments revealed

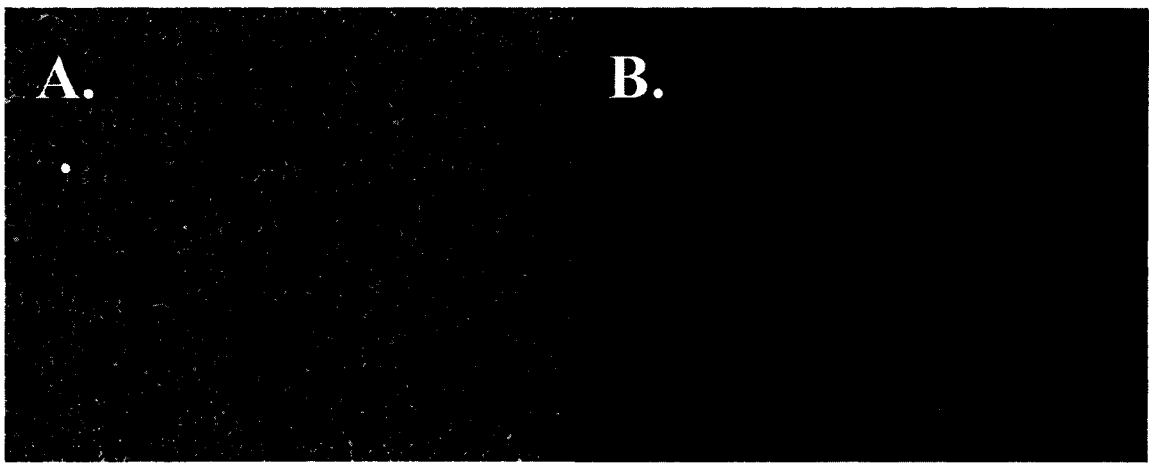



**Figure 5.** RPE cells grown on Transwell membranes for 10 days. Panel A shows 20x objective magnification. Panel B shows 80x objective magnification).

differences in the observed $H_2O_2$ $LD_{50}$ concentrations, which varied 60–800 μM. Many researchers did not establish the $LD_{50}$, and chose to work against a defined $H_2O_2$ level with degree of damage defined by loss of cell viability versus time. However, in order to investigate bilberry extract's potential protective properties against $H_2O_2$-induced oxidative stress, it was necessary to establish a stable and reproducible level of oxidative stress in RPE cell cultures. Defining culture conditions that would yield a reproducible or reliable oxidative stress level became crucial to the outcome of these studies. Therefore, studies were initiated to determine the conditions (primarily time required in culture after cell plating) that would result in a state of ARPE-19 cell differentiation of sufficient reproducibility for repeated oxidative stress experiments.

ARPE-19 cells can be cultured on plastic culture dishes or on Transwell membranes. When plated sub-confluent at densities of 20,000 cells/$cm^2$, as reported by Dunn et al. (1996), and grown for 3 days, the monolayer appeared confluent and exhibited a "cobblestone" pattern. Cell counts at this point were approximately 60,000 cells/$cm^2$; however, when maintained in culture an additional 7 days (total 10 days in culture) cell counts approached 80,000 cells/$cm^2$, and the cells appeared smaller and more tightly packed in the cobblestone pattern. The ARPE-19 cells were cultured per the American Type Culture Collection, (ATCC, Manassas, VA, USA); ATCC instructions called for a growth medium comprised of 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/Hams F12) medium containing 10% fetal bovine serum. While this seemed a high serum concentration, the cells tolerated it well and grew rapidly. After 3 days in culture, the medium's fetal bovine serum concentration was decreased to 5% to slow division and encourage differentiation. Figure 5 (above) shows

the typical RPE monolayer appearance similar to that described by the line originators (Dunn et al., 1996). The Transwell membrane pores are visible below the cell monolayer as small light dots (lower magnification) or as black holes (higher magnification). Cell nuclei are dark stained and oval in shape. During apoptosis, they exhibit "blebbing" prior to fragmentation.

**Determining the State of RPE Differentiation.** Evidence suggests that differentiated RPE cells *in vivo* remain quiescent, in $G_0$ phase, and that they do not undergo significant cell division (Ts'o & Friedman, 1967). When placed in culture, however, RPE cells do reenter the cell cycle and proliferate (Korte et al., 1994; Stroeva & Mitashov, 1983). While *in vitro* RPE cells do not exhibit the complete complement of *in vivo* phenotypic characteristics, enough RPE characteristics remain to permit study of transport, injury, and survival (Newsome, 1983). As mentioned above, most studies examining effects of oxidative stress on RPE have been performed *in vitro* with RPE cells actively proliferating. These cells cannot conduct many functions of their differentiated phenotype the moment they leave the $G_0$ phase. RPE cells play a critical role in the development and maintenance of adjacent photoreceptors by acting as a formed polarized epithelial barrier and by supporting the photoreceptors, where they provide nutrients and remove metabolic debris. When native "differentiated" and cultured dedifferentiated human RPE cells are compared in proteomic analysis, two proteins (a putative receptor for plasma retinol-binding protein (RPE-65) and cellular retinaldehyde-binding protein (CRALBP)) are present in native differentiated RPE cells (Alge et al., 2003). RPE cells exhibit differentiated properties when displaying cuboidal morphology, functional polarity, and expression of RPE-specific gene markers, including CRALBP and RPE-65.

Alizadeh et al. (2001) used northern blot analysis to show that ARPE-19 cells cultured for 3 months displayed messenger RNA (mRNA) expression of CRALBP and RPE 65 while those grown only 3 days in culture did not. RPE-65 is a 61-kDa protein originally identified in RPE cells (Bavik et al., 1992; Hamel et al., 1993; Nicoletti et al., 1995). Unlike observations by Alizadeh et al. (2001), our study detected human RPE-65 and zona occludens-1 (ZO-1), a protein associated with tight junctions, by western blot analysis in ARPE-19 cultures as early as 1 day in culture (Figure 6), though the levels were very low. By 12 days in culture, RPE-65 reached 85.6 ± 5.2% percent of maximal expression. The antibody used against RPE-65 was a polyclonal rabbit serum rather than monoclonal antibody; therefore, determination of RPE-65 levels in cultures of human umbilical vein endothelial cells (HUVEC), human intestinal epithelial cells (Caco-2), and human-derived colonic epithelial cells (NCM460) were used as controls. RPE-65 levels in these endothelial and epithelial cells were minimal and did not exceed levels seen in 1 day–old RPE cultures.

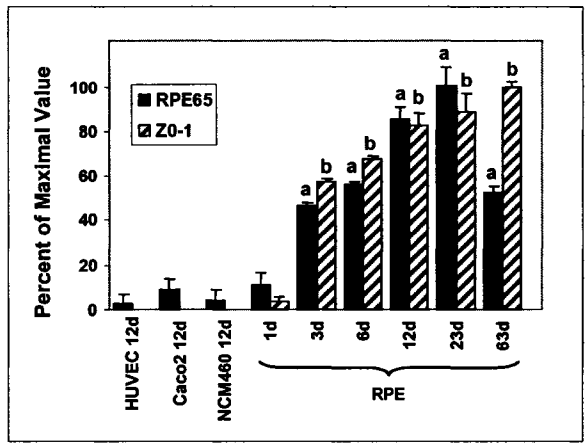



**Figure 6.** Western blot analysis of RPE-65 and ZO-1 in cultured RPE cells. RPE-65 and ZO-1 increase with time in culture. Other epithelial cells express little of these proteins. Human umbilical vein endothelial cells (HUVEC), human intestinal epithelial cells (Caco-2), and human-derived colonic epithelial cells (NCM460) were used as control endothelial and epithelial cells. Means ± standard deviation (S.D.) represent at least three cultures (n=3). For 12-day and 23-day values (n=6), no statistical significance was detected.
a and b = significance ($p \leq 0.05$) for RPE-65 and ZO-1, respectively, vs. 1 day mean and vs. means for other cell types.

Tight junctions are essential for establishing epithelial cell surface polarity and preventing lipid diffusion in the outer leaflet of the plasma membrane

between apical and basolateral membrane domains (Jin et al., 2002). To confirm the culture time during which ARPE-19 differentiated to establish intact tight junctions capable of barrier function, western blot analysis was conducted to determine the presence of ZO-1 (Tserentsoodol et al., 1998). Like RPE-65, ZO-1 was detected as early as 1 day in culture and increased in culture through day 12 (see Figure 5).

ZO-1's presence is required for, but does not prove the existence of, established tight junctions. When combined with transepithelial resistance (TER) results, the existence of functional tight junctions could be established with more certainty. Both RPE-65 and ZO-1 reached 80% of their maximal value 10–12 days in culture. Continued culture to 63 days did not significantly increase levels of ZO-1. However, at 63 days a statistically significant decrease was observed in RPE-65, which suggested a possible loss of some RPE functions with increasing RPE age in culture.

TER experiments were performed to establish the existence of RPE tight junctions capable of inhibiting ion flow. RPE cells were grown on Transwell membranes, which allowed them to be moved into an apparatus permitting the application of a potential across the membrane and any monolayer barrier growing upon it. When tight junctions were present and functioning, electrical resistance could thus be measured.

Figure 7 displays the results of TER experiments conducted in four independent cultures. TER was established and stabilized in RPE cultures by 10 days. TER measurements ranged from 50 to 120 ohms/cm$^2$ in these experiments; levels were lower than those reported by Kannan et al. (2001) for primary human RPE cultures, but in agreement with values reported by others for ARPE-19 cells (Dunn et al., 1996).

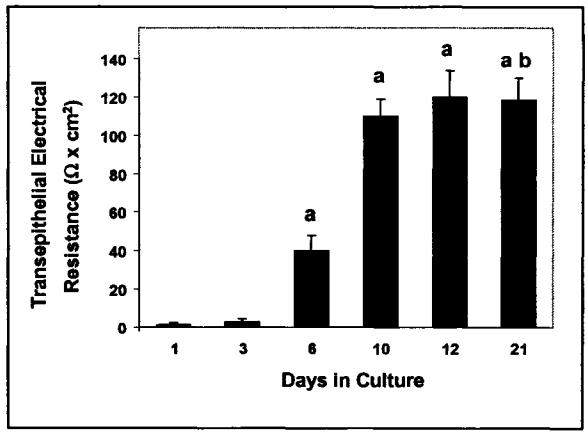



**Figure 7.** Transepithelial resistance of cultured RPE cells. Transepithelial resistance increases with time in culture. All values are mean ± S.D. of four independent cultures (n=4).
a = significant ($p \leq 0.05$) vs. 1 and 3 days;
b = significant vs. 1, 3, and 6 days and not significant (n.s.) vs 10, 12, and 21 days.

It has been suggested that when fully differentiated, RPE cells *in vivo* withstand oxidative stress levels among the highest in the mammalian body. The cellular redox potential of most cells depends upon GSH maintenance as the primary source for reducing cell equivalents (Cotgreave et al., 2002). Alteration of intracellular GSH levels has been associated with both the production of reactive oxygen intermediates and activation of signal transduction pathways critical to cellular activity (e.g., division and proliferation arising from an oxidative stress defense, apoptosis) (Armstrong et al., 2002). RPE cells produce among the highest GSH levels in the body, illustrated by the additional requirement for inclusion of L-glutamine at 2 mM in the media to support both the high-energy requirements and serve as precursor for high GSH production (Evans et al., 2003; Newsholme et al., 2003); however, little is said of it in the original manuscripts

regarding RPE culture. When sufficient precursor metabolites are present, RPE cells are normally well-protected against oxidative damage, and GSH levels are regenerated by recycling and sparing reactions through their antioxidant complements, such as catalase, superoxide dismutase, glutathione peroxidase, and vitamins E and C (Newsome et al., 1994). To determine if, upon full differentiation, RPE cells displayed the greatest resistance to oxidative stress by exhibiting higher levels of GSH production, GSH levels were monitored at varying times in culture (Figure 8).

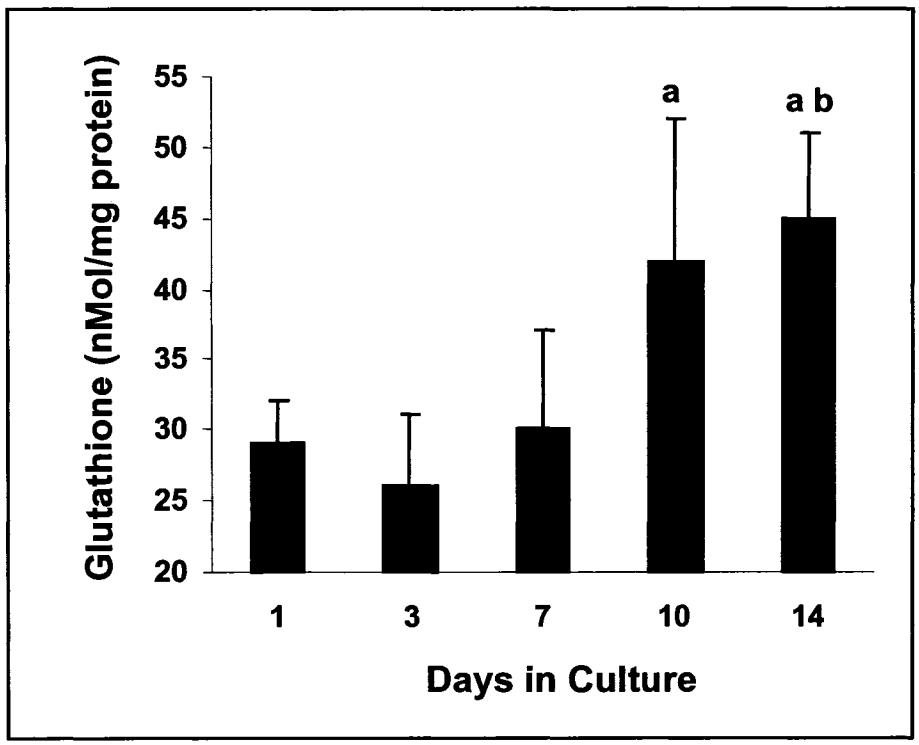



**Figure 8.** Effect of time in culture on GSH levels in cultured RPE cells. All values are mean ± S.D. of three independent cultures (n=3). a = significant vs. 1 and 3 days ($p \leq 0.05$); b = significant vs. 1, 3, and 7 days and n.s. vs. 10 days.

***In Vitro* Anthocyanin Uptake and Metabolism.** Transwell chambers were utilized to investigate the basal to apical anthocyanin transport across membranes exhibiting a minimum TER of 100 ohms/cm$^2$. Serum-free DMEM/Hams F12 media without phenol red and containing 1 µg/mL bilberry extract were placed in the lower chamber of the Transwell apparatus. The upper chamber was equalized to the media level in the lower chamber with media containing no anthocyanins. At 30, 60, 120, and 240 minutes after loading bilberry into the lower chamber of the Transwell, 0.1 mL was withdrawn from the upper and lower chambers for analysis by HPLC/ECD (Figure 9). At 4 hours, the integrity of the tight junctions was tested by TER measurement and found to be intact at approximately 100 ohms/cm$^2$.

Anthocyanin values in the upper chamber are plotted versus time (Figure 10) for Transwell chambers with and

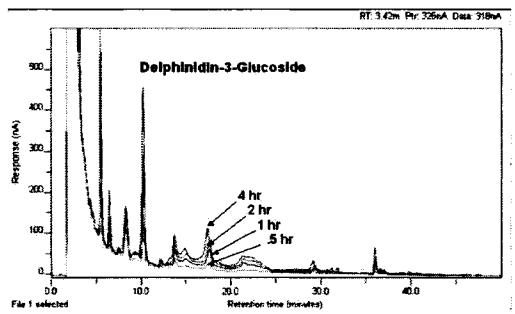



**Figure 9.** Representative anthocyanin movement across the RPE monolayer. Typical HPLC-ECD tracing of multiple analysis runs showing channel 6 (analysis at 350 mV potential) of samples collected from the upper Transwell chamber.

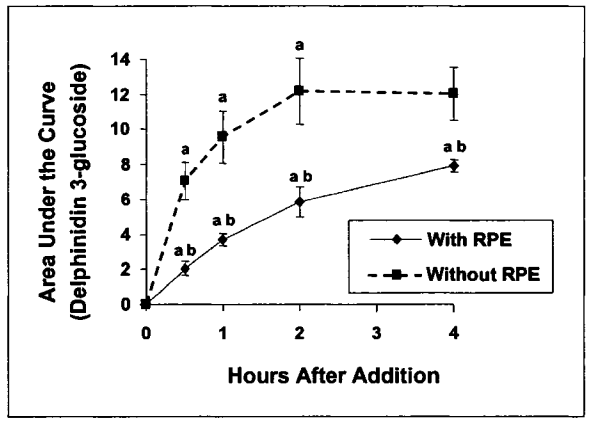



**Figure 10.** Delphinidin-3-glucoside crossing the RPE monolayer. Plot of Anthocyanin delphinidin-3-glucoside transport across the Transwell membrane with RPE cells (diamonds) and without RPE by passive diffusion through pores in Transwell membrane (squares). Data represent the mean ± S.D. of three independent cultures (n=3). a = significant vs. samples from the preceding time period ($p \leq 0.05$); b = significant vs. the corresponding time period samples from membranes without RPE cells. RPE cells maintained barrier function for over four hours.

without cells. Transwell chambers without cells represented passive diffusion across the transwell membrane and showed system equilibrium by 2 hours; differences between system equilibria by 2 hours and 4 hours were not statistically significant. Transwell chambers carrying membranes on which an intact RPE monolayer was grown displayed a barrier function, but permitted the anthocyanin passage albeit at a rate slower that that of passive diffusion. Equilibrium was not achieved between the two chambers within the 4-hour experimental period; this suggests that bilberry extract in the media at 1 μg/mL did not perturb the cell sufficiently to disrupt tight junction integrity. Instead, it shows only that bilberry anthocyanins can pass through the RPE monolayer. These results do not prove that bilberry anthocyanins are internalized.

To determine if anthocyanins were internalized in RPE, cells were grown for a total of 10 days (i.e., to confluence (3 days) plus 7 days) in T150 flasks and treated with bilberry extract. Although anthocyanins were detected in the apical chamber of the RPE transwell experiments within 30 minutes using 1 μg/mL bilberry extract, a concentration of 100 μg/mL bilberry extract was used to determine intracellular uptake levels. RPE cells exposed to this level for periods over 24 hours did not show signs of toxicity. Anticipated levels of intracellular anthocyanins were expected to be low.

Other cell types, such as enterocytes, take up and transport less than 5% of available anthocyanins in an *in vitro* system (McDougall et al., 2005). In this study, the combination of the larger cell numbers available from T150 flasks and use of 100 μg/mL bilberry extract resulted in sufficient anthocyanin extraction to permit HPLC/ECD and LC/MS/MS detection of individual anthocyanins from intracellular samples. Similar experiments have been conducted in bovine aortic endothelial cells *in vitro* using the

flavonol morin at a concentration of 200 μmol/L where flavonoid uptake was demonstrated after 8 minutes of incubation (Schramm et al., 1999).

Little evidence exists regarding flavonoid uptake and sequestration into cellular organelles. Elderberry anthocyanins have been shown to localize both within the cell membrane and the cytosol of vascular endothelial cells *in vitro* following supplementation; this occurred as quickly as 1 hour, though this was the shortest incubation time used (Youdim et al., 2000). Elderberry anthocyanins were used at 1 mg/mL, with incorporation levels at 1 hour over 50% of the maximum values observed after 4-hour incubations.

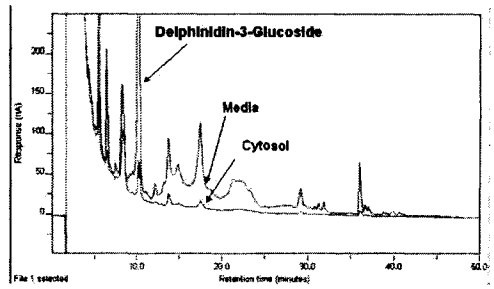



**Figure 11.** Analysis of media and cytosol from RPE cells exposed to bilberry. This figure shows analysis of media containing 100 μg/mL bilberry and cell cytosol from cells exposed to bilberry-containing media

Elderberry anthocyanin levels incorporated after incubation with 100 μg/mL were 60% of the maximum levels observed between 400 μg/mL and 1 mg/mL. Therefore, initial bilberry anthocyanin incubations were conducted at 100 μg/mL. Although RPE cells are not endothelial cells, calculations from evidence presented by Youdim et al. (2000) suggest that, either by pinocytosis or GLUT/SLGT1 transporters, RPE could internalize 1.5 μg anthocyanins/mg protein to the cell interior in 15 minutes. Indeed, anthocyanins were internalized and were sufficient for analytical detection by HPLC/ECD and LC/MS/MS.

RPE cells were exposed to DMEM/Hams F12 media without phenol that contained 100 μg/mL bilberry extract for 15 minutes, with the cells subsequently rinsed 3

times with DMEM/Hams F12 media free of bilberry. Cells were collected by careful scraping from the flask surface. The cells were lysed by the method of Youdim et al. (2000) and the cytosolic and microsomal components then isolated. Briefly, following incubation, culture plates were placed onto ice, the media removed, and the cells washed 3 times with cold phosphate-buffered saline (PBS). Cells were collected by scraping with a teflon policeman into 1 mL PBS and centrifuged for 10 minutes at 8,000 g in a microfuge, after which time the supernatant was removed.

Cells were washed twice more with PBS and the pellet finally reconstituted in 300 μL of 1% iced Triton X-100 in PBS. Cells were then disrupted by sonication in a chilled Eppendorf tube in an ice bath with a series of three 5-second pulses every 30 seconds (output 6, Sonicator M234; MSE). Cell membranes and debris were pelletted at 10,000 g using a table-top ultracentrifuge (TL-100; Beckmann Coulter, Inc., Fullerton, CA, USA) and allowed to stand at 4°C for 30 minutes. Cell membranes and cytosol were again separated by centrifugation at 10,000 g for 15 minutes. A small amount of cytosol was removed for protein determination. An aliquot (60 μL) of 1N HCl was added per 300 μL cytosol prepraration prior to centrifugation and injected onto HPLC/ECD. Membrane pellets were extracted with 300 μL acidic 50% methanol in PBS; this was dried and any residue resuspended in mobile phase for HPLC analysis.

Un-metabolized anthocyanins were detected via HPLC/ECD in the cytosolic preparation of cells exposed to bilberry-containing media (Figure 11). Although the membrane-containing pellet was not analyzed for anthocyanins, the pellet from cells exposed to anthocyanins exhibited a blue/red color that was not elutable or washed away with repeated rinsing using either PBS or 50% methanol in PBS. Anthocyanins were not

detected in the pellet's methanolic extract. This suggests that, in addition to localization in cytoplasm, anthocyanins may also bind tightly to membrane proteins. Samples were analyzed by LC/MS/MS. Data from a 15-minute exposure to bilberry-containing media showed no detectable methylated or glucuronated products in the cytosolic or media preparations. However, the detection limits of both the HPLC/ECD and the LC/MS/MS may have precluded results for metabolites at this time point.

In experiments conducted in phosphate buffer at physiological pH, some purified anthocyanins standards become unstable with losses evident in as little as 2 hours (Dr. Wilhelmina Kalts, Agriculture and Agri-Food Canada, personal communication). During the conduct of this research, malvidin glycoside standards appeared to be more stable than other anthocyanins; therefore, a malvidin metabolite was selected in the effort to detect the presence of.anthocyanin metabolism in RPE cells *in vitro*. Kay et al., (2004) have demonstrated these metabolites in humans after consumption of a chokecherry extract. The proposed metabolic pathway for malvidin glucuronide production begins with hydrolysis of cyanidin 3-galactoside by β-galactosidease or lactase-phlorizin hydrolase producing the aglycone cyanidin. Cyanidin then undergoes glucuronidation by UDP-glucuronosyltransferase. Addition of methoxy groups at the 3′ and 4′ positions by catechol-O-methyltransferase followed by oxidation by cytochromes-P450 yields mavidin glucuronide, with a molecular formula of $C_{23}H_{23}O_{13}$ and a m/z = 507/331. A mass/fragment table is shown in Table 2.

RPE-19 cells were cultured for 10 days in T150 flasks and exposed to 100 μg/mL bilberry extract in DMEM/Hams F12 media without phenol red for 4 hours. All media were collected and pooled for analysis by LC/MS/MS. An initial LC/MS scan of total

ions showed several compounds with m/e 507 as well as m/e equivalent to those seen for anthocyanin aglycones. To confirm that these could be glucuronidated anthocyanidins, samples were committed to LC/MS/MS analysis using selected ion monitoring at m/e 507. Chromatography revealed two compounds with molecular weights of 507 at retention times of 69.5 and 65 minutes and with fragments of 331.1 and 317.1 respectively (Figure 12). These molecular weights and fragmentation patterns were expected from malvidin glucuronides and from methyl petunidin glucuronide.

**Table 2.** M/Z chart for LC/MS/MS selected ion analysis of anthocyanin metabolites

| | **Aglycone** | **Gal or Glu Plus 162** | **Glucuronide Plus 176** | **Methyl Plus 14** | **Glucuronide And Methyl Plus 190** |
|---|---|---|---|---|---|
| **Pelargonidin** | 271 | 433 | 447 | 285 | 461 |
| **Cyanidin** | 287 | 449 | 463 | 301 | 477 |
| **Peonidin** | 301 | 463 | 477 | 315 | 491 |
| **Delphinidin** | 303 | 465 | 479 | 317 | 493 |
| **Petunidin** | **317** | 479 | 493 | 331 | **507** |
| **Malvidin** | **331** | 493 | **507** | 345 | 521 |

Comparison of compound retention times in feeding media exposed to cells and reserved versus *not* exposed to cells signifies that these compounds formed as a result of RPE cell metabolism. The compounds were not present in the original feeding medium or in the bilberry extract material.

These findings indicate that some bilberry anthocyanins are taken into the cytosol of RPE cells and metabolized by phase II enzymes. Both products are methylated, and of all the anthocyanins, malvidin is the most highly methylated anthocyanin. It is possible that other anthocyanins may have been hydrolyzed and subsequently glucuronidated and methylated, which would form malvidin glucuronides and methyl petunidin glucuronide products not immediately derived from malvidin or petunidin. If indeed malvidin products are the most stable, it is not surprising that they are found most easily in sufficient detectable quantities, especially if their concentrations are enhanced by derivation resulting from other anthocyanin methylation.

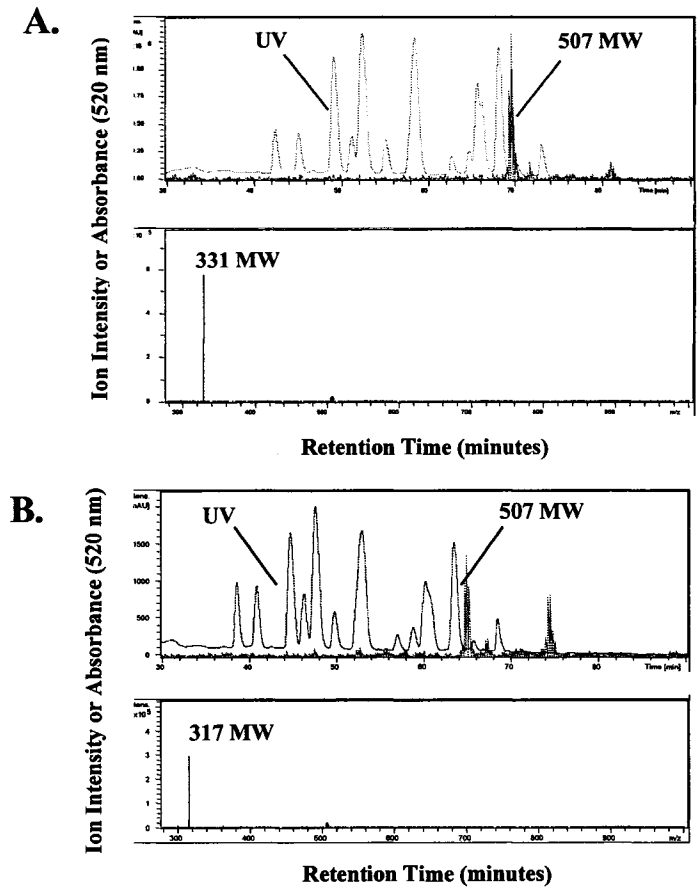



**Figure 12.** Phase II metabolites in media from RPE cells exposed to bilberry. Panel A shows malvidin glucuronide. Panel B shows methyl petunidin glucuronide. Each panel shows the total UV chromatogram, parent 507 ion scan at respective retention times, and the primary fragment ion on the lower portion of the panel.

## RPE Oxidative Stress and Apoptosis

**Characterization of the RPE Oxidative Stress Model**. As mentioned above, plating RPE cells at 20,000 cells/cm$^2$ produced RPE cultures that reached confluence by 3–4 days. Upon replacing the media, which contained 10% fetal calf serum, at 3 days with media containing 5% serum, RPE differentiated by 10 days in culture. As shown previously (Figures 6–8), by 10 days in culture RPE had developed tight junctions, expressed RPE-65, and reached near maximal GSH production. By 10 days in culture, RPE cultures also exhibited increased $H_2O_2$ tolerance and with a stabilized $H_2O_2$ $LD_{50}$ of aproximately 500 μM across all experiments conducted at 10 days (Table 3).

**Table 3.** RPE days in culture versus $H_2O_2$ $LD_{50}$ determined by MTT, JC-1, and ATP assays

| Day in Culture | Assay Type | H2O2 LD50 | Number of Dose Response Curves |
|---|---|---|---|
| 3 | MTT | 41 ± 14 | 8 |
| 3 | ATP | 108 ± 13 | 8 |
| 4 | ATP | 150 ± 34 | 4 |
| 7 | ATP | 119 ± 42 | 8 |
| 7 | ATP | 123 ± 38 | 8 |
| 9 | MTT | 342 ± 15 | 7 |
| 10 | MTT | 566 ± 14 | 8 |
| 10 | JC1 | 493 ± 39 | 8 |
| 10 | JC1 | 520 ± 34 | 8 |
| 10 | JC1 | 527 ± 34 | 8 |
| 10 | ATP | 473 ± 35 | 8 |
| 10 | ATP | 508 ± 21 | 8 |
| 10 | ATP | 519 ± 17 | 8 |
| 12 | ATP | 585 ± 13 | 8 |
| 35 | MTT | 583 ± 14 | 9 |

Cell viability can be determined using MTT, JC-1, or ATP assays, as well as many other assays that depend upon cellular reductive capability. The majority of cell viability tests performed in this study were accomplished using the MTT and ATP assays. A typical MTT assay conducted on a 9-day-old culture is shown in Figure 13. Values are expressed as percent absorbed at 485 nm for control cells (i.e., cells not treated with $H_2O_2$). Values were corrected for the amount of absorbance in wells not treated with bilberry extract and that had been exposed to 2000 μM $H_2O_2$ (the dose found to be 100% lethal in these experiments).

Figure 13 shows a typical result from RPE cells exposed to bilberry extract in decreasing doses (from 1 to $10^{-5}$ mg/mL extract material) for 4 hours followed by a 2-hour exposure to $H_2O_2$ (doses increasing from 0 to 1000 $\mu$M $H_2O_2$). Neither these individual data (within a single experiment) nor the combined data across the three independent experiments showed statistically significant differences in cell viability when comparing bilberry extract–treated cells and –non-treated cells at any $H_2O_2$ dose.

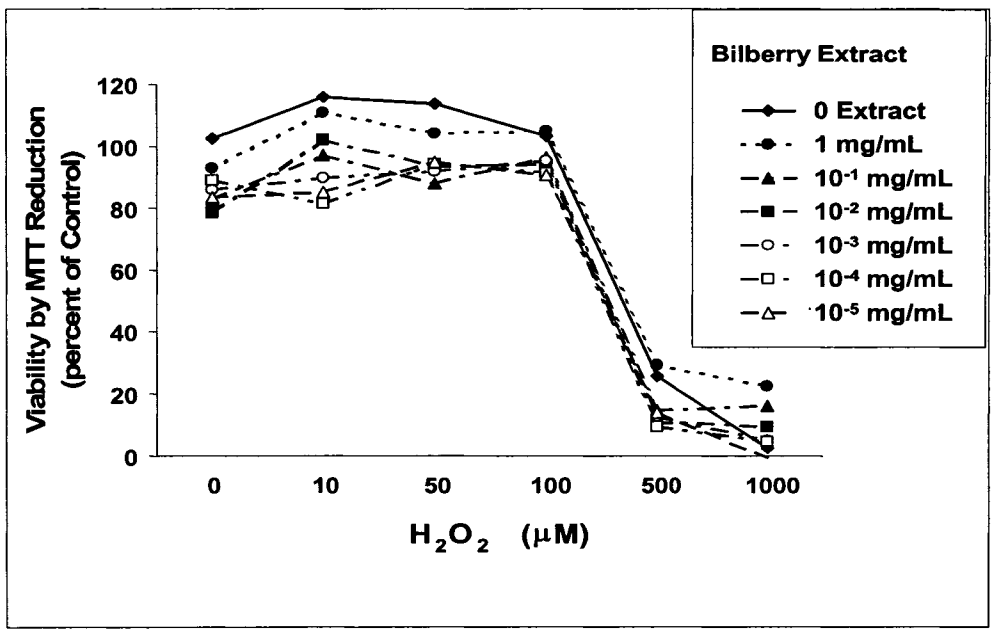



**Figure 13.** $H_2O_2$-induced glurathione decreases in RPE: a dose response. Viability is expressed as percent control absorbance at 485 nm. Values were corrected for the amount of absorbance in non-bilberry extract–treated wells exposed to 2000 μM $H_2O_2$ and expressed as means. S.D. bars were excluded for clarity. Controls (0 $H_2O_2$) included n=8 wells, while the comparator(s) included each combined condition (extract followed by $H_2O_2$) in n=4 wells. For 500 and 1000 μM $H_2O_2$, all conditions (0–1 mg/mL extract) were significant ($p \leq 0.05$) vs. all conditions at or below 100 μM $H_2O_2$. No bilberry treatment differed significantly from its corresponding control (0 bilberry) at any $H_2O_2$ concentration.

Whenever GSH utilization exceeds cellular capacity to produce it, levels of GSH decline. Glutathione-dependent enzymes glutathione peroxidase (GSH-Px) and glutathione reductase (GSHR), as well as the NADPH-producing enzymes glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) participate in the mechanism of defense against oxidation. GSH-Px reduces $H_2O_2$ to water or alkyl peroxides to alcohols, at the expense of reduced GSH. GSH oxidation provides, therefore, an important protection against endogenous and exogenous peroxides. Oxidized glutathione (GSSG) is reduced back to GSH by the NADPH-dependent reductase, GSHR. GSHR is important not only for the maintaining required GSH levels but also for reducing protein thiols to their native state.

While a trend toward decreasing cellular GSH levels is observed by 100 μM $H_2O_2$, no statistically significant difference ($p \leq 0.05$) was reached between control cells and exposed cells until 500 μM $H_2O_2$ (Figure 14). Oxidized GSSH levels show an increase over control values after treatmenmt with 100 μM $H_2O_2$. Of note, cellular GSSH levels are normally much lower than GSH levels.

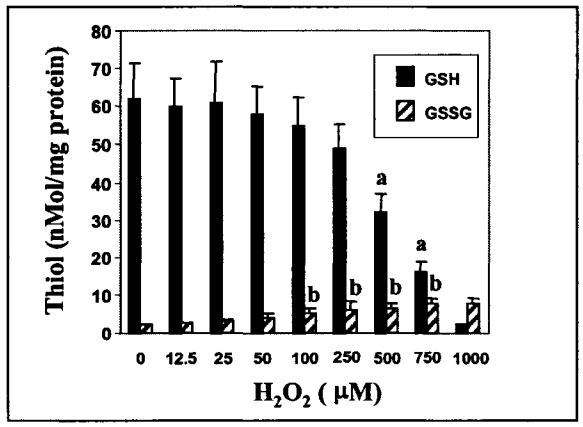



**Figure 14.** MTT assay for viability of cells exposed to $H_2O_2$. Here, $H_2O_2$ decreases GSH levels reaching significance at 500 μM. Data represent the mean ± S.D. of three independent experiments (n=3) except for 1000 μM $H_2O_2$ where n=1 precluding statistics. a = GSH significant ($p \leq 0.05$) vs. control (0 μM $H_2O_2$); b = Oxidized glutathione (GSSG) significant ($p \leq 0.05$) vs. control (0 μM $H_2O_2$).

**Bilberry Extract Effects on Radical Formation.** RPE cells are damaged by oxidant treatment and undergo apoptosis, a possible fate during the early phase of retinal pathologies (e.g., AMD). The main injury target is mitochondria, an organelle which undergoes increasing genomic damage in other post-mitotic tissues during aging. RPE oxidative stress is induced by generators of reactive oxygen species (ROS), including $H_2O_2$ (Jahngen-Hodge et al., 1997), high glucose concentrations (Du et al., 2003), UV radiation (Liu et al., 1995), and the industrial radical generators 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) (Ballinger et al., 1999). Anthocyanins are porent quenchers of radicals *in vitro*; however, in this project, pre-incubation with bilberry extract did not protect RPE cells from $H_2O_2$-induced mortality. Therefore, we sought to determine whether bilberry extract could inhibit $H_2O_2$-induced intracellular radical generation in ARPE-19. Using the dichlorodihydrofluorescein (DCF) assay, experiments were conducted to determine whether pre-exposure of RPE cells to bilberry extract resulted in an intracellular quenching of radicals.

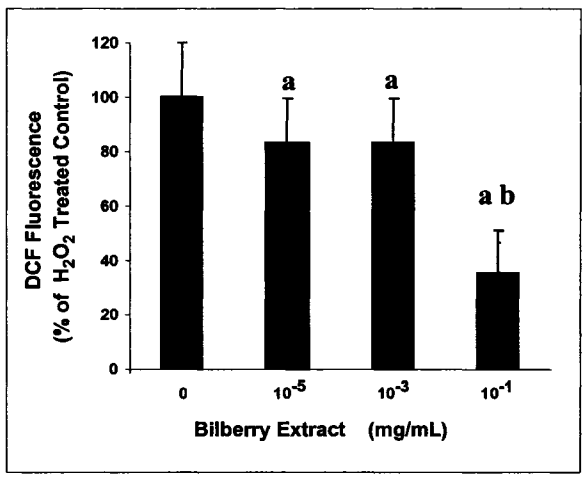



**Figure 15.** Bilberry prevention of $H_2O_2$-induced intracellular radicals. Here, ARPE-19 cells were pre-incubated with bilberry extract for 4 hours prior to exposure to 500 $\mu$M $H_2O_2$, showing lower levels of intracellular radical generation. Data represent the means ± S.D. of three independent experiments.
a = significant ($p \leq 0.05$) vs. control (0 extract);
b =significant ($p \leq 0.05$) vs. $10^{-3}$ and $10^{-5}$ mg/mL bilberry extract.

Confluent 10 day–old cultures were challenged with $H_2O_2$, the cells rinsed with PBS, and then loaded with 5 μM 2′,7′-dichlorodihydrofluorescein

diacetate (DCF-DA). At 30 minutes, fluorescence was measured. Pre-incubation with bilberry containing-media diminished intracellular radical formation induced by 500 $\mu$M $H_2O_2$. Low bilberry extract levels ($10^{-5}$ mg/mL) decreased radical formation by 18% (Figure 15); however, even high bilberry extract levels (0.1 mg/mL) failed to abolish radical formation induced by by 500 $\mu$M $H_2O_2$.

**Bilberry Extract Effects on Mitochondrial Function**. Oxidative stress–induced apoptosis involves alterations in the mitochondrial membrane pores that will result in pore opening and mitochondrial potential loss. When this happens, the mitochondria lose the ability to produce ATP. Figure 16 shows ATP levels in a representative experiment; here, RPE cells of differing times in culture were pre-incubated with varying doses of bilberry extract for four hours before a two-hour exposure to increasing $H_2O_2$ levels. As shown earlier, culture time affected RPE cell viability after $H_2O_2$ treatment. Comparing RPE cells grown to 10 days in culture to those grown to seven days in culture, Figure 16 reveals differing capability to resist $H_2O_2$-induced loss of

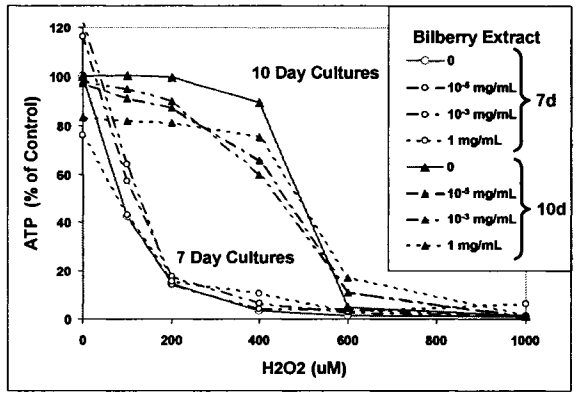



**Figure 16.** ATP levels in ARPE-19 cells after $H_2O_2$ treatment and after pre-incubation with doses of bilberry extract. ARPE-19 cells were pre-incubated with doses of bilberry extract for 4 hours and then exposed to $H_2O_2$ for 2 hours. Values are expressed as means. S.D. bars are excluded for clarity. Controls (0 $H_2O_2$) n=8 wells and each combined condition (extract followed by $H_2O_2$) n=4 wells. In 7-day cultures, values for all points >100 $\mu$M $H_2O_2$ were significantly different ($p \leq 0.05$) from control values. In 10-day cultures, values significantly different ($p \leq 0.05$) from controls were not achieved until 500 $\mu$M $H_2O_2$. No bilberry treatment was significantly different from its corresponding control (0 bilberry) at any concentration of $H_2O_2$, with the exception of 7-day cultures pre-incubated with $10^{-3}$ and $10^{-5}$ mg/mL extract which were different from untreated controls ($p \leq 0.05$).

mitochondrial function. In independent experiments (four at 10 days in culture, three at seven days in culture, and three at three days in culture), no significant protection against $H_2O_2$-induced loss of mitochondrial function was detected, no matter the bilberry extract concentration.

**Bilberry Extract Effects on Apoptosis.** The Bax/Bcl-2 ratio was altered in 10-day-old RPE cells by exposure to increasing $H_2O_2$ doses. As few as 2 hours of exposure to 500 $\mu$M $H_2O_2$ altered the Bax/Bcl-2 ratio in favor of apoptosis (Figure 17). By 22 hours post-treatment, cells that had not succumbed to the treatment and entered apoptosis established an anti-apoptotic Bax/Bcl-2 ratio. These cells were more apoptotis resistant upon a second $H_2O_2$ exposure. This is not an unrecognized phenomenon and it is referred to as preconditioning. Cells subjected to transient oxidative stress respond by up-regulating oxidative stress

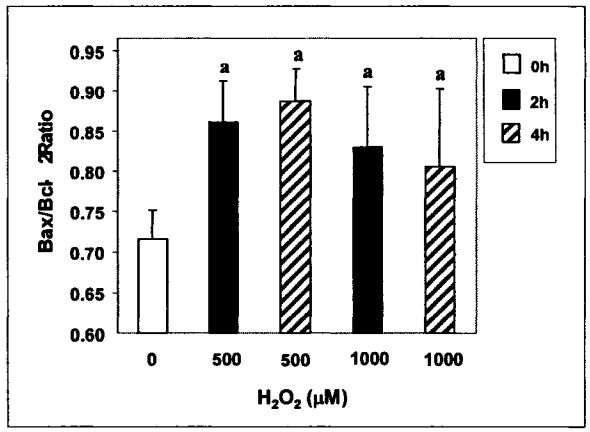



**Figure 17.** Bax/Bcl-2 ratio in RPE cells exposed to $H_2O_2$. Treatment with either 500 $\mu$M or 1000 $\mu$M $H_2O_2$ altered the Bax/Bcl-2 ratio in RPE cells in favor of apoptosis. Increasing the exposure from 2 hours to 4 hours did not enhance the effect. Data are expressed as the mean of three experiments ± S.D. a = significance ($p \leq 0.01$) vs. control.

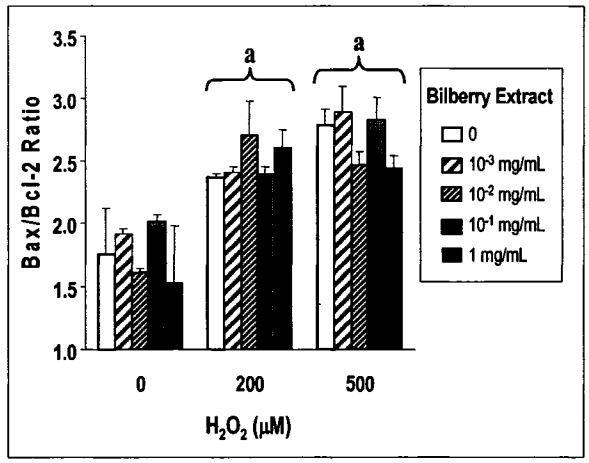



**Figure 18.** Bax/Bcl-2 ratio in RPE cells pre-incubated with bilberry extract prior to $H_2O_2$ exposure. Pre-incubating RPE cells for 4 hours with bilberry extract did not inhibit changes in the Bax/Bcl-2 ratio induced by 2-hour incubation with either 200 or 500 μM $H_2O_2$. Data represent the mean of three experiments ± S.D. No treatment with bilberry extract was different from its corresponding 0 bilberry control.
a = significance ($p \leq 0.05$) vs. cells untreated with $H_2O_2$.

defense systems (Jang et al., 2004). Increasing $H_2O_2$ concentration from 500 $\mu$M to 1000 $\mu$M, a dose producing 100% lethality, produced no additional change in the Bax/Bcl-2 ratio, nor was there increased change with additional exposure time (Figure 17). Bax protein levels remained stable and unaffected by $H_2O_2$ exposure. However, the Bax/Bcl-2 ratio was affected because Bcl-2 protein levels were down regulated by $H_2O_2$. Bilberry extract down regulated Bcl-2 protein level but only slightly and not significantly. By 24 hours, Bcl-2 levels recovered in those cells surviving $H_2O_2$ exposure.

Pre-incubating RPE cells with bilberry extract at concentrations between $10^{-6}$ mg/mL and 1 mg/mL failed to prevent $H_2O_2$-induced increase in Bax/Bcl-2 ratio (Figure 18). To determine whether mitochondria membrane pores were affected by $H_2O_2$, cells were exposed to 200–500 $\mu$M $H_2O_2$; GSSG levels increased while GSH levels showed no significant decrease from control levels.suggesting increased oxidation, but not proving decreased reductive power in the RPE cells.

By four hours after $H_2O_2$ exposure, cleavage of procaspase-3 to its active caspase-3 form is evident by western blot analysis. Only one experiment was conducted to verify that caspase cleavage occurred in response to $H_2O_2$ exposure. While just barely significant ($p<0.046$), pre-incubation with bilberry appeared to ameliorate but did not abolish procaspase-3 cleavage, even at 0.1 mg/mL bilberry extract.

## Bilberry Extract Induction of HO-1 and GSTP1

**Bilberry Extract Effects on Protein Levels.** Western blot analysis of protein collected 24 hours after treatment showed that RPE cell exposure to 500 μM $H_2O_2$ for 2 hours resulted in a 10-fold increase in HO-1 protein expression (Figure 19). RPE cells were cultured for 10 days and pre-incubated for four hours with either serum-free media or serum-free media containing 1 mg/mL bilberry extract. The media containing bilberry extract was washed from the cells with two washes of fresh media; the cells were then incubated an additional 2 hours with serum- and phenol red–free media containing the indicated $H_2O_2$ concentrations. Next, the media was replaced with media containing 2% serum, and 24 hours after treatment the cells were harvested and protein extracts prepared for western blot analysis. HO-1 protein values were adjusted to corresponding β-actin values for the sample obtained within the same gel. Results indicated that 24 hours after cell

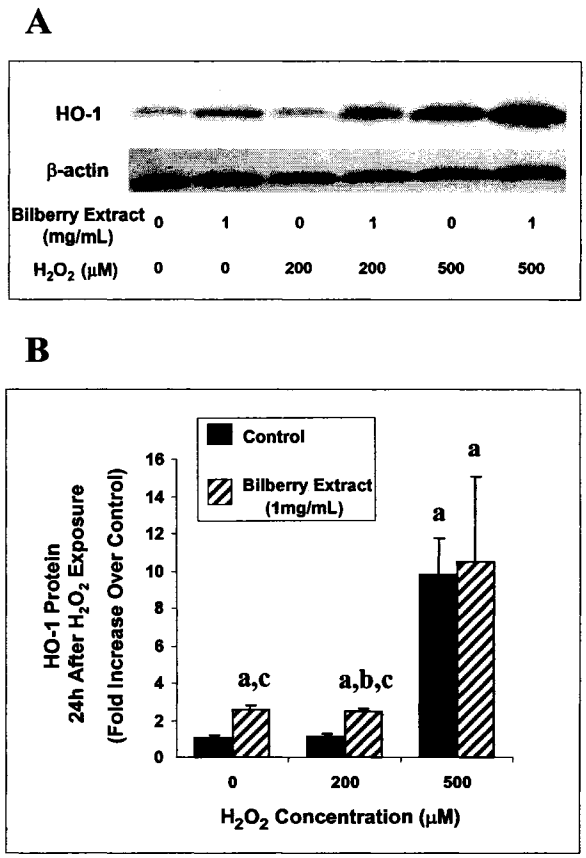



**Figure 19.** Western analysis of HO-1 protein in RPE cells cultured for 24 hours after exposure to bilberry extract and $H_2O_2$. Panel A shows an example of the western analysis gel. HO-1 levels were 10-fold higher than control levels when sampled 24 hours after $H_2O_2$ exposure. This HO-1 up-regulation was not prevented by pre-incubation for 4 hours with 1 mg/mL bilberry extract in media. Data represent the mean ± S.D. of three independent experiments. a = significant ($p \leq 0.05$) vs. control cells (no treatment); b = significant ($p \leq 0.05$) vs. cells treated with 200 μM $H_2O_2$; c = significant ($p \leq 0.05$) vs. control cells (untreated cells).

exposure to 200 μM $H_2O_2$, HO-1 protein had not increased. RPE cells treated with 500 μM $H_2O_2$ showed a 10-fold increase in HO-1 that was not prevented by bilberry extract pre-incubation. In the control and 200 μM $H_2O_2$ treatment groups, a 4-hour pre-incubation with 1mg/mL bilberry extract produced a significant increase in HO-1 protein expression at 24 hours.

Figure 20 shows a dose response relationship for up-regulation of HO-1 protein levels in RPE cells in response to 4 hours of incubation with bilberry extract when observed 6 hours after exposure. Samples were treated with bilberry extract for 4 hours, rinsed, and then remained 2 hours further in serum-free medium prior to sample collection for western blot analysis. At 6 hours after first exposure to bilberry extract, a 2- to 2.5-fold increase was noted for the highest levels of bilberry extract used (0.1 and 1 mg/mL). Extending time in culture to 24 hours did

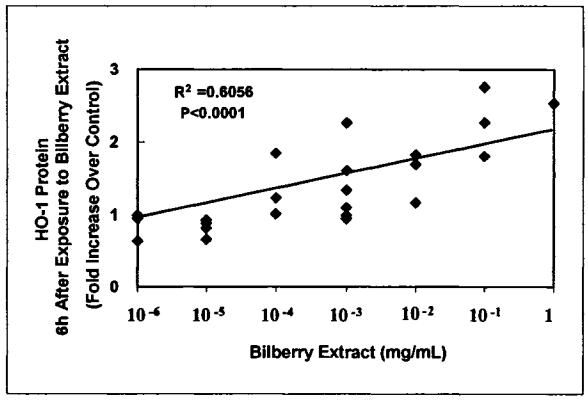



**Figure 20.** Bilberry dose response effect on HO-1 protein expression. Each displayed data point indicates the mean of at least three data points for a given dose of bilberry extract in different independent experiments. Only one experiment was conducted measuring HO-1, which contained a 1 mg/mL extract level. Collectively the scattergram displays a bilberry extract dose dependent increase in HO-1 protein.

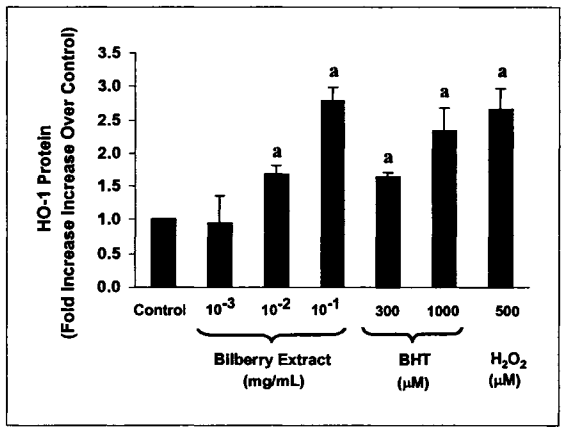



**Figure 21.** Bilberry, BHT, and $H_2O_2$-induced increases in HO-1 protein. The figure shows increased HO-1 protein levels by western blot analysis in response to treatment with bilberry extract media containing 300 and 1000 μM BHT or 500 μM $H_2O_2$. Bars represent mean ± S.D. of at least three independent experiments. a = significance ($p \leq 0.05$) vs. control (untreated cells).

increase the level of bilberry induced–up-regulation (see Figure 19). Exposure to bilberry extract did not add more than a 2.5-fold increase to any treatment group; therefore, in all subsequent experiments cells were exposed to bilberry extract or pooled fractions of the extract for 4 hours and harvested for analysis at 6 hours after time of first exposure.

HO-1 protein levels increased in a dose dependent manner when assessed after 4 hours incubation with $10^{-1}$ or $10^{-2}$ mg/mL bilberry extracts (Figure 21). Butylated hydroxytoluene (BHT) a known up-regulator of the antioxidant response element controlled genes, was used at 300 and 1000 μM as a positive control. RPE cells were also exposed to $H_2O_2$ at 500 μM for a 2-hour period. In similar experiments, these same treatments increased GSTP1 protein levels (Figure 22).

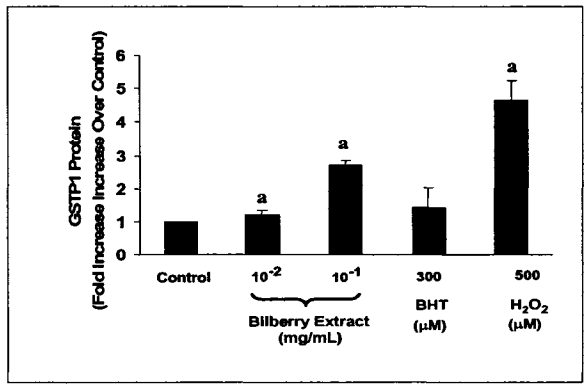



**Figure 22.** Bilberry-induced increase in GSTP1 protein levels in RPE. Data show an increase in GSTP1 protein by western blot analysis in response to treatment for 4 hours with media containing bilberry extract ($10^{-2}$ and $10^{-1}$ mg/mL), 300μM BHT, or 500 μM $H_2O_2$. Data bars represent mean ± S.D. of at least three independent experiments. a = significance ($p \leq 0.05$) vs. control (untreated cells).

**Bilberry Extract Effects on mRNA Levels.** RPE cells cultured for 10 days were pre-incubated with increasing doses of either bilberry extract or the known antioxidant response element (ARE) agonists BHT or β-naphthoflavone (BNF) at 300 μM and 10 μM, respectively, for 4 hours. Total RNA was immediately extracted, and RNA was analyzed by rt-PCR for human HO-1 mRNA, human GSTP1 mRNA, and normalized to both β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs.

Crosschecking against two housekeeping genes assured data were not inadvertently corrected to a gene that might be altered by the experimental treatments. Data is expressed as fold increases over untreated controls using the ΔΔCt method.

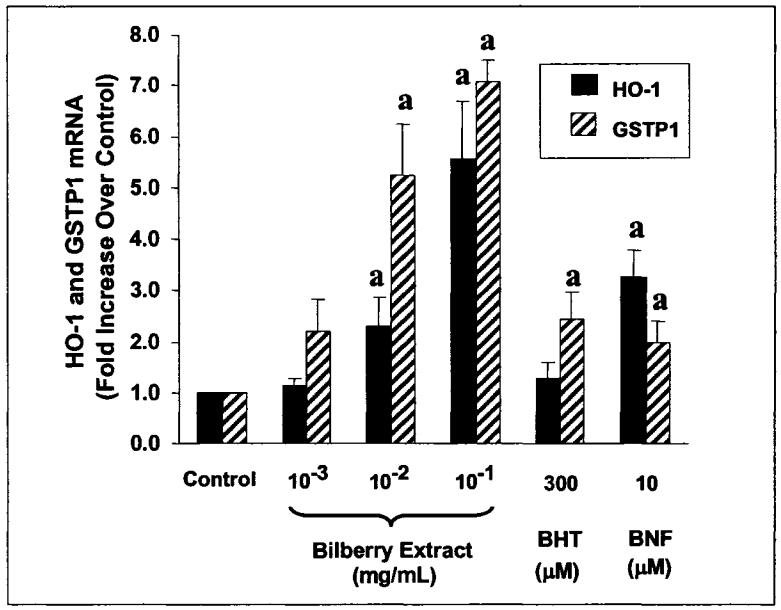



**Figure 23.** Effects of bilberry extract on HO-1 and GSTP1 mRNA levels in RPE cultures. Data bars represent the mean ± S.D. of three independent experiments. a = significance ($p \leq 0.05$) vs. control (untreated cells).

Rt-PCR analysis by RPE mRNA exposed to bilberry extract doses $10^{-1}$ to $10^{-3}$ mg/mL showed up-regulation of HO-1 and GSTP1 mRNA; however, while a general trend was observed, $10^{-3}$ mg/mL bilberry did not show a statistically significant up-regulation in these experiments (Figure 23). ARE agonists BHT and BNF also showed increases in mRNA for these two enzymes (Figure 23); however, BHT did not reach a significant difference ($p \leq 0.05$) from controls for HO-1 mRNA. While a trend is noted, lack of statistical significance was likely due to the limited number of experiments and a scatter in the data. It is also probable that an increased dose of BHT would have shown a difference. BNF, which activates antioxidant and xenobiotic response elements via the same mechanism but more potently than BHT, does show an up-regulation of HO-1 mRNA.

To determine the extent to which anthocyanins or other phenolics in the bilberry extract up-regulate HO-1 and GSTP1 mRNA, the anthocyanin and the phenolics pools that were chromatographically isolated from the original bilberry extract were tested in RPE cells grown 10 days in culture. Cells were pre-incubated with respective pools at concentrations of phenolics or anthocyanins representative of (i.e., contained within) the original bilberry extract material. The RPE cells were treated with $10^{-1}$ to $10^{-3}$ mg/mL bilberry extract equivalents for 4 hours and total RNA was extracted. Rt-PCR was conducted for human HO-1 mRNA and the housekeeping gene GADPH mRNA. HO-1 mRNA levels were normalized to GAPDH and expressed relative to that of untreated control culture samples using the ΔΔCt method.

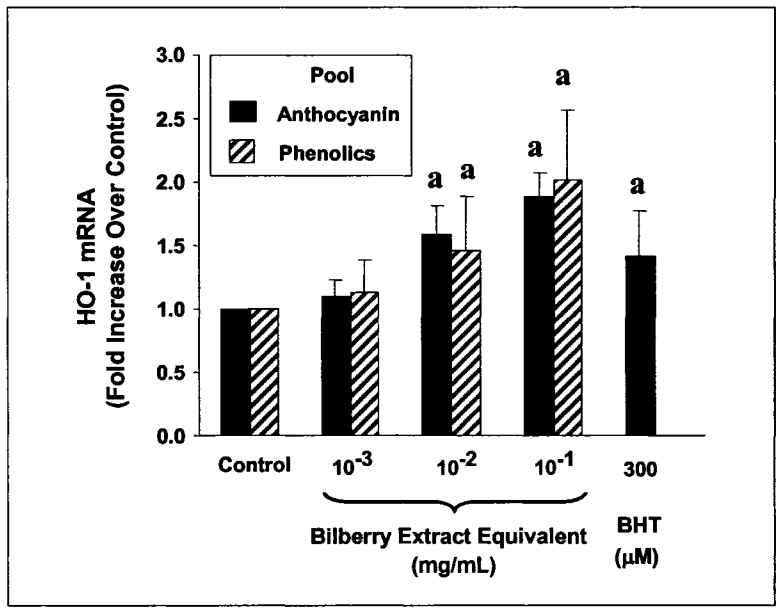



**Figure 24.** Effects of bilberry anthocyanins and phenolics pools on HO-1 mRNA levels in RPE cultures. Data bars represent the mean ± S.D. of three independent experiments. a = significance ($p \leq 0.05$) vs. control (untreated cells).

Both the anthocyanin and the phenolics pools were able to induce the up-regulation of HO-1 mRNA at concentrations contained within $10^{-1}$ to $10^{-2}$ mg/mL bilberry extract (Figure 24). Again, the $10^{-3}$ mg/mL equivalent level showed a trend but did not reach a statistically significant difference from untreated cells. The up-regulation

observed at 4 hours was not different between the anthocyanin and phenolics pools. In fact, up-regulation was roughly equivalent, which suggests that the HO-1 up-regulation observed at 4 hours for the original bilberry extract (Figure 23) represents additive effects of both anthocyanins and phenolics fractions. While the data for $10^{-1}$ mg/mL bilberry treatment suggest a possible synergistic effect in up-regulating HO-1 mRNA for anthocyanin and phenolics components when combined in the intact bilberry extract, this synergistic effect was not observed in $10^{-2}$ mg/mL bilberry treated RPE cells. More investigation is required; however, this data implies that HO-1 up-regulation results only from polyphenolics and phenolic components of the mixture and not to other ingredients within the extract.

# CHAPTER VI

# DISCUSSION AND CONCLUSIONS

Despite the high level of metabolic and environmental stresses to which retinal pigment epithelium (RPE) cells are subjected, some researchers do not believe that RPE undergo significant cell division (Ts'o & Friedman, 1967). Differentiated RPE cells are thought to be quiescent and to remain in $G_0$ phase for their lifetime *in vivo*. When placed in culture, however, it is clear that RPE cells re-enter the cell cycle and proliferate (Korte et al., 1994; Stroeva & Mitashov, 1983). While RPE cells *in vitro* have not yet been proven to fully complement *in vivo* functionalities, sufficient phenotypic characteristics are conserved in culture to make them extremely useful for study of RPE injury, survival, differentiation, and transport (Newsome, 1983).

Only a few studies examining the effects of oxidative stress on RPE have been performed with cultured, proliferating RPE cells. For example, tert-butylhydroperoxide (tBH), a chemical oxidant, induces cell death in cultured human RPE cells (Weigel et al., 2002). In proliferating cells, glutathione (GSH), its amino acid precursors, and dimethylfumarate (an inducer of GSH synthesis) protect RPE cells from tBH-induced injury (Nelson et al., 1999). When tBH reaches sufficient concentrations, the resulting damage induces apoptosis via a Fas-mediated pathway. While these studies demonstrate induced oxidative damage to the RPE, *in vivo* observation of proliferating cells may not accurately reflect apoptosis as it occurs in quiescent cells.

In the present study, fully differentiated, non-dividing, cultured RPE cells were used to study oxidant-induced injury and potential protective effects of bilberry anthocyanins. Differentiation was verified by monitoring the production of RPE-specific proteins and by observing maximum levels of antioxidant production (Figures 6, 7, and 8). Results demonstrated that RPE cells were not fully differentiated when they reached confluence. In fact, reduction in culture media serum and continued culture to 10 days (7 days beyond the point of visual confluence) were required to reach their optimal oxidant defense status as well as the highest levels of RPE-specific proteins (e.g., RPE-65). Cells grown 7 days in culture were easily damaged by relatively low levels of hydrogen peroxide ($H_2O_2$), while an additional 3 days in culture yielded cells containing higher levels of reduced GSH than most other mammalian cells. These cells can withstand extraordinary levels of $H_2O_2$, in the 4-500 μM range.

Anthocyanins have physiological effects that include inhibition of cell proliferation (Katsube et al., 2003; Marko et al., 2004; Seeram et al., 2004; Shih et al., 2005), antioxidant capacities (Faria et al., 2005), and modulation of inflammatory processes (Rossi et al., 2003). These physiological effects also arise from over-expression of heme oxygenase-1 (HO-1) (Morita et al., 1997; Otterbein et al., 2003; Poss & Tonegawa, 1997; Willis et al., 1996). Results from the present study suggest that bilberry anthocyanins either prevent or slow the radical damage that could lead to age-related macular degeneration (AMD) pathology via their ability to up-regulate HO-1 (Figures 19, 20, and 21) . The fact that anthocyanins can up-regulate genes for HO-1 and for glutathione S-transferase pi class P-1 form (GSTP1), a phase II metabolic enzyme, indicates they may also be able to affect important intracellular signal transduction

pathways, such as modulating phosphorylation events that could lead to other health benefits. At the very least, anthocyanin ability to up-regulate phase II metabolism hints that anthocyanins could serve as dietary anticancer agents (Bomser et al., 1996). This study supports the hypothesis that anthocyanins may be involved in mechanisms other than direct radical quenching. One possible mechanism includes enzyme system modulation normally regulated by redox status via signal transduction pathways.

## Effects on RPE Apoptosis and Phase II Up-regulation

Consumption of fruits and berries has been associated with decreased risk of developing age-related disorders. Moreover, epidemiological evidence suggests that anthocyanins in fruits and berries may contribute to this protective activity. *In vitro* evidence demonstrates that anthocyanins and their aglycones produce a wide range of physiological effects in mammalian cells (Middleton et al., 2000). Indeed, the epidemiological evidence discussed earlier in this dissertation hints at health benefits derived from the consumption of anthocyanin-bearing fruits and berries. Among berries, bilberries are highest in both anthocyanin content and anthocyanin variety (Wu & Prior, 2005).

Legend and traditional medical practice purport an association between health benefits and bilberry consumption. However, modern medicine has yet to establish unequivocally either health benefits or mechanisms of action for bilberry anthocyanins. In the 1960s, scientific observations from Europe suggested that bilberry extract consumption improved dark adaptation and nighttime visual acuity (Gloria & Peria, 1966; Jayle & Aubert, 1964; Terrasse & Moinade, 1964). Several recent mechanistic

studies suggest that anthocyanidins may stimulate rhodopsin regeneration (Bastide et al., 1968; Matsumoto et al., 2003). Conversely, a double-blind, placebo-controlled, crossover study conducted on 15 young and healthy United States Navy personnel found no statistically significant improvement in either night visual acuity or night contrast sensitivity in response to a three-week supplementation of thrice daily administrations of 160 mg bilberry extract containing 25% anthocyanin (Muth et al., 2000). A 2004 systematic review on bilberry use to improve night vision concluded that, to date, controlled studies fail to demonstrate vision improvement in healthy eyes (Canter & Ernst, 2004). The authors noted, however, an absence of rigorous clinical interventions with bilberry in patients with impaired night vision or diagnosed eye disease. A literature review also revealed a lack of intervention studies of sufficient duration to determine effects of anthocyanin use on degenerative eye disorders (e.g., cataracts, AMD, or other retinopathies). Despite these mixed results, more recent trials have not been undertaken because of anthocyanin's low bioavailability and the belief among researchers that no plausible mechanism of action has been presented for such low levels. The results of the present study, combined with the results of other cancer-related studies, suggest that anthocyanins could modulate protective enzyme systems at very low concentrations.

Several potential mechanisms exist regarding how bilberry anthocyanins could modify vision and long-term eye health. In addition to stimulating re-synthesis of rhodopsin, thereby aiding in dark adaptation and night vision, other proposed anthocyanin effects include: antioxidant activity, anti-inflammatory activity, retinal enzyme activity modulation, intracellular matrix stability, and microcirculation or

vascular integrity. Many of these proposed mechanisms require more rigorous research or clinical trials in subjects with impaired vision or early signs of retinopathies.

Confounding factors — including the age and visual function status of the subjects, the formulation of the supplement, and duration of the treatment — that were present in previous studies may have contributed to the mixed evidence presented above. While a deeper understanding of the biochemical effects of anthocyanins and other phenolics and polyphenolic antioxidants would improve future study design, little information exists regarding their distribution, metabolism, and action in the retina. Studies using tissues other than retinal tissue have demonstrated that anthocyanins suppress tumor growth (Kamei et al., 1998), inhibit the epidermal growth-factor receptor (Meiers et al., 2001), and reduce platelet aggregation and lipid peroxidation (Ghielli et al., 1998; Pawlowicz et al., 2000). Further, flavonoids have been shown to up-regulate phase II enzymes, including glutathione-S-transferase (GST), UDP-glucuronosyltransferase, NAD(P)H:quinone oxidoreductase 1, and epoxide hydrolase; however, these studies have not focused on anthocyanins (Zhang & Gordon, 2004).. The promoter regions of the genes for phase II enzymes are transcriptionally regulated by the xenobiotic response elements and antioxidant/electrophile response elements (ARE/EpRE) (Nguyen et al., 2003). In their quinone form, flavonoids, including anthocyanins, may influence redox-sensitive pathways by reacting directly with thiols (Boots et al., 2003) affecting GSH status (Carlsen et al., 2003l; Kessler et al., 2003) or inducing oxidative respiratory bursts in mitochondria (Hodnick et al., 1986). These actions could trigger signal mechanisms that activate the ARE/EpRE and xenobiotic response elements.

In our study, pre-incubation with bilberry extract did not significantly decrease hydrogen peroxide ($H_2O_2$) toxicity in fully differentiated RPE cells *in vitro* (Figures 14, 16, and 18). In contrast, pre-incubation with bilberry extract, even at low concentrations (10 ng/mL extract), decreased intracellular radical formation (Figure 15). Western blot analysis showed that 500 μM $H_2O_2$ (the observed $LD_{50}$ in 10-day-old RPE cultures in these experiments) induced an increase in HO-1 protein expression that was not inhibited by pre-incubation with bilberry extract (Figure 19). Indeed, pretreatment with bilberry extract alone induced an increase in HO-1 protein expression in a dose-dependent manner within 4 hours (Figures 20 and 21). It is possible, then, that while bilberry extract internalized by RPE cells can diminish intracellular radical formation induced by $H_2O_2$, the concentrations of anthocyanins are insufficient or ineffective in preventing apoptosis induced by radical damage to cell surface proteins and lipids or induced via death receptor mechanisms. It is also possible that oxidative damage to surface receptors by $H_2O_2$ could modulate signal mechanisms that would be unaffected by reductions in intracellular radicals by anthocyanin pre-incubation.

Studies on anthocyanin bioavailability show that plasma anthocyanin levels can reach $10^{-8}$ to$10^{-7}$ M concentrations; however, studies of anthocyanin pharmacological effects, such as inhibition of cancer cell proliferation, show anthocyanin and anthocyanidin bioactivities at concentrations of $10^{-5}$ to $10^{-4}$ M. Anthocyanins barely inhibit growth at concentrations below $10^{-4}$ M. They have been shown to affect enzyme systems of importance in cancer research (e.g., tyrosine kinase, cyclooxygenase (COX) enzymes, and phosphodiesterases), where anthocyanins behave as second messenger modulators (Cross & Dexter, 1991; Marko et al., 2004; Mestre et al., 1999; Seeram et al.,

2001; Wang et al., 1999). It is unknown whether anthocyanins, anthocyanidins, or their conjugate forms achieve *in vivo* concentrations sufficient to explain anti-carcinogenic, anti-proliferative, or pro-apoptotic mechanisms. The evidence that these same effects exist *in vitro* suggests a possible role for these compounds in AMD.

To date, numerous studies have been conducted on the potential health benefits of anthocyanidins, the aglycones of anthocyanins. However, these compounds, while produced by lactase phloridzin hydrolase (LPH) in the lumen before enterocyte absorption, are not likely to circulate or enter tissues in high concentrations relative to anthocyanins transported directly by the sodium-dependent intestinal glucose carrier (SLGT1) or relative to anthocyanin phase II metabolites circulating in plasma. Indeed, anthocyanidins are not the major form found in plasma after ingestion of anthocyanin-rich foods or extracts (Milbury et al, 2002; Kay et al., 2005).

Anthocyanins are unique among flavonoids in that they are absorbed and circulated in mammals as both parent plant-derived glycosides and as metabolites (Kay et al., 2004). However, at this time, little is known about concentrations in tissues such as the retina. Further study is necessary to determine which metabolic forms may be more active *in vivo* with regard to the cited effects. Due to the lack of purified phase II metabolites of bilberry anthocyanins, the studies cited cannot distinguish between the effects of parent glycosylated anthocyanins and RPE metabolites of these compounds with regard to quenching radicals or up-regulating HO-1 and GSTP1. However, this study's *in vitro* data do suggest that bilberry anthocyanins absorbed from the diet and transported in circulation could be taken up and metabolized by RPE cells (Figures 9, 10,

11, and 12). Bilberry anthocyanins were detected unchanged in cytosolic fractions from bilberry extract exposed to RPE cells (Figure 11).

Obstacles to determining physiological concentrations of anthocyanins in such transport experiments are primarily a problem of analytical detection limits. The relevance of high anthocyanin concentrations tested in the uptake experiments to potential physiological bioactivity as antioxidants or as modulators of signal transduction and gene expression mechanisms is unknown. High doses of bilberry extract (1 μg to 1 ng/mL) in these experiments were necessary for detection by high pressure liquid chromatography with electrochemical detection (HPLC/ECD) or liquid chromatographic mass spectrometry (LC/MS/MS). In all likelihood, such concentrations would not be achieved in eye tissue *in vivo* by dietary intake. The results do, however, show that anthocyanins can be internalized by RPE cells *in vitro*. Once radiolabeled anthocyanins become available to the research community, these experiments should be repeated to determine uptake at physiologically relevant levels. It is important to note that cell fractionation is seldom free from cross-contamination; it distinguishes only between what is inside the plasma membrane and what is bound to the membrane or external to the cells. For example, the "cytosolic" fraction from these experiments could also contain material that would have resided within vacuoles.

The key insight regarding RPE uptake of bilberry anthocyanins lies in phase II enzyme metabolism prior to media return. LC/MS/MS detection of anthocyanin metabolites in the culture media of RPE cells *in vitro* is evidence of such uptake, since glucuronide metabolites were not present in the media containing bilberry prior to the experiments (Figure 12). These glucuronide metabolites could only have been produced

by RPE cells via metabolism of parent plant–produced anthocyanins. This study's experiments do not identify whether anthocyanins or their metabolites serve as the active forms responsible for the observed effects, but they do establish that *in vivo*, if anthocyanins reach the retina, there is a reasonable chance that up-regulation of HO-1 and GSTP1 occurs.

This research project aimed to investigate whether bilberry anthocyanins have protective effects against oxidative stress–induced apoptosis in RPE cells as well as possible mechanisms of bilberry anthocyanidins involvement in AMD prevention or amelioration. Among anthocyanidins, delphinidin generally has been shown to possess the most potent growth inhibition and pro-apoptotic activity, suggesting the structural importance of hydroxyl groups on the anthocyanidin B ring. Nevertheless, several cancer cell lines have been shown to be more sensitive to malvidin, suggesting the possibility of separate mechanisms of action among anthocyanidins with regard to anti-proliferative and pro-apoptotic activity. A study by Zhao et al. showed that malignant colon cancer cells are more sensitive to anthocyanin-containing extracts of bilberries, chokeberries and grapes than are non-malignant colon-derived cells (Zhao et al., 2004). This suggests that the actions of anthocyanins may be dependent on cell state-dependent mechanisms. The RPE cells subjected to oxidative damage were not malignant; nor could they be considered normal, since they are injured and will succumb via apoptosis. At the start, it was unknown whether bilberry anthocyanins would protect RPE from oxidative damage, thereby reducing the number of cells succumbing to apoptosis, or whether anthocyanins would instead exacerbate apoptosis in stressed RPE cells. The results described here indicate that apoptosis is induced in fully differentiated RPE cells by treatment with 500

μM $H_2O_2$ (Figure 14, 16, 17, and 18). However, *in vitro*, pre- or co-treatment with even pharmacologic doses of bilberry anthocyanins did not alter the apoptotic demise of RPE cells (Figures 14, 16, and 18).

In cancer cells, it has been established that flavonoids, of which anthocyanins are a class, generally inhibit proliferation or cell growth at pharmacologically achievable doses without causing immediate cell toxicity (Rosenberg et al., 2002). Indeed, many of the studies cited here show the ability of the aglycones of anthocyanins (the anthocyanidins) to inhibit tumor cell proliferation. Ferguson et al. (2004) showed that flavonoids extracted from cranberries, another *Vaccinium* species, could induce both cell cycle arrest and apoptosis in human breast, skin, colon, lung, and brain tumor cell lines. While the authors did not fully define the exact polyphenolics composition of the cranberry fraction, anthocyanins are present in both cranberries and bilberries even if in different ratios. In addition, non-anthocyanin polyphenolic compounds present in the cranberry fractionation included quercetin and epigallocatechin gallate, which inhibit tumor cell proliferation (Kampa et al., 2000; Chen et al., 1998; Choi et al., 2001). Two triterpene hydroxycinnamate compounds found in cranberries also inhibit tumor cell proliferation *in vitro* (Murphy et al., 2003).

Of note, the Ferguson study described above did not attempt to ascribe activity to any particular component within the cranberry extract fraction; hence, this example of cranberry flavonoid activity in tumor cell lines illustrated the difficulties of working with berry extracts rather than purified individual compounds. When using extracts, observed activities could be attributed to anthocyanins, other non-anthocyanin polyphenolics, or to a combination of the two. For instance, Seeram et al. (2004) showed that the anti-

proliferative activity of cranberry extract was additive or synergistic when anthocyanins, proanthocyanidins, and flavonol glycosides fractions were combined. The effects of bilberry anthocyanins on pro-apoptotic pathways in RPE cells were unknown; however, the cited work regarding the effects of cranberry extracts on the cell cycle signified that both anthocyanin fractions and non-anthocyanin fractions of the bilberry extract would have to be checked for possible contribution to any observed activities in RPE cells. Given previous research, then, the present study aimed to separate and determine the individual contribution of anthocyanins and other phenolics to observed activities (Figures 23 and 24).

As mentioned in Chapter III, apoptosis is intimately linked to oxidative stress. Since oxidative stress appears to be a major process involved in AMD pathology, the approach to this project seemed at first to be relatively straightforward. After stressing RPE cells with $H_2O_2$ to induce oxidative stress, tests were conducted to determine whether bilberry anthocyanins altered apoptosis induced by the oxidative challenge. A series of indicators of mitochondrial dysfunction, which play a role in apoptosis, were also monitored. Indicators chosen include adenine tri-phosphate (ATP) production since it is highly dependent upon maintenance of mitochondrial membrane potential. When mitochondrial membrane potential is lost, so also is the ability of cells to reduce MTT; therefore, this cell viability assay is also a de facto measure of mitochondrial function. The Bcl-2/Bax ratio because it also indicates the opening of the mitochondrial membrane pores that lead to loss of mitochondrial membrane potential and release of mitochondrial factors (e.g., apoptosis-inducing factor (AIF) and cytochrome c); and activation of the caspase cascade and progression, resulting from mitochondrial factor release, to an

irreversible stage in the apoptotic process. All of the chosen indicators are markers of early stage apoptosis.

As noted above, the Bcl-2/Bax ratio serves as the "death checkpoint" beyond which opening of mitochondrial membrane pores occurs. This leads to the eventual cleavage of "death substrates" (e.g., poly(ADP-ribose)polymerase (PARP)) and quickly brings a cell to a point of no return. If bilberry anthocyanins can modulate expression of Bcl-2, then mechanisms in apoptosis modulation could involve binding and inhibition of pro-apoptotic family members, such as Bax. Although Yeh & Yen (2005) used an anthocyanidin rather than an anthocyanin, they demonstrated that delphinidin-induced apoptotic cell death was accompanied by up-regulation of Bax and down-regulation of Bcl-2 protein. Here, delphinidin-induced DNA fragmentation was blocked by N-acetyl-l-cysteine and catalase, suggesting that death signaling was triggered by oxidative stress. These data provided evidence that activation of c-Jun N-terminal kinase cascade was involved.

By monitoring the Bcl-2/Bax ratio, any observed action of bilberry anthocyanins in modulating apoptosis can be limited to signaling pathways converging directly at apoptotic-related mitochondrial involvement (e.g., c-Jun NH2-terminal kinase (JNK) or tyrosine kinase phosphorylation mediated mechanisms). It has been shown that the anthocyanidins cyanidin and delphinidin serve as potent inhibitors of epidermal growth-factor receptor, since they shut off downstream signaling cascades, including epidermal growth factor receptor (EGFR) protein tyrosine kinase activity and the MAP kinase pathway. Cyanidin 3-galactoside and malvidin 3-glucoside appear much less potent in this regard (Meiers et al., 2001). However, anthocyanins extracted from bilberry, such as

delphinidin glycosides and cyanidin glycosides, have been shown to induce apoptosis in HL-60 cells (Katsube et al., 2003). Activity varies substantially and is highly dependent on anthocyanidin structure and sugar and hydroxyl group placement (Hou et al., 2004). Whether for the anthocyanins or their aglycones, presence of the ortho-dihydroxyphenyl structure on the B ring appears essential for apoptotic induction.

In cancer cells, anthocyanidins block kinase phosporylation in the MAP kinase–pathway; they also inhibit cyclooxygenase (COX-1 and -2) at transcriptional levels by interfering with the signal pathways, blocking LPS-induced I$\kappa$B degradation, and suppressing NF-$\kappa$B activation and COX-2 gene expression. Here again, delphinidin has been shown to be most potent. These anticancer mechanisms are of interest because they suggest that anthocyanins may interfere with pathways capable of affecting apoptosis in oxidatively injured cells.

Delphinidin treatment has been found to increase the levels of intracellular reactive oxygen species (ROS) in leukemia cells and to induce apoptosis through ROS/c-Jun $NH_2$-terminal kinase (JNK)–mediated caspase activation (Hou et al., 2003). N-acetyl-L-cysteine (NAC), an antioxidant, blocks delphinidin-induced apoptosis in leukemia cells. However, it is counterintuitive that as a potent antioxidant itself, delphinidin may induce apoptosis via an oxidative stress–mediated JNK signal transduction mechanism.

This research project, which used anthocyanins, observed no increase in intracellular radicals resulting from bilberry treatment (Figure 15). This suggests that, while anthocyanidins may well be pro-oxidant in cancer cells, anthocyanins are not pro-oxidant in RPE. Moreover, because most *in vitro* mechanistic studies have been

conducted using anthocyanidins rather than anthocyanins, relatively little is known about the effects of anthocyanins on cell signal mechanisms.

The study's approach toward apoptosis in RPE cells involved several steps. First, we developed a predictable model in which apoptosis could be induced by oxidative stress. Using this model, bilberry extracts could be tested for their ability to prevent or exacerbate apoptosis. It proved more difficult than originally anticipated to establish a stable apoptosis model using ARPE-19 cells. Mimicking prior studies of oxidative stress in RPE cells in culture (Ballinger et al., 1999), RPE cells were grown to confluence at 3 days in culture and then tested. The observed $LD_{50}$ values in these cells (i.e., the cells reaching confluence and contact inhibition) ranged widely between 25 and 200 μM $H_2O_2$. Evidence emerged revealing that beyond confluence, cellular age and state of differentiation play a role in the robustness of defense mechanisms that RPE mount against $H_2O_2$ exposure *in vitro*. The literature regarding the $LD_{50}$ of $H_2O_2$ also shows a wide range of values; however, upon closer examination of this study's results, a weak correlation emerged between $H_2O_2$ levels RPE cells can withstand and the point after plating at which various investigators chose to conduct their experiments.

In order to test the ability of bilberry anthocyanins to either promote or inhibit RPE apoptosis, a stable and predictable oxidative stress model was required. The early experiments in this series did not afford a sufficiently stable platform for performing the apoptosis studies required. To stabilize the model and achieve a predictable $H_2O_2$ dose that would produce apoptosis, RPE was cultured beyond confluence and differentiated by lowering the serum providing growth factors in the media. RPE cells cultured to confluence, followed by 10 days in culture, reached defensive capability at least 80% of

their maximal capability. Culture durations at least 2–3 times longer (i.e., up to a month in culture) were required to reach the highest levels of resistance to $H_2O_2$-induced oxidative damage. However, the extra time, expense, and contamination risk associated with maintaining RPE in culture for each three-week experiment were not justified; in fact, it was determined that only 10 days in culture were required to obtain a reliable and predictable $LD_{50}$ of $H_2O_2$ between 450 and 500 μM. In addition, the 10 day–culture allowed for repeatable results, as evidenced by the shape of the $LD_{50}$ curve, or the range over which mitochondrial function was lost and apoptosis ensued.

As noted previously, little uniformity exists in the literature concerning the length of time in culture or the level of $H_2O_2$ used in oxidative stress studies. Once contemplated, the realization that fully differentiated RPE cells are more resistant to oxidative stress than dividing cells was not surprising. However, the dispersed body of experimental results supporting this realization raises the possibility that oxidative damage may be more likely, and RPE cells more vulnerable, during turnover than when fully differentiated and quiescent. If RPE cells are damaged during turnover to a degree insufficient to cause apoptosis, and if this damage is not adequately repaired, damaged RPE cells may mishandle transport and therefore deposit lipofuscin or drusen, leading to AMD. Such an endpoint is also possible and indeed likely to occur in fully differentiated RPE. The literature on oxidative stress suggests that differences exist not only in the degree of resistance to oxidative stress among proliferating, arrested, and differentiated RPE, but also in the forms of damage and mechanisms of cellular response. One study found that long-term confluent culture creates non-dividing RPE cells more sensitive to oxidant-induced apoptosis than their proliferating counterparts (Jiang et al., 2002). The

authors concluded that non-dividing RPE cells may be superior to proliferating and de-pigmented cells when studying oxidative injury of RPE cells. Results from this project, while conducted on non-transformed RPE cells, were not in accordance with the Jiang study. ARPE-19 cells cultured past confluence into quiescence at 10 days exhibited an $LD_{50}$ of 500 μM $H_2O_2$, while cells just reaching confluence and contact inhibition succumbed to apoptosis with much less $H_2O_2$ (Table 2).

The ATP experiments conducted here provide evidence that bilberry anthocyanin might protect undifferentiated RPE cells from $H_2O_2$-induced mitochondrial membrane permeability (Figure 16). However, it is more likely that observed ATP levels, which remained slightly above control levels in 7-day cultures, resulted from a slight pro-proliferative effect of anthocyanins, especially at very low concentrations. Indeed, in 7-day cultures treated with 10 ng/mL and 1 μg/mL bilberry extract but not treated with $H_2O_2$, ATP levels were 120% of controls. Apoptotic protection was modest at best and not evident as concentrations of $H_2O_2$ increased beyond 400 μM. These effects were lost entirely as RPE cells aged past 10 days in culture and bilberry anthocyanins appeared to induce apoptosis; however, these effects did not reach statistical significance at $p \leq 0.05$.

As in this *in vitro* study, cultured RPE cells can be triggered to undergo apoptosis. Several means can be utilized to induce RPE apoptosis, such as protein kinase inhibitors, peroxynitrite or $H_2O_2$, experimental ischemia/reperfusion, or tumor necrosis factor (Behar-Cohen et al., 1996; Jorgensen et al., 1998; Kimura et al., 1997; Osborne et al., 1997). This implies that multiple intrinsic apoptotic pathways are expressed by RPE cells in culture.

Evidence also indicates that oxidative stress–induced apoptosis involves a signaling mechanism generated from mitochondria. An early event in the apoptotic process involves loss of mitochondrial membrane potential; this loss occurs prior to caspase activation and DNA fragmentation (Cai et al., 1999) and strongly implies a regulatory role for the mitochondria in apoptosis (Cai et al., 1998; Yang et al., 1997). Indeed, cytochrome c released from oxidatively damaged mitochondria has been implicated in the formation of cytosolic apoptosomes and consequential caspase activation (Li et al., 1997; Mancini et al., 1998; Susin et al., 1999a; Susin et al., 1999b; Zou et al., 1999).

Release of cytochrome c involves the mitochondrial permeability transition (MPT) pore (Zoratti & Szabo, 1995). One MPT component is the adenine nucleotide transporter (ANT). Bax (a pro-apoptotic member of the Bcl-2 family of proteins) appears to interact with ANT (Marzo et al., 1998) to irreversibly open the mitochondrial permeability transition (MPT) pore, thereby decreasing mitochondrial membrane potential. The MPT pore also appears to be redox-sensitive and -responsive to the oxidative state of the thiol groups associated with the adenine nucleotide transporter (ANT) within its structure (Bernardi et al., 1994). Under states of oxidation, the MPT pore is opened (Majima et al., 1995; Zoratti & Szabo, 1995), facilitating other signals to promote apoptosis (Cai et al., 1999). Bilberry anthocyanins do not appear to directly interfere with this process. In fact, bilberry extract appears to modify expression of Bcl-2, but only slightly and in a manner insufficient to offer protection against mitochondrial permeabilization induced by $H_2O_2$ treatment.

Data presented here demonstrate that cultured RPE cells, under conditions of high oxidant exposure, can undergo mitochondrial depolarization. Unlike data reported by Zhang et al. (2003), pro-caspase-3 cleavage was observed in western blot analysis of proteins harvested from ARPE-19 cells after $H_2O_2$ treatment. For lack of additional primary antibody, the experiment was not repeated. The observation suggests that once $H_2O_2$ oxidative stress induces mitochondrial depolarization in RPE, the caspase cascade proceeds as in other apoptotic cells. Nevertheless, even though bilberry anthocyanins decreased intracellular radical formation (Figure 15), they could not prevent apoptosis induced by 500 μM $H_2O_2$. This result implies that simply reducing $H_2O_2$-induced intracellular radical formation is insufficient to prevent apoptosis. It is possible that $H_2O_2$-mediated damage leading to apoptosis begins by disrupting or modulating a signal transduction pathway and leads to disruption of thiol status and loss of mitochondrial potential via a pathway unaffected by anthocyanins.

In some cancer cells, such as human gastric adenocarcinoma cells (Shih et al., 2005), human promyelocytic leukemia cells (Katsube et al., 2003) and human monocytic leukemia cells (Hyun & Chung, 2004), anthocyanidins inhibit proliferation and induction of apoptosis. In cancer cells, anthocyanins have been shown to induce apoptosis via loss of mitochondrial membrane permeability through a mitogen-activated protein kinase (MAPK) pathway. Furthermore, the anthocyanidin malvidin mediates continued reduction of mitochondrial membrane potential at the same time as it elevates the ratio of Bax/Bcl-2 expression, which represents pro-apoptotic functions. In RPE cells, we did not observe Bax elevation, and bilberry anthocyanins did not appear to modulate pathways

affecting mitochondrial permiabilization. Results from this research suggest RPE does not respond to anthocyanins in the same manner as the cancer cells mentioned above.

Other signal transduction pathways potentially modulated in RPE by bilberry anthocyanins may have preventive AMD effects. As discussed in chapter III, oxidative stress is implicated in the pathogenesis of AMD (Beatty et al., 2000; Caiet et al., 2000). Supplementation with β-carotene, vitamins C and E, and zinc (AREDS Research Group, 2001), as well as dietary intake of these nutrients (van Leeuwen et al., 2005), is associated with reduced AMD risk; this implies that antioxidant mechanisms may play a role in preventing AMD progression.

Flavonoids are potent antioxidants *in vitro* (Rice-Evans et al., 1996) and may have a role in visual function (Schonlau & Rohdewald, 2001). However, little information is available regarding the antioxidant capacity or other mechanisms of flavonoid action in the retina. The relatively poor bioavailability and low cellular concentrations of most flavonoids, including anthocyanins, suggest that their most significant bioactivity may result less from quenching radical species than from modulating redox status indirectly (Fursova et al., 2005; Katsube et al., 2003; Kong et al., 2003; Wang et al., 1999). Recently, Fursova et al. (2005) found that bilberry anthocyanins decreased lipid peroxides in serum and retina, as well as slowed cataract development and macular degeneration in senescence-accelerated OXYS rats. The literature shows that anthocyanins are absorbed and excreted un-metabolized in their intact glycosylated form, although they are also biotransformed *in vivo* to glucuronidated and methylated derivatives (Kay et al., 2004; Kay et al., 2005; Milbury et al., 2002). Further studies are

required to characterize anthocyanin distribution and retention in the retina *in vivo*, as well as to determine the bioactivity of parent and metabolized anthocyanins.

Up-regulation of stress proteins is a universal, protective response to adverse conditions, including oxidative stress (Jaattela, 1999). HO-1 is a small heat-shock protein (Hsp32) that catalyzes the rate-limiting first step in the catabolism of heme to biliverdin, free iron, and carbon monoxide (CO) (Tenhunen et al., 1968). Biliverdin is subsequently converted to the antioxidant bilirubin via biliverdin reductase (Stocker et al., 1987). Three isoforms of heme oxygenase (HO), each the product of individual genes, have been identified: the inducible HO-1 and the constitutive heme oxygenase-2 (HO-2) and heme oxygenase-3 (HO-3) (Maines et al., 1986). Both HO-1 and HO-2 are found in human RPE cells (Kutty et al., 1994; Frank et al., 1999; Schwartzman et al., 1987; Hunt et al., 1996). HO-1 in retina appears to be up-regulated by light exposure and is increased in RPE from eyes with neovascular AMD (Miyamura et al., 2004). While the impact of these changes in RPE is not understood, in other cells HO-1 is induced by a wide variety of stress stimuli including hyperoxia and heavy metals, and it acts to protect against oxidative damage in cell cultures and *in vivo* (Lee et al., 1996). HO-1 shares regulatory genomic mechanisms with other enzymes, including GST and NAD(P)H:quinone oxidoreductase (NQO1), which function to detoxify by-products of oxidative stress (Nguyen et al., 2003).

Three distinct genes encode HO-1, HO-2, and HO-3 (Willis, 1999; Maines, 1997). HO-3 encodes a protein whose function is unknown (Hayashi et al., 2004). HO-1 and HO-2 encode enzymes that catalyze the rate-limiting step in heme catabolism to produce equimolar amounts of biliverdin, $Fe^{2+}$ and CO (Tenhunen et al., 1968). HO-2 is

constitutively expressed in most cell types while HO-1 is inducible only in selected tissues. Increased cellular expression of HO-1 is generally thought to be a defense mechanism against oxidative stress (Jaattela, 1999; Keyse & Tyrrell, 1989; Maines et al., 1993). Indeed, HO-1 messenger RNA (mRNA) and protein levels are elevated by known stress agents in human neuronal and retinal pigment epithelium cells in culture (Alizadeh et al., 2001; Kutty et al., 1992; Kutty et al., 1994; Ulyanova et al., 2001). HO-1 is up-regulated in rat retina exposed to visible light and exists in the retina at higher levels during daylight hours, suggesting a role in limiting light-induced radical damage (Organisciak et al., 2000; Kutty et al., 1995).

On the other hand, products, including $Fe^{2+}$, biliverdin, and CO, of HO-1 heme degradation can all be cytotoxic. Whether these molecules are toxic or cytoprotective may depend upon whether they are generated at appropriate levels within a tissue. In some cells, high levels of HO-1 may be damaging, while induction at lower levels of expression can confer some resistance to oxidative stress (Kapitulnik, 2004; Tomaro & Batlle, 2002) and to apoptosis (Liu et al., 2004). In other instances, experimental protein suppression with HO-1 antisense transfection (Kushida et al., 2002) has been shown to increase oxidative injuries. In contrast, over-expression of HO-1 may elicit toxicity from excessive production of heme-derived free $Fe^{2+}$ (Suttner & Dennery, 1999).

During oxidative stress, HO-1 mRNA is induced rapidly and results in increased levels of biliverdin; biliverdin, in turn, is converted to bilirubin by biliverdin reductase, a protein with antioxidant properties (Singleton & Laster, 1965; Stocker et al., 1987). In addition to its capability to scavenge radicals, bilirubin can also scavenge degraded low density lipoprotein (LDL) cholesterol (Wagner et al., 1993) which may limit the pro-

inflammatory effects of these molecules. Additionally, bilirubin inhibits activation of cell signal transduction pathways influenced by oxidative stress (i.e., activation of nuclear transcription factor kappa B (NF-κB)) (Soares et al., 2004). Inhibition of NF-κB inhibits cytokine tumor necrosis factor-α (TNF-α)–mediated expression of E-selectin and VCAM-1 receptors, thus decreasing extravasation of neutrophils, monocytes, activated T-helper and T-cytotoxic, and memory T and B cells to inflammation sites.

HO-1 activity also frees $Fe^{2+}$ from heme, since $Fe^{2+}$ can cause oxidative damage via the Fenton reaction (Borg, 1993). However, heme-derived $Fe^{2+}$ may also induce ferritin expression and activate the ATPase $Fe^{2+}$–secreting pump to decrease intracellular free $Fe^{2+}$ (Baranano et al., 2000).

CO, the third product of HO-1 activity, plays a significant role as an endogenous anti-inflammatory signaling molecule (Wagener et al., 2003). CO acts *in vivo* similar to nitric oxide (NO) as a signal transduction agent but absent its radical properties (Marks et al., 1991). Like NO, CO can mediate vasodilation, inhibit platelet aggregation, and suppress cytokine production, all factors beneficial in ameliorating AMD pathophysiology (Wu & Wang, 2005). Further, CO generated in the retina may play a role in dark adaptation and light sensitivity (von Restorff & Hebisch, 1988). In this study, we observed that bilberry extract induced HO-1 expression in RPE. While studies of the efficacy of bilberry in improving night vision have produced mixed results and have not been thoroughly conducted in populations with impaired vision (Canter & Ernst, 2004), anthocyanins may stimulate rhodopsin regeneration through increased retinal CO production via HO-1 up-regulation (Matsumoto et al., 2003).

An age-related decrease in HO-1, localized in the macula, has been demonstrated in human RPE cells (Frank et al., 1999; Miyamura et al., 2004). Further, a study of Asian Indian patients with Type 2 diabetes (especially those with microangiopathy) showed that oxidative damage was associated with increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and decreased HO-1 gene expression (Adaikalakoteswari et al., 2006). Short-term experimental diabetes in rats has been shown to increase heme oxygenase (HO) expression in the retina (Cukiernik et al., 2003). However, hyperglycemia has also been shown to inhibit HO-1 protein and HO activity in human endothelial cells in culture (Abraham et al., 2003), as well as in diabetic rats; this condition may reflect a lower level of antioxidant defense (Abraham et al., 1988; Bitar & Weiner, 1984). Experiments in rats and mice suggest that HO up-regulation decreases diabetes-induced dysfunction and injury (Goodman et al., 2006; Quan et al., 2004). Just as age and oxidative stress are associated with AMD and other retinopathies, diet or drug interventions that up-regulate HO-1 may be associated with diminished oxidative stress in the retina, especially in diabetics.

HO-1 induction at low levels of expression is associated with protection against oxidative stress. Similarly, HO-1 pre-induction in different injury models has been shown to confer increased resistance to oxidative stress (Kapitulnik, 2004; Tomaro & Batlle, 2002). But the evidence is mixed. For example, suppressing HO activity using antisense transfection or inhibitors worsens oxidative stress (Kushida et al., 2002). Moreover, high levels of HO-1 expression may be toxic, due to excessive cellular levels of heme-derived free iron (Suttner & Dennery, 1999).

Of the three isoforms transcribed from separate mammalian tissue genes, HO-1 is the primary ubiquitous, inducible isoform under conditions of oxidative stress (Cruse & Maines, 1988). A common cellular response to oxidant stress is apoptosis; therefore, HO-1 up-regulation in response to oxidative stress raises the question of whether HO-1 is a potential anti-apoptotic molecule. Indeed, HO-1 up-regulation in cancer cells has been shown to confer apoptotic resistance (Liu et al., 2004). It is unknown whether HO-1 up-regulation also confers apoptotic resistance in RPE cells.

Data exists suggesting that flavonoids, possibly including anthocyanins, may significantly up-regulate phase II enzymes, including GST, UDP-glucuronosyltransferase, NQO1, and epoxide hydrolase (Zhang & Gordon, 2004). The gene promoter regions for these detoxifying enzymes are transcriptionally regulated in common by gene response elements, including the xenobiotic response elements and the antioxidant/electrophile response elements (AREs/EpREs) (Nguyen et al., 2003). Flavonoids, when in their quinone forms, may also influence redox-sensitive pathways by reacting directly with thiols (Boots et al., 2003), influencing cellular glutathione redox status (Kessler et al., 2003), or inducing mitochondrial respiratory bursts with resultant increased hydrogen peroxide and superoxide anion production (Hodnick et al., 1986). Recently, an elegant transgenic mouse imaging study showed that gamma-glutamylcysteine synthetase promoter activity increased with administration of berry flavonoids (Carlsen et al., 2003). The degree to which dietary anthocyanins can modulate cellular GSH concentrations among humans and the degree to which they influence regulation of major cellular signaling pathways *in vivo* is unknown.

Studies suggest an association between oxidative stress–induced depletion of reduced GSH and HO-1 up-regulation (Lautier et al., 1992). Treating cells with compounds that complex easily with thiols results in HO-1 up-regulation (Foresti et al., 1997; Hiwasa & Sakiyama, 1986; Keyse & Tyrrell, 1989). In contrast, increasing N-acetyl-L-cysteine, a precursor to GSH, inhibits HO-1 up-regulation in stressed cells (Borger & Essig, 1998), further supporting a role for GSH in HO-1 regulation.

Although its exact role is unclear, HO-1 appears to function with other enzymes such as GST and NQO1 in the detoxification process of oxidative stress (Bao et al., 1997; Ketterer & Meyer, 1989; Ryter & Tyrrell, 2000; Talalay & Benson, 1982). The enhancer region of these enzymes' genes, responding to oxidative stress, contains the antioxidant response element (ARE), so named because it is responsive to phenolic antioxidants such as butylated hydroxytoluene (BHT) (Rushmore & Pickett, 1990). At first, this may seem a contradiction; however, ARE mediates transcriptional responses to both oxidative stress–induced alterations in cellular redox status and pro-oxidant xenobiotics that are thiol reactive and mimic an oxidative insult. Further, ARE-regulated genes encode a wide variety of proteins that help control cellular redox status, defending against oxidative damage in phase II metabolism (Hayes & McLellan, 1999). Although we expected $H_2O_2$ to increase oxidative stress and up-regulate HO-1 in our model, we observed that bilberry extract elicited a significant increase in HO-1 expression (Figures 19 and 21). Though it has been suggested that flavonoids can induce cellular radical formation, incubation with bilberry extract did not produce radicals in these experiments and even diminished those generated by $H_2O_2$ exposure (Figure 15).

The observed HO-1 up-regulation in response to bilberry anthocyanins in this study appeared in the low over-expression range. Hence, bilberry-induced HO-1 up-regulation may confer protection against oxidative stress–induced damage in RPE. Furthermore, these observations suggest that while $H_2O_2$, bilberry polyphenolics, and bilberry anthocyanins induce HO-1 up-regulation, they do so by distinctly different mechanisms and pathways. The ability of bilberry anthocyanins to up-regulate both HO-1 and GSTP1 implies that anthocyanins can stimulate pathways leading to ARE stimulation, thereby boosting an array of endogenous antioxidant protective systems. We know that HO-1 up-regulation in response to conditions of oxidative stress has excess potential, and the iron release from heme degradation can exacerbate oxidative injury. Our study revealed that bilberry anthocyanins elicit a moderate HO-1 up-regulation and, therefore, a potentially protective response in RPE cells.

HO-1 shares regulatory genomic mechanisms with other enzymes, including GST and NQO1, both of which function to detoxify by-products of oxidative stress (Nguyen et al., 2003). Inducing enzymes involved in GSH biosynthesis provide further antioxidant protection in cells (Hayes & McLellan, 1999). Age-related declines in GSH are associated with increased AMD risk (Samiec et al., 1998). The purpose of the present study was to investigate the effects of phenolic antioxidants, primarily anthocyanins, from bilberry (*Vaccinium myrtillus*) on RPE cell cultures under quiescent conditions and oxidative stress.

As mentioned earlier, HO-1 induction at low levels of expression confers some resistance to oxidative stress (Tomaro & Batlle, 2002) and in so doing may also confer apoptotic resistance (Liu et al., 2004). Conversely, experimental suppression of the HO-1

protein with anti-sense transfection or inhibitors such as Tin mesoporphyrin (SnMP) increases oxidative injuries (Kushida et al., 2002). However, over-expression of HO-1 may elicit toxicity from excessive production of heme-derived free iron (Suttner & Dennery, 1999). In rats, HO-1 up-regulation promotes Muller cell survival after retinal ischemia by increasing intraocular pressure (Arai-Gaun et al., 2004). A similar study showed that sour cherry seed flavonoid-rich extract protects against reperfusion-induced injury; this occurs through reduced changes in retinal ion concentrations via HO-1–related endogenous CO production in the ischemic/reperfused retina (Szabo, 2004).

Sacca et al. (2003) investigated photic regulation of heme oxygenase activity in golden hamster retinas and found statistically significant differences in light-related enzymatic activity differences between midday and midnight. *In vitro*, inhibitor studies of this light-induced increase in HO-1 activity suggest that hamster retinal HO-1 activity is regulated by the photic stimulus, probably through a dopamine/cAMP/PKA–dependent pathway. Investigators also found that low bilirubin concentrations decreased retinal thiobarbituric acid reactive substances (TBARS) levels (an index of lipid peroxidation) in basal conditions and after exposing retinal cells to $H_2O_2$. These studies suggest that HO-1 up-regulation serves as a response to oxidative stress in the retina, both *in vivo* and *in vitro*. Flavonoids also up-regulate HO-1 in intact retinas, and HO-1 up-regulation appears protective against radical-induced damage. Evidence does not, however, address whether HO-1 up-regulation occurs in RPE cells or whether it is protective in RPE.

One study by Jang et al. (2005) investigated the extent to which plant-derived anthocyanins modulate adverse effects of pyridinium bisretinoid A2E, an autofluorescent pigment accumulating in RPE cells with age and, in some disorders, in ARPE-19 cells.

This study showed that anthocyanins serve as antioxidants that suppress photo-oxidative changes induced by short wavelength light in RPE cells via the lipofuscin fluorophore A2E. In so doing, anthocyanins thus reduce loss of RPE cell viability. These results may be specific to damage induced by singlet oxygen.

Tsuda et al. (1994) showed that cyanidin 3-galactose was more potent in inhibiting lipid peroxidation at neutral pH than either vitamin E or Trolox. It is hypothesized that antioxidant activity of anthocyanins involves many structural elements, including the ability to form stable quinonoidal anhydro bases, a C ring possessing a conjugated diene system capable of quenching singlet oxygen, hydroxyl groups on the B ring, and amphipathic behavior with a tendency toward hydrophobicity. Anthocyanins are polar in nature (Lamikarna, 1987). The results reported here support Jang et al. (2005) in that bilberry extract can be taken up by the RPE cell (Figures 9, 10, 11, and 12) and can act to quench intracellular radicals (Figure 15). Although $H_2O_2$ itself is not a free radical, it serves as an intermediary in the formation of highly reactive free radicals and reactive oxygen species, such as hydroxyl radicals (Fridovich, 1997). It is likely that the damage inflicted on RPE in studies using light differs from that induced by $H_2O_2$ exposure; nevertheless, $H_2O_2$ exposure is a relevant oxidative stress, considering the retinal levels generated by photoreceptor reactions and phagocytic processes.

Unlike the results described above , this study showed that despite the ability of bilberry anthocyanins to up-regulate phase II enzymes (Figures 19–24) and quench intracellular radicals, bilberry extract did not protect against $H_2O_2$-induced apoptosis. This suggests that $H_2O_2$-induced apoptosis in our RPE model may be mediated through damage to cell surface receptors or membranes that are unaffected by intracellular radical

quenching or up-regulation of xenobiotic metabolism and endogenous antioxidant defenses.

GSH depletion through oxidative stress or agents that complex thiols is known to up-regulate HO-1 (Foresti, et al., 1997; Lautier et al., 1992). These effects were seen in this study as well (Figures 13, 19, and 21). A role for GSH regulating HO-1 is also evident by the ability of N-acetyl-L-cysteine, a GSH pro-drug, to inhibit HO-1 up-regulation in hypoxic cardiomyocytes (Borger & Essig, 1998). HO-1 also functions in antioxidant defenses in coordination with GST (Ketterer & Meyer, 1989; Ryter & Tyrrell, 2000; Talalay & Benson, 1982). Data presented here show that GSTP1 and HO-1 protein and mRNA are up-regulated in a coordinated manner. BHT and beta-naphthoflavone (BNF) have been reported to modify the expression of ARE-containing genes (Rushmore & Pickett, 1990), including those involved in phase II metabolism (Hayes & McLellan, 1999), and they were used as positive controls in our quantitative real-time polymerase chain reaction (rt-PCR) experiments. Dietary antioxidant flavonoids, such as quercetin and epicatechin, are capable of inducing detoxifying enzymes by pathways mediated by ARE/EpRE (Benson et al., 1978, Gordon et al., 1991). While anthocyanins have not been tested for ARE/EpRE activation, their structural similarity to flavonoids and phenolics known to modulate ARE make the expectation of ARE/EpRE reasonable. A role for anthocyanins as polyphenolic xenobiotic compounds would not be surprising, considering their evolutionary history in plants as bitter deterrents for herbivores. The ability of anthocyanins to modulate ARE/EpRE requires more study, especially in light of their potential anticancer properties.

The observation presented here of increases in HO-1 and GSTP1 expression in response to bilberry extract in RPE indicate a coordinated protective mechanism against oxidative stress. Both the HO-1 and GSTP1 genes are induced by reactive oxygen species, ultraviolet-radiation, and xenobiotics, all of which induce changes in cellular redox potential. These disturbances of intracellular redox equilibrium represent an adaptive stress response that ultimately changes gene expression to re-establish cellular homeostasis. Depletion of reduced GSH, either via oxidative stress or utilization in xenobiotic metabolism, appears to underlie the initiation of this response. GSH depletion has been shown to play a role in the translocation of NF-E2-related transcription factor (Nrf2) that activates ARE/EpRE gene transcription (Hayes & McLellan, 1999). In addition to kinase action, a protein designated Kelch-like-ECH–associated protein 1 (Keap1) has been shown to repress Nrf2 activity within cells. It has been postulated that sulfhydryl group chemistry, modified by the cellular levels of reduced glutathione, may play an important part in the regulation of cell signaling pathways and in protein-protein interaction, such as between Keap1 and Nrf2 (Nguyen et al., 2003). Anthocyanin interaction with redox-sensitive protein kinase cascades (e.g., mitogen-activated protein kinases) may serve as an initial mechanism whereby anthocyanins can up-regulate HO-1 and GSTP1. These data imply that anthocyanins can modulate a pathway, or pathways, that lead to ARE stimulation, thereby stimulating an array of endogenous antioxidant protective systems potentially protective of RPE cells.

Anthocyanins have physiological effects, including inhibition of cell proliferation (Katsube, 2003; Marko et al., 2004), antioxidant capacities (Faria et al., 2005), and modulation of inflammatory processes (Rossi et al., 2003). These physiological effects

also arise from over-expression of HO-1 (Morita et al., 1997; Willis et al., 1996). The results from this study suggest that the ability of bilberry anthocyanins to increase HO-1 may provide beneficial effects in preventing or slowing other aspects of advanced AMD pathology (e.g., angiogenesis and inflammation). If such actions are substantiated *in vitro* and extended to *in vivo* models, anthocyanins and other flavonoids may promote retinal functions and/or treatment of degenerative retinal conditions such as AMD. In conclusion, this study supports the hypothesis that although anthocyanins are excellent radical quenchers *in vitro* and can quench radicals *in vivo*, they do not prevent apoptosis induced by high $H_2O_2$ doses. While radical quenching may play a role at lower concentrations of radical induction, low anthocyanin levels relative to other dietary and endogenous antioxidants make their role as a major contributor unlikely. However, these data suggest that other mechanisms involved in redox regulation may provide cellular benefit from consumption of berry anthocyanins.

## Future Direction of Research

In recent *in vitro* experiments, bilberry anthocyanins were shown to serve as antioxidants to suppress photo-oxidative processes mediated by lipofuscin fluorophore A2E in RPE cells (Jang et al., 2005). Results of this project correspond with Jang et al.: that is, pre-incubation with bilberry extract quenches intracellular radicals in RPE that are oxidatively stressed with $H_2O_2$. Unlike evidence from cancer cell studies demonstrating anthocyanin-induced apoptosis (Nichenametla, 2006), our results show that in non-transformed, differentiated RPE cells *in vitro,* bilberry anthocyanins and phenolics do not interfere with $H_2O_2$-induced apoptosis. However, the same treatments with $H_2O_2$ up-

regulated protective enzyme systems are modulated by oxidative stress, antioxidants, and xenoboitic compounds. This disconnect between the inability to modulate oxidative stress–induced apoptosis in "normal" cells and the ability to quench intracellular radicals and modulate protective enzymes needs further investigation. Indeed, the more important functions of anthocyanins may lie in their ability to restore pathways that permit apoptosis in those cells having lost or blocked apoptotic capability.

Apoptosis and cell-cycle arrest are two common outcomes induced by dietary anti-proliferative compounds effective against the promotion and progression of pre-neoplastic or neoplastic cells. In contrast to death receptor–mediated cell apoptosis, chemopreventive compounds generally induce oxidative stress; oxidative stress, in turn, down-regulates anti-apoptotic molecules (e.g., Bcl-2 or Bcl-x) and up-regulates pro-apoptotic molecules (e.g., Bax or Bak). In this project, bilberry extracts neither increased intracellullar radicals nor altered Bcl-2 or Bcl-x in the non-transformed RPE cells. Indeed, the extract performed as an antioxidant in ARPE-19 cells in culture. Future experiments could be performed using a carcinoma cell line to determine whether bilberry extract, rather than acting as an antioxidant, instead induces oxidative stress, down-regulates anti-apoptotic Bcl-2 or Bcl-x, and up-regulates pro-apoptotic Bax or Bak. Observation of anthocyanin response differences between ARPE-19 cells and carcinoma cell lines could lead to better understanding of the pathways involved.

Cancer-associated retinopathy (CAR) is an ocular manifestation of a paraneoplastic syndrome that has been linked to aberrant expression of recoverin, a retina-specific $Ca^{2+}$-binding protein, that leads to retinal degeneration. Evidence also suggests involvement of G-protein–coupled receptor kinases (Miyagawa et al., 2003).

Monitoring apoptotic re-establishment as well as G-protein–coupled receptor kinases could provide insight into whether this pathway is involved in anthocyanin-induced re-establishment of cancer cell apoptosis. Since many cancers show aberrant recoverin expression, inhibiting associated retinopathies may be one way in which bilberry anthocyanins can assist in maintaining visual function. Implications for cancer treatment more generally could be extrapolated.

It has been established that the imbalance between anti-apoptotic and pro-apoptotic proteins affects the maintenance of mitochondrial potential and can lead to apoptosis (wherein the release of factors to the cytosol leads to activation of the caspase cascade, and, eventually, apoptosis). Flavonoids in general can activate JNK, inhibit anti-apoptotic NF-κB signaling pathways, and block growth factor–mediated anti-apoptotic signals by inhibiting growth factor binding to the receptor. Flavonoids also inhibit the downstream phosphatidylinositol 3-kinase (PI3K)–Akt pathway. These pathways have been defined in large part by studying flavonoid influence on cancer cells *in vitro* and are thought to be mediated by stress signals. In light of the evidence presented in this dissertation and by Jang et al. (2005), anthocyanins do not appear to induce oxidative stress signals within normal retinal cells. It is unclear whether anthocyanins induce cancer cell apoptosis. However, answering this question could help explain the differences observed between the actions of anthocyanin on normal and pre-neoplastic or neoplastic cells.

Before proceeding into animal models, however, more research is required on anthocyanin uptake into tissues. *In vivo* evidence suggests that bilberry anthocyanin supplementation affects neurological function and memory (Andres-Lacueva et al., 2005;

Ramirez et al., 2005). Recent research also indicates that berry anthocyanins pass through the blood-brain barrier (Youdim et al., 2003), and that anthocyanins are found in the eye after blueberry ingestion (Milbury et al., 2005; Milbury et al., 2006). Determining dietarily achievable concentrations of individual anthocyanins in the eye and retina will be essential to determining whether bilberry consumption will affect physiological changes in RPE cells *in vivo*. However, analytic techniques are just emerging to allow analysis of low anthocyanin levels within tissue. This task of determining tissue distribution of both anthocyanins and their metabolites *in vivo* would be enhanced should individual radiolabeled anthocyanins become available. Discussions to address the issue are underway.

Future research could easily proceed *in vivo* on the subject of phase II induction, testing the effects of bilberry extract or anthocyanin by monitoring *in vivo* and *ex vivo* imaging of promoter activity in transgenic mice (Moskaug et al., 2005). Mice carrying reporter genes and injected with luciferin after feeding or lavaging with bilberry extracts or anthocyanins could be imaged and the luminescence emitted by each transgenic mouse could be quantified. This technique has been applied to investigate the effects of quercetin on transgenic mice strains expressing luciferase regulated by various transcription factor binding sites regulated by NF-κB, AP-1, or several binding sites in the gGCSh gene promoter (Moskaug et al., 2004). The approach could be adapted to study the anthocyanin effects on apoptosis-related or ARE-regulated genes.

In addition to verifying the results of anthocyanin effects in OXYS rats, studies of the effects of anthocyanins in preventing AMD in an Cu, Zn-superoxide dismutase deficient mouse model can be undertaken. Recent investigations show that senescent

Sod1$^{-/-}$ mice have drusen, thickened Bruch's membrane, and choroidal neovascularization that increase with age, and exposure to excess light (Imamura, et al., 2006). The retinal pigment epithelial cells of Sod1$^{-/-}$ mice showed oxidative damage in a manner that affects junctional proteins necessary for RPE barrier integrity. These observations strongly suggest that oxidative stress may play a causative role in age-related retinal degeneration. The ability of bilberry anthocyanins in preventing this damage could easily be tested in this animal model.

As mentioned at the beginning of this thesis, anthocyanins are members of a diverse and profuse class of phytochemical: the flavonoids. They are ubiquitous in human diets, and possess potential health benefits that have yet to be unequivocally proven. Alone or in combination, these compounds appear to exhibit many cellular effects; however, it is difficult to predict the overall combinatorial effect of these compounds on gene expression *in vivo*. The emerging field nutrigenomics uses genomic techniques, such as gene chip analysis, to study the integrated effects of nutrients on gene regulation. However, use of *in vivo* bioluminescence imaging of gene expression presents a unique opportunity to directly observe effects of bioactive dietary compounds, such as anthocyanins, on biological systems where preliminary evidence of activity exists. In the case of anthocyanins, it would be tempting to study next the up-regulation of xenobiotic metabolism in the protection against retinal damage or in the defense against cancer. Uptake and metabolism of xanthophylls carotenoids in the retina is thought to be mediated by specific binding proteins and GSTP1 has been shown to be a zeaxanthin-binding protein in the retina (Bhosale et al., 2004). It is possible that long-term bilberry consumption or other anthocyanin-rich foods may elevate levels of

xenobiotic enzymes, including GSTP1; as a secondary consequence, xanthophyll concentrations could increase. While intervention trials are required to fully determine this possibility, our results suggest that the mechanism is plausible.

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