EFFECTS OF ANTHOCYANIDINS ON INTESTINAL ADENOCARCINOMA CELLS UNDER OXIDATIVE STRESS

by

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(Under the Direction of Joan G. Fischer)

ABSTRACT

Non-nutritive phytochemicals may reduce risk of chronic disease via antioxidant activity. It is possible that higher concentrations may act as prooxidants and stimulate apoptosis. To determine whether the aglycone anthocyanins malvidin and peonidin act as antioxidants or prooxidants in vitro, we treated HT-29 cells at varying concentrations. Cells were incubated with 1, 5, 10, 25, or 50 µg/mL malvidin or peonidin for two hours at 37°C, 5% CO2, 95% air. Cells were then treated with H_2O_2. The Comet assay determined DNA damage, and apoptosis was assessed using a DNA fragmentation ELISA kit. Peonidin decreased H_2O_2-induced DNA damage at the lowest tested concentration ($p < .05$). Compared to positive control malvidin at 50 µg/mL significantly increased ($p<.05$) H_2O_2 induced DNA damage. Our data suggests that at concentrations of 5-50 µg/mL malvidin and peonidin did not decrease H_2O_2 induced DNA damage, and malvidin at 50 µg/mL increased H_2O_2-induced DNA damage in HT-29 cells.

INDEX WORDS: Berries, Anthocyanins, Anthocyanidins, Malvidin, Peonidin, Cancer, Colon, Oxidative stress, In vitro

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B.S. The Ohio State University, 2006

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2008

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DEDICATION

To Uncle Dan, for editing his niece’s thesis pieces.

ACKNOWLEDGEMENTS

Thanks to Dr. Joan Fischer for her constant guidance and support. Thanks also to my committee members Dr. Arthur Grider and Dr. Silvia Giraudo. Special thanks to Sharon Hanson and the Foods and Nutrition office staff, for always keeping me well supplied. To Joyce Powers, thanks for helping in every lab.

Rachel Dulebohn and Lisa Kobs- thanks for the memories and for keeping me sane.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS v

LIST OF TABLES viii

LIST OF FIGURES ix

CHAPTER

I INTRODUCTION 1

References 3

II BACKGROUND 5

Cancer 5

Apoptosis 8

Oxidative Stress 9

Oxidative Damage & Polyphenols 11

Measures of Oxidative Stress 12

Use of Hydrogen Peroxide to Create Oxidative Stress 13

Polyphenols 14

Anthocyanins 15

Measures of Total Antioxidant Capacity 16

Antioxidant Capacity of Anthocyanins 18

Intake and Bioavailability 19

Prior Studies 20

Hypothesis 21

References 23

III EFFECTS OF ANTHOCYANIDINS ON INTESTINAL ADENOCARCINOMA CELLS UNDER OXIDATIVE STRESS 31

Abstract 31

Introduction 32

Methods 34

Comet Assay 35

Apoptosis 38

Lipid Peroxidation 39

Cell Viability 40

Statistics 40

Results 41

Discussion 42

Limitations 45

References 56

IV SUMMARY AND CONCLUSIONS 59

References 61

APPENDICES 63

A Anthocyanin Serial Dilution 64

B Comet Assay with H_2O_2 Treatment for Trevigen kit 66

C DNA Fragmentation using ELISA Cell Death Detection kit 70

LIST OF TABLES

Page

Table 1: Proposed mechanisms of action by which dietary antioxidants may prevent cancer.....28

Table 2: Anthocyanin content of selected foods on a per serving basis 29

Table 3: DNA Damage in H_2O_2 exposed HT-29 cells treated with malvidin and peonidin at concentrations of 10-50 µg/mL 49

Table 4: DNA Damage in H_2O_2 exposed HT-29 cells treated with malvidin and peonidin at concentrations of 1-50 µg/mL 50

Table 5: Apoptosis in H_2O_2 exposed HT-29 cells treated with malvidin and peonidin at concentrations of 1-50 µg/mL 53

Table 6: Concentrations of anthocyanidins tested 55

LIST OF FIGURES

Page

Figure 1: Basic structure of anthocyanidins.....30

Figure 2: DNA Damage in H_2O_2 exposed HT-29 cells treated with malvidin and peonidin 51

Figure 3: DNA Damage in H_2O_2 exposed HT-29 cells treated with malvidin and peonidin 52

Figure 4: Apoptosis in H_2O_2 exposed HT-29 cells treated with malvidin and peonidin 54

CHAPTER I

INTRODUCTION

Whole berries and berry extracts contain a number of potential chemopreventive agents. These include vitamins A, C, and E, folate, calcium, selenium, B-carotene, a-carotene, lutein, polyphenolics, phytosterols, and triterpene esters (Seeram, 2008b; Duthie, 2007). Among these, the anthocyanin subclass of polyphenols are potentially among the most bioactive in cancer prevention. Berries, berry extracts, and anthocyanins have all been investigated for anti-carcinogenic ability. These compounds may act as anti-cancer agents by reducing inflammation, facilitating carcinogen excretion, upregulating DNA repair enzymes, and also as antioxidants to reduce DNA damage (Duthie, 2007; Seeram, 2008a).

The role of anthocyanins as antioxidants is of particular interest, as reactive species are inherent to aerobic metabolism and are potent genotoxins. We are exposed to free radicals, reactive oxygen and reactive nitrogen species throughout the lifespan. Free radicals cause damage to lipids, proteins, and nucleic acids (Prior and others 1998). The cumulative damage to DNA, in the forms of mutations, strand breaks, and base damage may be a determining factor in the development of cancer. Reactive species may also affect DNA damage indirectly, by inhibiting DNA repair enzymes (Halliwell, 2007). Concomitant lifetime exposure to potent phytochemicals from fruits and vegetables in the diet may counteract this damage, preventing the disease and onset of cancer.

108,070 new diagnoses of colon cancer and 49,960 colon cancer deaths are projected for 2008 in the United States (ACS, 2008). Diet may play an important role in the prevention of colon cancer; as much as eighty percent of colorectal cancer incidence is attributed to diet (Johnson, 2004; WCRF/AICR, 2007; ACS, 2007).

Cancer is characterized by uncontrolled cell growth, replication, resistance to apoptosis, and tissue invasion (WCRF/AICR 2007). In the pathology of cancer, one or more events occur which allow the tumor cell to forgo regulatory mechanisms normally in place. Oxidative damage is proposed as one of these mechanisms, as oxidative stress is known to damage proteins, lipids, and nucleic acids (Prior and others, 1998).

Oxidative damage is both an event that initiates cancer and a regulatory mechanism that can be bypassed by cancer cells. Damage may cause a cell to mutate or allow tumor promoter genes to be expressed; conversely, damage may be inflicted by the immune system with the intent of removing the offending cell, but a cancer cell may be able to survive the insult (Loo, 2003). The polyphenols found in fruits and vegetables, especially berries, may interfere with these processes (Seeram, 2008a; Duthie, 2007). Polyphenols may serve as antioxidants, block the initiation stage of cancer and facilitate excretion of pro-carcinogens by altering phase I and II enzymes, stimulate DNA repair enzymes, inhibit cell proliferation, and induce cell apoptosis (Scalbert and others 2005).

It remains to be seen which of the many compounds in berries exerts the anti-cancer effect, or whether the effects of multiple anthocyanins are synergistic. As extracts and polyphenols are singled out for antioxidant capacity, the quantity required for a therapeutic dose is still unknown. Another question facing researchers is whether the same compounds would act differently in different cell types- for example, to the benefit of healthy cells while to the detriment of tumor cells.

Two such compounds identified for their antioxidant capacity are malvidin and peonidin. Based on previous work in our lab these two anthocyanidins were chosen for further study in a cell model of human colon cancer. HT-29 human adenocarcinoma cells were exposed to a range of anthocyanidin concentrations to assess their effects in the presence of an added stressor, hydrogen peroxide. I tested the hypothesis that anthocyanidins had a prooxidant effect at higher concentrations while acting as antioxidants at lower concentrations. This was assessed using measures of DNA damage, apoptosis, lipid peroxidation, and cell viability.

The results of our study are inconclusive for the prooxidant effect of malvidin, as it was significantly induced at higher concentrations, but only in one of two studies. Malvidin showed no antioxidant effect at the ranges tested. Conversely, peonidin showed no prooxidant effect at the ranges tested here, but did show an antioxidant effect at the lowest concentration. These results further the debate of the role of anthocyanidins in cancer prevention and treatment.

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CHAPTER II

BACKGROUND

Cancer

The National Institutes of Health National Cancer Institute definition of cancer is "a term for diseases in which abnormal cells divide without control; cancer cells may invade nearby tissues and can spread through the bloodstream and lymphatic system to other parts of the body" (Hance, 2008). Though it is possible to develop cancer at any age, one prevailing hypothesis is that following a lifetime of exposure to endogenous and exogenous oxidative stress, mutagenic DNA is likely to result. Incidence of cancer increases with age; approximately 35% of people will have cancer by age 85 (Halliwell, 2007b).

The pathogenesis of cancer is a multi-stage process and is influenced by many factors; however the process can be understood in terms of initiation, promotion, and progression (WCRF/AICR, 2007). In the initiation stage of cancer normal cells suffer genetic or epigenetic alterations to become initiated. This may be the result of DNA damage, exposure to carcinogens, or DNA adducts that cause mutation. Strategies to prevent initiation of healthy cells are to alter carcinogen metabolism, enhance carcinogen detoxification, scavenge reactive oxygen species, and enhance DNA repair enzymes (WCRF/AICR, 2007; Hance, 2008).

Once initiated, the cells replicate and bypass regulatory mechanisms that would otherwise suppress proliferation and induce apoptosis. As cells replicate they may proceed to a preneoplastic lesion, and finally progress to a neoplastic tumorous growth (WCRF/AICR, 2007). If left unchecked, the cancer will metastasize to other locations in the body. Mechanisms which

may prevent promotion and progression are to scavenge reactive oxygen species, decrease inflammation, inhibit cell proliferation and induce apoptosis, enhance immune response, and inhibit angiogenesis (Hance, 2008).

There are six characteristics consistent among all types of cancer, known as the hallmarks of cancer (Hanahan and Weinberg, 2000). These hallmarks are growth signal autonomy, insensitivity to antigrowth signals, limitless replicative potential, evasion of apoptosis, sustained angiogenesis, and tissue invasion and metastasis. The first two are closely related - normal cells must receive signals to grow and replicate, these signals may be from within the cell, from nearby cells, or hormone signaling (WCRF/AICR, 2007). In cancer cells, this process is not needed and tumor cells replicate without regulation. Cancer cells also adapt to ignore anti-growth signals that would otherwise instruct a normal cell to stop replicating. Even if healthy cells continued to replicate, after approximately 60-70 replications they reach the end of their telomeres, and apoptosis begins. Healthy cells cannot replicate indefinitely, and will undergo programmed cell death when they reach senescence. Cancer cells adapt or mutate to avoid this regulatory mechanism, and are characterized by their endless ability to replicate.

Again, healthy cells are stopped from endless replication and by the process of programmed cell death, apoptosis. Cancer cells evade apoptosis, even though it is intended to remove damaged cells. For continued growth, cancer cells need a large supply of nutrients, which is facilitated by angiogenesis. The development of new blood vessels provides tumors with the nutrients needed to progress, and as such, some cancer drugs may act to suppress angiogenesis (WCRF/AICR, 2007). The sixth hallmark of cancer is tissue invasion and metastasis, at which point cancer cells spread throughout the body through the blood and lymph. Metastasis is the primary cause of mortality in cancer (WCRF/AICR, 2007).

The cancer process is intricate and complicated, and current therapies for cancer treatment include radiation therapy, chemotherapy, and surgical removal of tumors. The success of these therapies varies by cancer site and stage, and for some cancers the five-year survival rate is grim. While research is ongoing as to novel and improved methods of cancer treatment, cancer prevention is also under study.

Cancer chemoprevention can be no more succinctly stated than as the use of chemically active, non-toxic compounds to "reverse, suppress or prevent progression of disease from pre-invasive cancer to frank malignancy" (Sporn and others 1976). The ultimate goal of cancer treatment then, is to establish a treatment that is differential- lethal to cancer cells, but non-toxic to healthy cells and tissue (Kraft and others 2005). It has been estimated that 35% of cancer incidence can be attributed to diet (Willett, 1995; Steinmetz and Potter, 1996; ACS, 2007) and that diets high in fruits and vegetables (more than 5 servings per day) could prevent 20% of all cancer incidence (Van Duyn and Pivonka, 2000). Lifetime exposure to these protective compounds from food or supplements may counteract the unavoidable sources of oxidative stress and DNA damage, which may cause carcinogenesis.

Apoptosis

Apoptosis is the process of cell suicide, or programmed cell death. Intrinsic and extrinsic signaling mechanisms instruct the cell to carefully self destruct, and the cell is then engulfed by phagocytes for removal. Apoptosis and ensuing phagocytosis avoids cell lysis, which would otherwise cause damage to surrounding cells and elicit an inflammatory response (Johnstone and others 2002). Though it may seem counterintuitive, apoptotic cell death is essential to healthy tissue. A balance between cell growth and proliferation and cell death must be maintained. Apoptosis allows the organism to carefully regulate cell number and tissue size, crucial to prevention of tumor development (Yi and others 2006a; Yi and others 2005a).

Though there are a number of intrinsic and extrinsic pathways which regulate apoptosis, the p53 tumor suppressor gene is well studied and mutations of it are found in nearly all human tumors (Johnstone and others, 2002). The p53 gene activates apoptosis within a cell, and is frequently silenced in tumorigenesis. As such, agents that activate apoptosis through other means are desirable for control and elimination of cancer cells. Direct DNA damage can activate apoptosis (Johnstone and others, 2002). Other apoptotic stimuli include reactive oxygen species, ion fluctuations, and cytokines (Sun and others 2004).

Apoptosis is considered the preferred method of cell death in cancer treatment, and potentially the most potent (Hou, 2003; Kuo and others 2005; Yi and others, 2006a; Johnstone and others, 2002; Sun and others, 2004). Necrosis is another form of cell death, but it is uncontrolled. In necrosis the cell may burst, releasing harmful metal ions and toxins that damage surrounding cells and increase oxidative stress (Halliwell, 2007a). While the goal of cancer treatment is to kill the cancerous cells, necrotic cell death does significant damage to all cells, healthy and cancerous, surrounding it. Necrosis and the resulting cell lysis also cause inflammation, a known risk factor for cancer (World Cancer Research Fund / American Institute for Cancer Research, 2007; Hance, 2008).

Tumors may be controlled by cytostatic therapies which cause stagnation of tumor growth, but cancer may return or tumors relapse if cells are not removed. Apoptosis terminates the cell and removes it from the organism, reducing the possibility of relapse (Johnstone and others, 2002). Also, rapid removal of tumor cells from the organism by apoptosis could reduce treatment length and side effects experienced with treatments that act to promote cytostasis or cell differentiation (Sun and others, 2004). Apoptosis also aids in cancer prevention as it can remove genetically damaged, pre-initiated, or neoplastic cells (Seeram and others 2006). Even slight improvements in apoptotic control of cells may help maintain a cell growth balance, and potentially decrease the risk of carcinogenesis (Yi and others, 2005a; Hanahan and Weinberg, 2000).

Oxidative Stress

One proposed cause of cancer and most chronic diseases including cardiovascular disease is increased reactive oxygen species and oxidative stress (Jacob and Burri, 1996). Oxidative stress is the result of more pro-oxidant (electron or hydrogen atom taking) molecules being

present in a tissue or sub-cellular organelle than antioxidant (electron or hydrogen atom donating) molecules. This can be due to a deficiency of antioxidants or an excess of pro-oxidants (Halliwell, 1999). Pro-oxidants are produced through various metabolic processes including the electron transport chain, the cytochrome P450 system in the liver, and the immune system (Cai and Harrison, 2000). Pro-oxidant molecules are also produced by exogenous factors such as cigarette smoking, radiation, UV light, and inflammation. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are pro-oxidants (Halliwell, 2007b). The term "reactive species" encompasses reactive oxygen, nitrogen, halogen, and sulfur species (Halliwell, 2007b).

Reactive species and antioxidants are ever present *in vivo*. Pro-oxidants are not inherently "bad" as often portrayed; rather they are natural by-products of aerobic metabolism and necessary components of immune function. Reactive species can create a "respiratory burst" that kills pathogens or executes programmed cell death. The ability to destroy pathogens or cells that have ceased to function normally is essential in preventing diseases caused by bacteria, viruses, or cancer cells (Halliwell, 2007b).

Antioxidants exist *in vivo* in many forms. Enzymatic antioxidants are referred to as preventative antioxidants because they inhibit the formation of reactive species (Ou and others 2002). Superoxide dismutase, catalase, and glutathione peroxidase are enzymatic antioxidants and are dependent upon metal ions of selenium, manganese, zinc, copper, and iron (Cotelle, 2001).

Non-enzymatic antioxidants are referred to as chain-breaking antioxidants because they scavenge oxygen radicals and break chain sequences of oxidation after they have begun (Ou and others, 2002). Glutathione is a critical antioxidant compound as it is the major contributor to the

redox state of the cell and is involved in many aspects of metabolism and regulation (Wu and others ; Moskaug and others 2005). Glutathione is synthesized from glutamate, cysteine, and glycine and exists in two states, reduced glutathione (GSH), and oxidized glutathione (GSSG). The cell cytosol contains 80-95% of all glutathione present (Wu and others, 2004). The GSH:GSSG ratio is determined by the redox state of the cell, and glutathione may be up-regulated by dietary factors or depleted by hydrogen peroxide (Wu and others, 2004; Loo, 2003; Moskaug and others, 2005).

The Institute of Medicine Food and Nutrition Board defines antioxidants as "a substance in foods that significantly decreases the adverse effects of reactive oxygen species, reactive nitrogen species, or both on normal physiological function in humans" (Food and Nutrition Board, 1998). Polyphenols are non-enzymatic antioxidants, in addition to vitamins C and E (Huang and others 2005).

Oxidative Damage & Polyphenols

When the ratio of pro-oxidants to antioxidants becomes too high or imbalanced, pro-oxidants begin to cause damage. An excess of pro-oxidants in the system can oxidize and cause damage to many biomolecules including lipids, proteins, and nucleic acids (Halliwell, 1999). In short, too many reactive species can potentially lead to cancer (Halliwell, 2007a). An excess of antioxidants can be damaging as well, as found when an antioxidant reduces a reactive species. The antioxidant then becomes a weak oxidant itself and must be regenerated by another antioxidant. To prevent this, polyphenols may act synergistically with other antioxidants. Polyphenols may regenerate other antioxidants in the body that have become pro-oxidants, such as vitamin E (Scalbert and others 2005).

The imbalance among different pro-oxidants and antioxidants is involved in many disease processes including atherosclerosis, cancer, cataracts, arthritis, diabetes and neurological diseases (Halliwell, 1994). The hypothesized mechanisms in which polyphenols protect against cancer are: acting as a blocking agent at the initiation stage of cancer by decreasing activity of phase I enzymes which would otherwise produce carcinogenic compounds, facilitating the excretion of pro-carcinogens by up-regulating phase II enzymes, stimulation of DNA repair, suppression of tumors, inhibition of cell proliferation, and induction of cell apoptosis (Scalbert and others, 2005). This last mechanism may be due to polyphenols acting as pro-oxidants rather than antioxidants, as some antioxidants have pro-oxidant properties in the presence of transition metal ions or alkalis, and some have pro-oxidant properties regardless. This can be beneficial when the target of the activity is a cancer cell or cancer DNA (Lee and others 2005).

Measures of Oxidative Stress

Oxidative stress takes on a variety of forms as it can attack a multitude of different biomolecules. As such, many biomolecules can be analyzed as markers of oxidative stress. Some of the more frequently used markers include products of oxidation such as malondialdehyde and F_2-isoprostanes as measures of lipid peroxidation; protein carbonyls as a measure of protein oxidation, and lipid hydroperoxide (Duthie and others 2006). In neurodegenerative diseases, nitrotyrosine formed by peroxynitrite and 8-hydroxy-2'-deoxyguanosine have been proposed as measures of oxidative damage to the brain and DNA (Esposito and others 2002). Nuclear factor-kappa B (NF-kB) is activated by oxidative stress, and is another measure (Esposito and others, 2002). Another means of measurement is the presence of antioxidants or antioxidant enzymes such as superoxide dismutase, glutathione, the GSH:GSSG ratio.

While each of these markers has its merits, some argue that none can elucidate the effects of antioxidants to alleviate oxidative stress given how we define oxidative stress (Jones, 2006). Jones argues that rather than assuming there is a global increase in pro-oxidants in a disease state and that the administration of antioxidants should ameliorate this; what should be measured is the disruption of redox signaling and control. Both GSH:GSSG and cytseine/cystine (Cys:CySS) should be measured as each represent the tissue oxidative state and the extracellular oxidative state, respectively (Jones, 2006). This he contends would allow for study and development of organ and disease-specific antioxidant therapies (Jones, 2006). There is clearly no single measure of oxidative stress or oxidative damage in vivo (Halliwell and Whiteman, 2004).

Use of Hydrogen Peroxide to Create Oxidative Stress

Hydrogen peroxide (H_2O_2) causes damage to cells by the formation of hydroxyl radical (OH·) via the Fenton reaction in the presence of metal ions or through the action of lipid peroxides (Duthie and Collins, 1996). While hydrogen peroxide may not react with all biomolecules, hydroxyl radicals can attack most any biomolecule including DNA (Halliwell and Whiteman, 2004). This is measured by the mutagenic products of such attack. An example is 8-hydroxy-2'-deoxyguanosine, frequently measured in urine to determine the extent of DNA damage. Hydrogen peroxide is created endogenously as a by-product of aerobic metabolism, and has been used repeatedly as an added stressor in cell models (Yamamoto and others 2004).

Hydrogen peroxide is a normal by-product of metabolism and is normally eliminated by catalase and glutathione peroxidase. However, in excessive amounts, H_2O_2 has been shown to induce oxidative damage, specifically to DNA (Wijeratne and others 2005). Though necessary to normal functioning, H_2O_2 is considered an active carcinogen (Shih and others 2007).

Interestingly, H_2O_2 is present at higher concentrations in cancer cells than normal cells and may be necessary for cancer growth (Loo, 2003). H_2O_2 may act as a second messenger, as it increases the activity of protein tyrosine kinase (PTK) which phosphorylates epidermal growth factor receptor (EGFR). EGFR signals cell growth, crucial to carcinogenesis. The enzymatic antioxidant glutathione is largely responsible for the redox state of a cell and can scavenge H_2O_2 before it forms the hydroxyl radical. In a cancer cell with a high concentration of H_2O_2, glutathione is depleted. Without glutathione present, PTKs become over-activated and stimulate redox-sensitive transcription factors and genes that promote cancer growth (Loo, 2003). The amount of H_2O_2 present in the cell must also be considered, as there may be a threshold upon which H_2O_2 no longer acts as a second messenger and instead causes sufficient damage to DNA to induce apoptosis (Loo, 2003).

Here polyphenols may intervene, though through several different mechanisms. Phenolic compounds may protect the body as either antioxidants by scavenging H_2O_2, removing it as a second messenger and stopping the cell signaling cascade, or as pro-oxidants. The H_2O_2 produced by polyphenols added to the high ROS concentration present in a cancer cell may induce cell death (Loo, 2003; Lee and others, 2005). The high H_2O_2 concentration in a cancer cell may leave it susceptible to cancer drugs as compared to normal cells, as the cell is already near a reactive species threshold (Loo, 2003).

Polyphenols

Polyphenols are one class of phytochemicals; substances in plants that are non-nutritive but have a beneficial function in the body (Liu, 2004). Polyphenols in the diet may come from a number of fruits and vegetables including onions, apples, berries, fruit juices, green and black teas, red wine, chocolate, chocolate drinks, coffee, beer, dry legumes, and cereals (Scalbert and

Williamson, 2000). Polyphenols are categorized as phenolic acids, isoflavones, coumarins, tannins and flavonoids (Liu, 2004). Thousands of polyphenols have been identified and extensively researched *in vitro* and *in vivo* for their impact on chronic disease as they are the most abundant antioxidants from the diet (Liu, 2004; Skibola and Smith, 2000; Scalbert and Williamson, 2000). Flavonoids make up two-thirds of all polyphenols in the diet (Cotelle, 2001).

Flavonoids confer many benefits and are antiallergenic, antiviral, anti-inflammatory, and vasodilators (Cotelle, 2001). Additionally, flavonoids chelate free metals such as copper and iron which would otherwise cause oxidative damage (Jovanovic and Simic, 2000). Dietary polyphenols not only neutralize ROS directly, but also increase the synthesis of the antioxidant compound glutathione. Glutathione also scavenges ROS, and the up-regulation of glutathione then leads to the up-regulation of glutathione dependent processes, such as the detoxification of xenobiotics, glutathionylation of proteins, regulation of redox switching of protein functions, signal transduction and gene expression, DNA and protein synthesis, cell proliferation and apoptosis, cytokine production and immune response, and mitochondrial function and integrity (Wu and others; Moskaug and others, 2005). In this manner, dietary polyphenols activate a cascade of events that are implicated in the prevention of disease (Kris-Etherton and others ; Moskaug and others, 2005).

Anthocyanins

Studies in our lab have found anthocyanins to be the most bioactive constituent of all the polyphenols tested from blueberries in control of cell proliferation (Yi and others 2005a). Berries contain a variety of polyphenols, and flavonoid anthocyanins have been the focus of many other studies (Neto, 2007). Anthocyanins are a sub-category of flavonoids and predominate in the current literature as flavonoids of interest for their multitude of proposed

anticarcinogenic properties (Hou, 2003). Anthocyanins act at the initiation, promotion, and progression stages of cancer development (Duthie, 2007; Hou, 2003). Anthocyanins have been studied in vitro and in vivo, as they have been shown to have greater antioxidant effect than vitamins C and E (Duthie, 2007). The trolox equivalent antioxidant capacity assay (TEAC) of anthocyanins showed three to six fold greater antioxidant capacity than Trolox, a water soluble vitamin E analog and antioxidant standard (Cooke and others 2005). Anthocyanins have two to six times the activity of known antioxidants ascorbate and glutathione (Prior and others 1998).

Berries have been shown to modulate phase I and II enzymes and inhibit cell proliferation, malignant transformation, invasiveness, and angiogenesis. Seeram et al. (2006) showed a variety of anthocyanin-rich berries, including blueberries, to be effective growth inhibitors in cell models of human oral, breast, colon, and prostate cancer in a dose dependent manner (Seeram and others, 2006). They may also reduce cell adhesion (Duthie, 2007). Anthocyanins chelate transition metal ions, and inhibit epidermal growth factor receptor (EGFR) (Hou, 2003; Beattie and others 2005). In regards to neural function, anthocyanins have been shown to stabilize connective tissue, promote collagen formation, and help prevent oxidative damage to blood vessels (Andres-Lacueva and others 2005).

Measures of Total Antioxidant Capacity

Assays measuring antioxidant capacity of a food extract or whole food can be divided into two categories, those based on the transfer of hydrogen atoms (HAT) and those based on the transfer of electrons (ET) (Huang and others, 2005). The HAT assays are based on a competitive reaction scheme, in which the antioxidant under study and a substrate compete for available radicals, and the amount of radicals reduced by each is measured. This category includes the oxygen radical absorbance capacity (ORAC) and total radical trapping antioxidant parameter

(TRAP) assays (Huang and others, 2005). The ET assays also measure the capacity of an antioxidant to reduce an oxidant *in vitro*. The oxidant present in the assay changes color when reduced, and the degree to which the color changes following exposure to the antioxidant is the measure of the antioxidant capacity. This category includes Folin-Ciocalteu reagent (FCR), Trolox equivalence antioxidant capacity (TEAC), and ferric ion reducing antioxidant power (FRAP) assays (Huang and others, 2005). HAT based assays quantify the hydrogen atom donating capacity of an antioxidant, and ET based assays measure an antioxidant's reducing capacity (Huang and others, 2005).

The results from each of these assays are difficult to generalize, however, because each uses a specific substrate and cannot be said to measure "total antioxidant capacity". For example the ORAC assay measures peroxyl radical scavenging ability, while the FRAP assay measures Fe^{3+} reducing activity (Ou and others, 2002). Additionally, dietary sources contain more than one antioxidant, and may contain multiple antioxidants (such as vitamins C and E) that confer their protective effects in different systems. The activity of an antioxidant in the presence of many oxidants, such as *in vivo*, is also difficult to measure. Cell culture models cannot emulate the *in vivo* environment as they do not have the same temperature, enzymes, and microflora as the human digestive tract and do not take into account the bioavailability of polyphenols (Liu and Finley, 2005).

The effect of any given polyphenol on chronic disease varies by both the dose in which it is consumed and its structure; even the same compound may have multiple stereoisomers or be esterified to different sugars (Lee and others, 2005; Liu and Finley, 2005; Scalbert and Williamson, 2000). Nonetheless, many investigators have called for the development of a "comprehensive" assay, as one test is not enough to show all that antioxidants do in vivo (Frankel, 2000; Liu and Finley, 2005; Schlesier and others ; Lee and others, 2005; Halliwell and Whiteman, 2004).

Antioxidant Capacity of Anthocyanins

Blueberries have been tested for polyphenolic content and antioxidant capacity, including use of the ORAC assay. While there is no gold standard for assessing total antioxidant capacity in foods, the ORAC is widely used and reference values for a variety of foods are available. The ORAC compares the food in question to a standard antioxidant, Trolox, a water soluble vitamin E analog (Huang and others, 2005). The ORAC quantifies the peroxyl radical scavenging ability of a substance. Commercially available blueberries were found to have 24.0 ± 2.0 μmol Trolox equivalents (TE) per gram of fresh weight (Prior and others, 1998). By comparison, strawberries had 15.4 μmol TE/g, and spinach had 12.6 μmol TE/g (Prior and others, 1998). In assessing foods and food components, both blueberries and anthocyanins rank among the highest for antioxidant capacity (Kraft and others, 2005; Prior and others, 1998; Sellapan and others 2002). Although polyphenol content varies by the cultivation conditions of the blueberry, compared with all food items tested in their lab, Prior et al (1998) found blueberries to have the highest peroxyl radical scavenging capacity on a fresh-weight basis.

Intake and Bioavailability

Daily dietary intake of anthocyanins has been estimated anywhere from 20 to 215 mg/day (Cooke and others, 2005; Hou, 2003). Recent estimates for consumption of anthocyanins in the United States are as low at 12.5 mg/day. Of the anthocyanins consumed, cyanidin is estimated to comprise 45%, delphinidin 21% and malvidin 15% of total anthocyanin intake (Wu and others 2006). As shown in **Table 2**, anthocyanin content of selected fruits and vegetables varies greatly and is highest among berries, especially blueberry. Bioavailability of anthocyanins has been assessed in humans in several trials. In one such trial, twelve healthy volunteers were fed a controlled, anthocyanin free diet with one of three treatments. In the crossover design, subjects consumed 250 g raw purple carrots, 250 g cooked purple carrots, or 500 g cooked purple carrots. Peak plasma total anthocyanin concentrations reached 5.8 ± 1.7, 5.3 ± 1.9, and 5.0 ± 1.4 nmol/L respectively (Kurilich and others 2005).

Another bioavailability trial used cooked red cabbage at 100, 200, and 300 g doses in twelve human volunteers fed an otherwise anthocyanin free diet. In this study a threshold for absorption of anthocyanins appeared, as although there was a linear dose-response, anthocyanins recovered were not doubled or tripled above that which was recovered at 100 g. Additionally, non-acylated anthocyanins were recovered in urine at significantly higher concentrations than the acylated forms (Charron and others 2007).

Investigators found a number of anthocyanins in brain tissue of 19 month old F344 rats fed a control diet or diet supplemented with 2% blueberry. This demonstrates that the anthocyanins were absorbed, and they were able to cross the blood brain barrier and localize in regions related to cognitive function (Andres-Lacueva and others, 2005).

The absorption of anthocyanins by intestinal cells was examined by Yi et al (2006). Caco-2 cells were used in this experiment to best imitate small intestine cells as they have been shown to develop brush border characteristics *in vitro*. Using this model, anthocyanins were effectively absorbed by the cells, although efficiency varied depending on the structure of anthocyanin tested (Yi and others 2006).

Anthocyanin dose, structure, and acylation of the anthocyanin backbone can affect bioavailability (Charron and others, 2007). A major factor affecting bioavailability of anthocyanins in systemic circulation and urine is their metabolism in the gut. The metabolites of many of the anthocyanins are unknown, and as such cannot be measured in plasma or urine (Seeram, 2008b). Additionally, anthocyanins are known to bind to proteins, further complicating their measurement (Seeram, 2008a). Individual nutrigenomic and nutrigenetic differences may also impact bioavailabilty in ways not yet understood (Seeram, 2008b).

The colon presents an ideal model for study as it is exposed to these bioactive compounds prior to and during digestion. Anthocyanins may be able to elicit their protective benefits in the colon before being metabolized. After a bolus feeding of 500 mg of polyphenols the concentration in the gut was 3 mmol/L, higher than the levels achieved in plasma (Scalbert and Williamson 2000).

Prior Studies

Previous research done in our laboratory has shown that phenolic compounds extracted from blueberries reduce proliferation and induce apoptosis in HT-29 and Caco-2 colon cancer cell lines, and in the HepG2 liver cancer cell line. In a 2005 study of muscadine grapes and cancer, Yi et al (2005a) examined four types of muscadine grape, a family of grapes selected for their high antioxidant content. Polyphenols were extracted from the skin and seeds of the grapes,

as the pulp or flesh of the grape contributes very few antioxidants. The study examined the effects of various types of polyphenols: phenolic acids, anthocyanins, flavonols, and tannins (Yi and others, 2005a). These extracts from the skin and seeds of muscadine grapes increased apoptosis and decreased tumor cell growth in HT-29 and Caco-2 cell lines (Yi and others, 2005a).

In a parallel study, Yi et al (2005b) examined the effects of rabbiteye blueberry fractions on colorectal cancer cells again using HT-29 and Caco-2 cell lines. At a concentration of 1000 μg/mL, phenolic acid fractions inhibited cell proliferation by 50% compared to control; furthermore inhibition by flavonol and tannin fractions occurred at concentrations of 70-100 μg and 50-100 μg / mL; and anthocyanins inhibited growth at concentrations of only 15-50 μg/mL (Yi and others 2005b). Of the phenolic compounds tested, the anthocyanin fraction exhibited the greatest potency on growth inhibition and apoptosis. Both colon cell lines showed a 50% growth inhibition with blueberry anthocyanin concentrations of 15-50 μg/mL; HepG2 liver cell line showed similar reduction at 70-150 μg/mL. Peak levels of apoptosis were found at concentrations of 40-80 μg/mL in HT-29 and Caco-2 cells, and at 100 μg/mL in HepG2 cells (Yi and others, 2006a; Yi and others, 2005a).

In vivo, Sprague-Dawley rats fed a diet supplemented with 1% blueberry flavonoids (anthocyanins and tannins) showed decreased liver DNA damage after three weeks as compared to rats fed the control diet (Dulebohn, 2007).

Watjen et al (2005) previously provided evidence of a dose-dependent relationship between cytoprotection against DNA damage or cytotoxicity with flavonoid compounds in rat H4IIE hepatoma cells. At low concentrations, 10-25 μmol/L, the flavonoids quercetin and fisetin reduced H_2O_2-induced strand breaks by 50% as compared to control ($p<0.05$). However, in the range of 50-200 μmol/L these same compounds demonstrated a dose-response curve. Quercetin increased comet tail length and DNA damage, and both quercetin and fisetin increased apoptosis by 100-500% ($p<0.05$) (Watjen and others 2005).

A final study of note is that of Srivastava et al (2007). In this study a variety of blueberry cultivars were tested: Tifblue, Powderblue, Brightblue and Brightwell. The primary anthocyanin found in three of the four cultivars was malvidin, though in the fourth, Brightwell, the predominant anthocyanin was peonidin (Srivastava and others 2007). Because differences were observed among the cultivars in terms of apoptosis, and the primary difference among them was the predominant anthocyanin, malvidin and peonidin were chosen for further study *in vitro*.

Hypothesis

Based on previous research by others and in our lab, we tested the hypothesis that the effects of the anthocyanidins malvidin and peonidin would be dependent on concentration in human intestinal adenocarcinoma cells. We hypothesized that low concentrations of the anthocyanidins would act as antioxidants, scavenging H_2O_2 and preventing DNA damage; and at higher concentrations the anthocyanidins would induce oxidative stress and increase DNA damage. We further hypothesized that the effects of DNA damage would induce apoptosis, such that low concentrations of anthocyanidins would decrease or have no effect on apoptosis, while higher concentrations would increase apoptosis.

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TABLE 1 Proposed mechanisms of action by which dietary antioxidants may prevent cancer[1,2]

antioxidant activity:
-scavenge free radicals
inhibition of cell proliferation*
induction of cell differentiation
inhibition of oncogene expression
induction of cell cycle arrest*
induction of apoptosis*
inhibition of signal transduction pathways*
enzyme induction and enhanced detoxification:
-phase II enzyme
-glutathione peroxidase
-catalase
- superoxide dismutase
enzyme inhibition:
-phase I enzymes (block activation of carcinogens)
-cyclooxygenase-2 (COX-2)
-inducible nitric oxide synthase
- xanthine oxide
enhancement of immune functions and surveillance
anti-angiogenesis*
inhibition of cell adhesion and invasion*
inhibition of nitrosation and nitration
prevention of DNA binding
regulation of steroid hormone metabolism
regulation of estrogen metabolism
antibacterial and antiviral effects

[1] Reprinted with permission from Liu and Finley 2005. Copyright 2005 American Chemical Society.

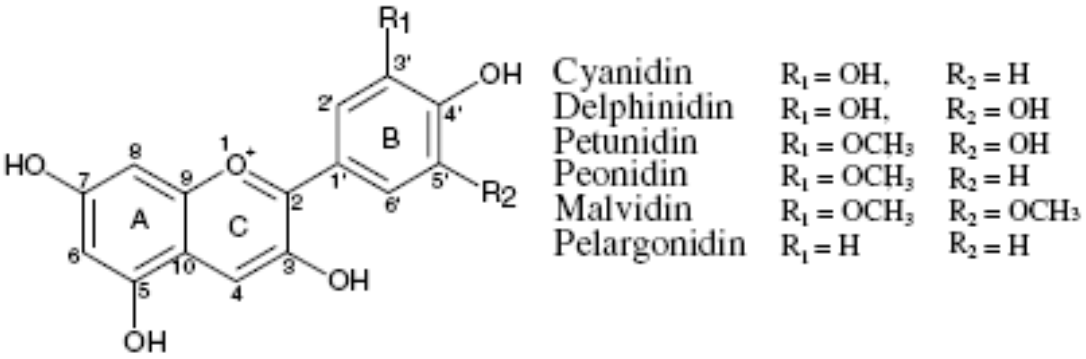
[2] Items marked * are the six hallmarks of cancer; each of these is bypassed in the pathogenesis of all cancers

TABLE 2 Anthocyanin content of selected foods on a per serving basis[1]

Food	mg Anthocyanins
black raspberry	845
wild blueberry	705
cultivated blueberry	529
blackberry	353
sweet cherry	177
cranberry	133
red raspberry	116
red radish	116
red cabbage	113
strawberry	69.2
concord grape	42.7
black bean	23.1
red delicious apple	17

[1]Serving size from USDA Nutrient Database (Wu and others, 2006)

FIGURE 1 Basic Structure of Anthocyanidins [1]



[1] Basic structure of an aglycone anthocyanin. When glycosylated, the sugar moiety most often attaches at the 3 position of the C ring.

CHAPTER III

EFFECTS OF ANTHOCYANIDINS ON INTESTINAL ADENOCARCINOMA CELLS UNDER OXIDATIVE STRESS

Abstract

Non-nutritive phytochemicals such as anthocyanins may reduce risk of chronic disease in part via antioxidant activity. However it is possible that at higher concentrations they may act as prooxidants and thus stimulate oxidative stress and apoptosis. The goal of our study was to determine whether the aglycone anthocyanins malvidin and peonidin act as antioxidants or prooxidants in vitro, at varying concentrations. HT-29 human adenocarcinoma cells were plated in 12-well plates and incubated with 1, 5, 10, 25, or 50 μg/mL malvidin or peonidin for two hours at 37°C, 5% CO_2, 95% air. Cells were then incubated with 75 μmol/L H_2O_2 for 5 minutes. The Comet assay was performed to determine DNA damage, and apoptosis was assessed. Peonidin decreased H_2O_2 -induced DNA damage at 1 μg/mL ($p< .05$). Compared to positive control, malvidin, but not peonidin, at 50 μg/mL significantly increased ($p<.05$) H_2O_2 induced DNA damage (control (8.5 ± 2.7); H_2O_2 alone (13.7 ± 2.4); Malvidin 50 μg/mL, (20.4 ± 3.3)) during one of two studies. Our data suggests that at concentrations of 5-50 μg/mL malvidin and peonidin did not function as antioxidants to decrease H_2O_2 induced DNA damage, and malvidin at 50 μg/mL may increase H_2O_2 -induced DNA damage in HT-29 cells. Apoptosis was not affected by malvidin, but increased above control levels at 5 and 10 μg/mL.

Introduction

Anthocyanins have been the subject of intense study as their potential chemopreventive qualities have been determined (Neto 2007). These water soluble secondary plant metabolites impart blue, red, and purple colors to the many fruits and vegetables in which they are found; and offer protection to the plant against environmental stressors (Prior and others 2008). They are found in large quantities in fruits, especially berries (Seeram, 2008a). In humans these same compounds may act in a variety of ways to confer physiological benefits, such as scavenging free radicals to prevent DNA damage. It is also possible however, that these same compounds may increase oxidative stress in certain cell types, inducing DNA damage and causing the cell to undergo apoptosis, a favorable outcome in cancer cells (Yamamoto and others 2004).

Cancer is one of the many disease states hypothesized to be influenced by oxidative stress and oxidative damage. Anthocyanins may interfere with many carcinogenic events in vivo. Anthocyanins are notable among the constituents of berries for their antioxidant potential *in vitro* and *in vivo* (Kraft and others 2005). As compared to known antioxidants ascorbate and glutathione, Prior et al (1998) found anthocyanins to have 2-6 times more antioxidant activity (Prior and others 1998). Aside from antioxidant potential anthocyanins may inhibit tumor development through a number of other mechanisms. Anthocyanins interrupt the cell cycle and decrease proliferation of cancer cells (Cooke and others 2005). Anthocyanins are anti-mutagenic and bind directly to mutagens such as heterocyclic amines, and bind to metal ions to prevent oxidative damage (Hou, 2003). Cyclooxygenases are inhibited by anthocyanins, reducing inflammation which is a known risk factor for cancer development (World Cancer Research Fund / American Institute for Cancer Research, 2007). The activity of anthocyanins seems to vary by structure; as number of hydroxyl groups increases so does biological activity (Hou,

2003). As cancers of the alimentary tract are most responsive to dietary modification, further study of these potential chemopreventative agents is warranted (Coates and others 2007).

Markers of oxidative stress and oxidative damage in vitro include DNA strand breaks, apoptosis, and lipid peroxidation. DNA damage can be measured in vitro using the Comet assay which quantifies the number of single and double strand breaks in DNA through the measurement of comet tail length following alkaline single cell gel electrophoresis. This assay along with assays for apoptosis and cell viability can determine the degree of DNA damage caused by an insult, such as hydrogen peroxide (Choucroun and others 2001). Hydrogen peroxide reliably induces DNA damage by increasing oxidative stress in cell models, likely due to the formation of hydroxyl radical (OH·) (Halliwell, 2007)

Information as to a dose-response effect of anthocyanidins and colon cells is lacking, specifically whether different anthocyanidins can elicit different responses in cancer cells at varying concentrations. Evidence for a dose-dependent relationship between cytotoxicity and cytoproection with flavonoid compounds was demonstrated by Watjen et al (2005). Using quercetin and fisetin, Watjen et al (2005) treated rat H4IIE hepatoma cells with a range of concentrations from 10 to 250 μmol/L. From 10-25 μmol/L, the flavonoids protected against H_2O_2 induced cytotoxicity, DNA strand breaks, and apoptosis (Watjen and others 2005). Above 50 μmol/L however, quercetin and fisetin induced cytotoxicity, DNA strand breaks, and apoptosis.

Previous studies in our laboratory found that anthocyanin fractions were the most bioactive of blueberry fractions tested in inducing cell proliferation (Yi and others 2005a). An anthocyanin fraction extracted from whole blueberries exerted 3-7 fold higher increases in DNA fragmentation and apoptosis in HT-29 human colon adenocarcinoma cells than control (Yi and

others, 2005a). The greatest increase in DNA fragmentation and apoptosis was observed at 40 μg/mL. In a similar study Yi et al (2006) showed a decrease in apoptosis in liver cancer HepG2 cells at concentrations beyond 100 μg/mL of blueberry anthocyanins, suggesting that beyond this point necrosis occurs (Yi and others 2006a).

We tested the hypothesis that the effects of anthocyanidins malvidin and peonidin would be dependent on concentration in human intestinal adenocarcinoma cells. At low concentrations the anthocyanidins would act as antioxidants, scavenging H_2O_2 and preventing DNA damage; and at higher concentrations the anthocyanidins would induce oxidative stress and increase DNA damage. We further hypothesized that the effects of DNA damage would induce apoptosis, such that low concentrations of anthocyanidins would decrease or have no effect on apoptosis, while higher concentrations would increase apoptosis.

Methods

The HT-29 colon adenocarcinoma cell line was chosen for study because although anthocyanin bioavailability is low, the colon is exposed to anthocyanins from the diet. Human colon adenocarcinoma cells were grown in 75 cm^2 flasks and maintained at 37°C, 5% CO2, controlled humidity. Medium was changed every 2-3 days and cells were passaged when confluent.

Bilberry anthocyaninidins malvidin (3,5,7,4'-tetrahydroxy-3',5'-dimethoxyflavylium chloride) and peonidin (3,4',5,7-tetrahydroxy-3'-methoxyflavylium chloride) were purchased from Chromadex (Irvine, CA) . These extracts are the product of homogenized, whole bilberries that are then separated into fractions and freeze dried. Cells were seeded at 1.5 x 10^5 /mL in twelve-well plates and incubated 24 hours. Following this 24 hour incubation period the media was removed and replaced with media containing anthocyaninidins, malvidin and peonidin, to

incorporate the compounds into the cells. Anthocyanins are characterized for their preventative effects against oxidative damage, and so were added before the stressor, H_2O_2. Briefly, 5 mg anthocyanidin was suspended in 1 mL media containing 10% DMSO, and then diluted to final concentrations by serial dilution to 50, 25, 10, 5, and 1 μg/mL. Anthocyanidin concentrations were chosen based on previous studies by Yi et al., 2005.

Initially, cells plated in 12-well plates were treated with anthocyaninidins malvidin and peonidin at 1-50 μg/mL for two hours. The anthocyanidin incubation period was selected as two hours are sufficient for cells to incorporate anthocyanin fractions (Youdim and others 2000). Recent literature suggests that the longer, 24-hour incubation period may be too long as bioactive compounds are metabolized to an inert state before any insult is added (Ghosh and others 2006). The incubation period was followed by trypsinization for the Comet assay or 1 hour treatment with 100 μM H_2O_2 for apoptosis, lipid peroxidation, and cell viability assays. Hydrogen peroxide treatment concentrations and times were chosen based on similar studies (Coates and others, 2007), and tested in our lab to reaffirm that hydrogen peroxide would reliably increase DNA damage without causing necrosis. An additional H_2O_2 positive and negative control were tested.

All cells were assessed for DNA damage and apoptosis using the Comet and DNA fragmentation assays. Choucroun (2001) has recommended that investigators conduct DNA fragmentation and viability assays along with the Comet assay because apoptosis contributes to the comet tail length and thus the Comet assay does not only reflect genotoxicity as all cells undergo some degree of apoptosis, regardless of stress (Choucroun and others, 2001).

Comet Assay

DNA damage was assessed using alkaline single-cell gel electrophoresis, also termed the Comet assay, using a silver staining kit (Trevigen). Two studies with the Comet assay were

performed. During the first study, malvidin and peonidin were tested at three concentrations, 10, 25, and 50 µg/mL on HT-29 cells. The second study examined malvidin and peonidin at five concentrations, 1, 5, 10, 25, and 50 µg/mL. Sample size was n=4 per treatment group. The results of each study are presented.

The Comet assay was completed with overhead lights turned off to reduce UV damage to the cells. Thirty minutes prior to the end of anthocyanidin treatment, the lysis solution was prepared with 120 ml lysis solution (provided in COMET kit) and 12ml DMSO. The solution was then kept chilled at 20° C until use. The low-melting point agarose to be used for the slides was held in a boiling water bath with loosened cap for approximately 5 min or until the agarose was liquid. The agarose was then placed in a 42^{o} C water bath until needed. Twenty minutes prior to the end of anthocyanidin fraction treatment, trypsin was placed in a 37 ^{o} C water bath for ten minutes. Eppendorf tubes and Comet slides were labeled and prepared for use.

Cells were harvested from the 12-well microplates by removing the media, rinsing the plates with 0.2 ml of trypsin, and then adding 0.3 ml of fresh trypsin. Trypsinization was then stopped by the addition of 0.7 ml of media. 100 µL of cells were added to 400 ul of phosphate-buffered saline (PBS) in Eppendorf tubes to achieve the desired concentration of 1-2X10^{5}cells/ml. Cells were then centrifuged for five minutes at 5000 rpm (125 x g). Following centrifugation, cells were washed with and then suspended in PBS.

Using a new set of Eppendorf tubes, 20 µL of cells were added to 200 µL agarose and rapidly mixed while held in a 42 ^{o} C water bath. 80ul of this mixture was then pipetted onto each of the two slide wells and carefully spread with the pipette tip without touching the slide, so as not to scratch the surface. Slides were allowed to dry for ten minutes, until a clear ring around the edge was visible.

The hydrogen peroxide to be added to the slides was prepared using a serial dilution. The slides were placed on ice for H_2O_2 treatment to prevent action of DNA repair enzymes (Duthie and Collins, 1996). 100 µL of 75 µmol/L H_2O_2 was added to the slides at ten-second intervals and left on the slides for five minutes. Hydrogen peroxide was then tapped off of the slides and placed in lysis solution, again at ten-second intervals. Cells were then placed in the refrigerator at 2-8 ^{o}C for four hours to allow lysis of the cells.

Thirty minutes prior to the end of the lysis incubation, a sodium hydroxide (NaOH) solution was prepared and allowed to chill. The solution contains 24 g NaOH, 0.744g EDTA, and 2 L distilled water. The electrophoresis tank was placed in the refrigerator to chill. At the end of the four hour lysis, the Comet slides were placed in the electrophoresis tank with sodium hydroxide solution for 30 min prior to electrophoresis at 25 V and 295 mA for 30 min. At the end of the electrophoresis, the slides were removed from the tank and excess buffer tapped off of the slides before being placed in 70% ethanol for 5 min. Slides were then allowed to air dry overnight and stored at room temperature with desiccant in the dark.

After drying overnight, the Comet slides were incubated in fixation solution (Trevigen) for twenty minutes. Slides were then rinsed with distilled H_2O for thirty minutes, and finally stained with staining solution (Trevigen) for 5-20 minutes; the staining reaction was observed under the microscope using 10x and was stopped with 5% acetic acid when the cells were easily visible. Slides were again rinsed, allowed to dry, and stored in the dark until visual scoring was performed. Slides were visually examined using an Olympus microscope to determine tail length. DNA damage was quantified as per Zhao et al (2006). Visual assessment has been validated as a means of determining comet tail length, without computerized assistance (Collins and Dusinska, 2002). All cells were blinded during visual examination and the code was not

broken until after quantification. The percentage DNA in the tail [(2.5 x $cells_0$ + 12.5 x $cells_1$ + 30 x $cells_2$ + 60 x $cells_3$ + 90 x $cells_4$)/ sum of cells] was calculated to express the amount of DNA damage (Zhao and others 2006).

Apoptosis

Apoptosis (n=4 per treatment group) was tested 24h after H_2O_2 treatment to allow the cells to undergo apoptosis. DNA fragmentation was conducted using a Cell Death detection enzyme-linked immunosorbant assay (ELISA) kit (Roche). This kit is specific to an endonucleotide which indicates whether the cell is undergoing or has undergone apoptosis, rather than necrosis. This assay is based on a quantitative sandwich-enzyme-immunoassay-principle which uses mouse-monoclonal antibodies directed against DNA and histones. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates (Roche).

Day 1 cells were incubated with media containing 100 µmol/L H_2O_2 for one hour, then fresh media were placed in the twelve-well plates and the cells allowed to incubate for 24 h. Cells were harvested from the well plates by gently detaching them with a rubber spatula. Cell counts were then performed and samples then diluted to the appropriate concentration, 1 x 10^5. 10 µL cells were added to 90 µL phosphate-buffered saline in Eppendorf tubes, and centrifuged for ten minutes at 200 x g in the eppendorf centrifuge.

Following centrifugation, the supernatant was removed and the pellet re-suspended in 200 µL lysis buffer, with an incubation period of 30 minutes at 15-25°C. During incubation, solutions for the ELISA kit were prepared using doubly-distilled water. At the end of the incubation period, cells were centrifuged again at 200 x g for ten minutes.

20 μL of supernatant was then transferred from the Eppendorf tubes to the streptavidin coated microplate for analysis, without disturbing the cell pellet. Positive, negative and background controls were provided by Roche. All samples for analysis were added without touching the bottom or sides of the microplate wells, so as not to disturb the coating.

Using a multipipettor, 80 μL of Immunoreagent (Roche) were added to each well. The microplate was then covered with adhesive cover foil and left to incubate for two hours at 15-25°C on a plate shaker set to 250 rpm. During the incubation period, the anti-histone antibody binds to the histone-component of the nucleosomes and captures the immunocomplex to the streptavidin-coated MP via its biotinylation. Additionally, the anti-DNA-POD antibody reacts with the DNA-component of the nucleosomes (Roche).

Thirty min prior to the end of this incubation period, the ABTS solution was brought to room temperature. At the end of incubation cells were washed with 100 μL incubation buffer three times each, and all liquid removed. The washing step removes the unbound components, the antibodies. Following the wash step all wells were treated with 100 μL ABTS solution prepared as indicated by the kit. Samples were left to sit for approximately six minutes before ABTS stop solution was added, 100 μL per well. The plate was then read by the absorbance microplate reader, ELx800^{TM} (Biot-Tek). Results were expressed as enrichment factors and calculated per the formula (treatment-background control) / (negative control – background control).

Lipid Peroxidation

Lipid peroxidation of cells treated with malvidin and peonidin was assessed using the thiobarbituric acid reactive substances (TBARS) assay (Buege and Aust, 1978). An overall treatment effect for malvidin or peonidin (n=5 samples) was examined because no concentration

effect was apparent. Lipid peroxides can be measured indirectly by the formation of TBARS. Reagents were prepared by mixing 1.88 g thiobarbituric acid and 75 g trichloroacetic acid in approximately 400 mL dH_2O, with an additional 10 mL concentrated HCl, final solution diluted to 500 mL. .5 mL of the TBA-TCA-HCl reagent was added to 5 mL glass test tubes, and an aliquot of sample was added. Each tube was then vortexed, heated in boiling water for fifteen minutes, and cooled in room temperature water. Samples were then centrifuged at 4000 x g in a HS4 rotor centrifuge. Absorbance was recorded using a Beckman DU 650 Spectrophotometer at 535 nm. Standards contained a derivative of malondialdehyde (MDA) and were prepared at 10 nmol MDA/mL. Protein was assessed as described by Lowry et al (1951) and TBARS was expressed per mg protein (Lowry and others 1951).

Cell Viability

Cell viability was assessed with the trypan blue assay. 1mL aliquots of cells for each treatment were centrifuged for five minutes at 100 x g, and supernatant discarded. Using 1 mL phosphate buffered saline (PBS) the cell pellet was re-suspended, after which 500 µL were added to 500 µL 0.4% trypan blue. Samples were left to incorporate trypan blue for three minutes, at which point 10 µL of each sample was placed on a hemocytometer for counting. Mixing of sample and trypan blue were staggered to allow for reading each sample prior to ten minutes of incubation with trypan, at which point the staining agent induces cell death. Percent viable cells were calculated as (total number of viable cells) / (total number of cells).

Statistics

Results are expressed as the mean plus/minus standard error. The difference between the control and each experimental test condition was determined using analysis of variance with Fisher's least significant difference test used for post-hoc tests. A value of $p < 0.05$ was

considered to be statistically significant. Statistical analysis was conducted using SAS 9.1 (Cary, NC).

Results

The effects of malvidin and peonidin on DNA damage at concentrations of 10, 25, and 50 μg/mL are shown in Table 3 and Figure 2. The overall treatment effect was significant ($p<0.05$). Post-hoc tests indicated that malvidin increased percent DNA damage above that of the positive control, suggesting a pro-oxidant effect on DNA damage at the highest concentration, 50 μg/mL. Malvidin showed neither an antioxidant nor prooxidant effect at lower concentrations as compared to positive control. Peonidin had no effect on DNA damage at concentrations of 10-50 μg/mL. Table 4 and Figure 3 show the effects of 5-50 μg/mL malvidin and 1-50 μg/mL peonidin on DNA damage in H_2O_2 treated HT-29 cells. There was a significant overall treatment effect ($p<0.02$). Peonidin significantly decreased percent DNA damage as compared to positive control at 1 μg/mL, while no prooxidant effect was observed at any concentration. In this study malvidin at 5-50 μg/mL did not show antioxidant or prooxidant effects compared to positive control.

Table 5 and Figure 4 show the effects of malvidin and peonidin on apoptosis in the range of 1-50 μg/mL. The effects on apoptosis were dependent on anthocyanidin type ($p< 0.01$) and concentration ($p< 0.001$). Peonidin showed greater induction of apoptosis than malvidin, and certain concentrations induced greater apoptosis than others. There was also an interaction between anthocyanidin type and concentration ($p< 0.05$). Specifically, peonidin at 10 μg/mL significantly increased apoptosis ($p<0.05$). In the first study malvidin tended to increase apoptosis non-significantly at higher concentrations.

Using the average of all concentrations for each anthocyanin treatment, malvidin induced significantly more damage (0.43 ± .071 TBARS per mg protein) than peonidin (0.18 ± .012 TBARS per mg protein) ($n=5$, $p < .01$). No difference in cell viability was found due to malvidin or peonidin treatment.

Discussion

The results of our study indicate that the anthocyanidins malvidin and peonidin have different effects on DNA damage and apoptosis in HT-29 colon adenocarcinoma cells exposed to oxidative stress. Anthocyanin prooxidant or antioxidant effects depend upon both structure and concentration. Malvidin may have a greater prooxidant effect at high concentrations, though this was demonstrated in only one of two studies. While the data in our study is inconclusive, the work of others shows similar variability among anthocyanidins of different structures.

As suggested by Duthie (2007) different compounds within the same berry may act at different points in the carcinogenic pathway. In a study by Marko et al (2004) HT-29 cells were treated with anthocyanidins cyanidin, delphinidin, pelargonidin, malvidin and peonidin. Using micromolar concentrations ranging from 0 to 300, malvidin and delphinidin had the greatest growth inhibition as compared to the other treatments, and peonidin had an intermediate effect on growth inhibition as compared to the other anthocyanidins (Marko and others 2004).

The investigators also examined the effects of the anthocyanidins on cAMP-hydrolyzing phosphodiesterases (PDEs); an inhibition of PDEs leads to apoptosis. In this experiment malvidin had the highest PDE inhibitory activity among anthocyanins tested, followed by peonidin, pelargonidin, cyanidin, and delphinidin (Marko and others, 2004).

In a separate cell model, A431 human vulva carcinoma cells, cyanidin exhibited the greatest inhibition of epidermal growth factor receptor (EGFR) activity. Inactivation of EGFR

shuts down a kinase cascade essential to cell growth, and is a known mechanism by which anthocyanins can inhibit carcinogenesis (Hou, 2003). Cyanidin showed the greatest inactivation of EGFR, followed by delphinidin, pelargonidin, peonidin and least of all malvidin. These studies indicate that the structure of the anthocyanidin affects its function. Each anthocyanidin affected different cellular signaling pathways involved in cell proliferation (Marko and others, 2004).

In an ex vivo study of human lymphocytes the flavonoids quercetin, morin, naringenin, and hesperitin all acted as prooxidants and increased DNA damage as measured by the Comet assay. Using a range of 25-200 μM, the investigators demonstrated a dose-response effect at thirty minutes incubation. 200 μM of each flavonoid significantly increased percent DNA in the comet tail; for the control it was 6.9%, quercetin 34%, morin 31.2%, naringenin 30.9%, and for hesperitin 34.9% (Yen and others 2003). Watjen et al (2005) previously demonstrated in their study of flavonoids that cytoprotective concentrations of quercetin and fisetin are 5-10 fold lower than the concentrations at which they damage DNA and promote apoptosis (Watjen and others, 2005). At 10-25 μmol/L, the flavonoids quercetin and fisetin reduced H_2O_2-induced strand breaks by 50% as compared to control ($p<0.05$). However, in the range of 50-200 μmol/L these same compounds demonstrated a dose-response curve increasing damage. Quercetin increased comet tail length and DNA damage, and both quercetin and fisetin increased apoptosis by 100-500% ($p<0.05$) (Watjen 2005). As shown in Table 6, these micromolar concentrations are comparable to the concentrations tested in our study (5.26-287.5 μmol/L).

Shih et al (2007) investigated the effects of various anthocyanins on cytotoxicity and apoptosis in rat liver cells (Clone 9). To test for cytotoxicity, 50 μmol/L concentrations of cyanidin, kuromanin, delphinidin, and malvidin, cells were incubated with anthocyanin for 24 h

and then stressed with 70 μmol/L H_2O_2 for 12 h. As compared to control with H_2O_2 treatment only, cyanidin and delphinidin significantly decreased H_2O_2 induced cytotoxicity ($p< 0.05$) (Shih and others 2007). For apoptosis, cells were treated with 50 μmol/L for 24 h and with H_2O_2 for 6h. The investigators demonstrated significant decreases in early apoptosis as compared to control ($p< 0.05$). Percent of cells undergoing apoptosis for control was (30 ± 3.3), whereas for cyanidin it was reduced to (10 ± 2.9), for delphinidin (15 ± 4.2) and malvidin (19 ±1.9). This represents approximately 60% reduction in apoptosis with cyanidin treatment, and 50% reduction with delphinidin treatment. Interestingly, in our study 10 μg/mL peonidin (equivalent to 57.5 μmol/L) significantly increased apoptosis.

Coates et al (2007) used a study design similar to our own but with different anthocyanins and observed very different results. This study used HT-29 human colon adenocarcinoma cells to test the effects of colon-available raspberry polyphenols (CARE) with and without the added stress of H_2O_2. The CARE extracts were tested for anthocyanin content, and the primary anthocyanins were cyanidin and pelargonidin. Coates et al (2007) tested CARE at concentrations of 0, 3.125, 6.25, 12.5, 25, and 50 μg/mL for 24 h prior to harvesting for Comet assay, and treated cells with 75 μM H_2O_2 on ice. As compared to H_2O_2 treated control, CARE demonstrated a dose-dependent decrease in DNA damage; higher concentrations of CARE resulted in lower percentages of DNA damage. At 50 μg/mL, DNA damage was reduced by 50% ($p< 0.05$). Further, the investigators found no evidence of genotoxic activity at any of the concentrations tested (Coates and others, 2007).

Other classes of polyphenols have shown concentration dependent effects on other parameters related to cancer control. Using SEG-1 and BIC-1 acid-responsive human esophageal adenocarcinoma cells, Kresty et al (2008) pretreated cells with 12.5, 25, 50, 100, 200,

or 400 µg/mL cranberry proanthocyanidins for twenty-four hours prior to acid insult. At concentrations of 25 µg/mL or greater cell viability was significantly inhibited after forty-eight hours, and at 100 µg/mL significant inhibition was seen at twenty-four hours. Cranberry proanthocyanidins also inhibited cell proliferation at concentrations of 25 µg/mL or greater, though no additional effect was seen beyond 50 µg/mL (Kresty and others 2008).

There have been repeated calls for studies examining the effects of polyphenols in different cell types and tissues, as this too may influence the cell outcome (Duthie, 2007; Watjen and others, 2005). One such study by Zhao et al (2004) examined the effects of anthocyanin-rich extracts (ARE) in both colon cancer and healthy colon cell lines. HT-29 human colon adenocarcinoma and NCM-460 non-tumorigenic cells were incubated with 25, 50, and 75 µg/mL grape and bilberry ARE, as well as with 10, 25, and 50 µg/mL chokeberry ARE (Zhao and others 2004). Each extract varied the number and types of anthocyanins it contained, and in its effects on both cell lines. 25 µg/mL bilberry ARE significantly inhibited cell proliferation at 48 and 72 h, by 35 and 55% respectively.

Bilberry ARE had no growth inhibitory effect on NCM-460 cells at 24 or 48 h, but was significantly inhibited by all concentrations tested at 72 h (Zhao and others, 2004). These results demonstrate the effect of AREs on cell growth are cell-type specific. However, the assay used to assess cell growth did not differentiate between apoptosis and necrosis, so the cause of cell death is not known.

Limitations

Despite the original design of this experiment to use a fetal human colon (FHC) healthy cell line, only one adenocarcinoma cell line was used. The need for studies comparing the effects of the same treatments on different cell types is well recognized (Duthie, 2007).

Attempts were made to culture the CRL-1831 cell line (ATCC) but despite multiple trials and adjustments to the media mixing protocol, the cells did not proliferate.

In vitro studies have many limitations; one such is the growing environment inside an incubator. Critics assert that the 5% CO2 95% air environment inside an incubator is grossly hyperoxic, and inherently stressful for cells (Halliwell 2007). While this may be, in vitro models are useful for determining mechanisms of action that may be present in vivo (Kuo 2005). Other limitations are cell specificity, as HT-29 human colon adenocarcinoma cells are only one model of colon cancer, and certainly not representative of all alimentary tract cancers.

The range of concentrations tested may also be a limitation, though as the cell line was of the intestine it is reasonable to assume a similar concentration could be achieved in the colon in vivo. Some may assert that the exposure time was chosen was inadequate to observe an effect, but it was based on recommendations from current literature (Youdim and others, 2000).

Hydrogen peroxide may have played more than one role in this cell model as well, as cancer cells may actually benefit from the presence of H_2O_2 (Loo, 2003). Halliwell (2007) argues that many anti-cancer effects observed with extract treatments are actually the product of H_2O_2 induced damage, where H_2O_2 is the causative agent, albeit though it may have been produced by the presence of the extracts (Halliwell 2007). Lee et al (2005) studied the amount of H_2O_2 produced in Dulbecco's Modified Eagles Medium with the addition of gallic acid and quercetin, without cells present (Lee and others 2005). Both were found to increase the amount of H_2O_2 present in the media, and as is this a potential means by which polyphenols may induce apoptosis, is a desirable effect (Loo, 2003).

Because it is an *in vitro* study the results cannot be immediately translated to dietary recommendations. When consumed as whole food, blueberries contain multiple antioxidants

including vitamins, which may confer their protective effects in different systems. Once consumed, how an antioxidant operates in the presence of many oxidants is also difficult to measure. Cell culture models cannot perfectly emulate the *in vivo* environment as they do not have the same temperature, enzymes, and microflora as the human digestive tract and cannot take into account the bioavailability of polyphenols (Liu and Finley). The effect of any given polyphenol on chronic disease varies by the dose in which it is consumed and its structure; even the same compound may have multiple stereoisomers or be esterified to different sugars (Scalbert and Williamson, 2000; Liu and Finley, 2005; Lee and others, 2005). Even so the *in vitro* model is useful for examining mechanism of action.

Processing of blueberries does affect the bioactivity of its antioxidants. In a study examining fresh, quick frozen, and heat treated blueberries Schmidt et al (2005) found that while all blueberries retained a high antioxidant capacity and high total phenols after treatments, those that were heat treated had slightly lower anti-proliferation activity (Schmidt and others).

Digestion may change the structure of the polyphenol from that found in a crude extract, and may change its bioactive properties. An advantage however to using colon cells is that polyphenols are found in the highest concentration in the gut, prior to digestion and absorption and may be able to elicit their protective benefits before being broken down. The concentration of polyphenols in the colon after a bolus feeding of 500 mg polyphenols was found to be 3mM, much higher than can be achieved in plasma (Scalbert and Williamson). While the concentrations of anthocyanin fractions used in this study will be well above physiological levels, this study is intended to determine dose-response. Assessment of bioavailability of anthocyanins is further complicated by the possibility that these compounds are metabolized prior to entering the bloodstream. These metabolites are largely unindentified, but could

nonetheless be active in the body (Seeram, 2008a). Anthocyanins may also bind to proteins in the bloodstream, rendering them undetectable by current measures (Seeram, 2008a). Another variable to consider in the bioavailabilty debate is the effect of nutrigenomics and nutrigenetics, and how these compounds may affect individuals differently (Seeram, 2008b).

Interactions between compounds found in whole berries as well as in the diet as a whole may be necessary to see benefit. These interactions may be synergistic, concomitant or additive (Kraft and others, 2005). No specific dietary recommendations for anthocyanin intake can be made at this time, nor will they be feasible following this study. Clinical trials and human studies must be conducted before such recommendations can be made.

TABLE 3 DNA Damage in H_2O_2 exposed HT-29 cells treated with malvidin and peonidin at concentrations of 10-50 μg/mL [1, 2]

Treatment	DNA Damage	n
Negative Control	8.53 ± 2.71 [a]	4
Positive Control	13.74 ± 2.40 [a, b]	5
Malvidin 10	18.98 ± 2.51 [b, c]	4
Malvidin 25	17.87 ± 1.99 [b, c]	3
Malvidin 50	20.41 ± 3.31 [c]	4
Peonidin 10	18.54 ± 1.56 [b, c]	4
Peonidin 25	18.16 ± 1.99 [b, c]	4
Peonidin 50	14.63 ± 0.83 [a, b, c]	4

[1] Values are mean ± SE. The negative control treatment received no anthocyanidin and no H_2O_2. Positive control received no anthocyanidin, but did receive H_2O_2. Results with different letters are statistically significant ($p < 0.05$)

[2] Cells were scored into 5 categories based on tail size. Percent DNA in the tail was calculated as: $(2.5*cells_1 + 12.5*cells_2 + 30*cells_3 + 60*cells_4 + 90*cells_5)/n$ where n=total number of cells. Percent DNA in the tail corresponds to percent DNA damage.

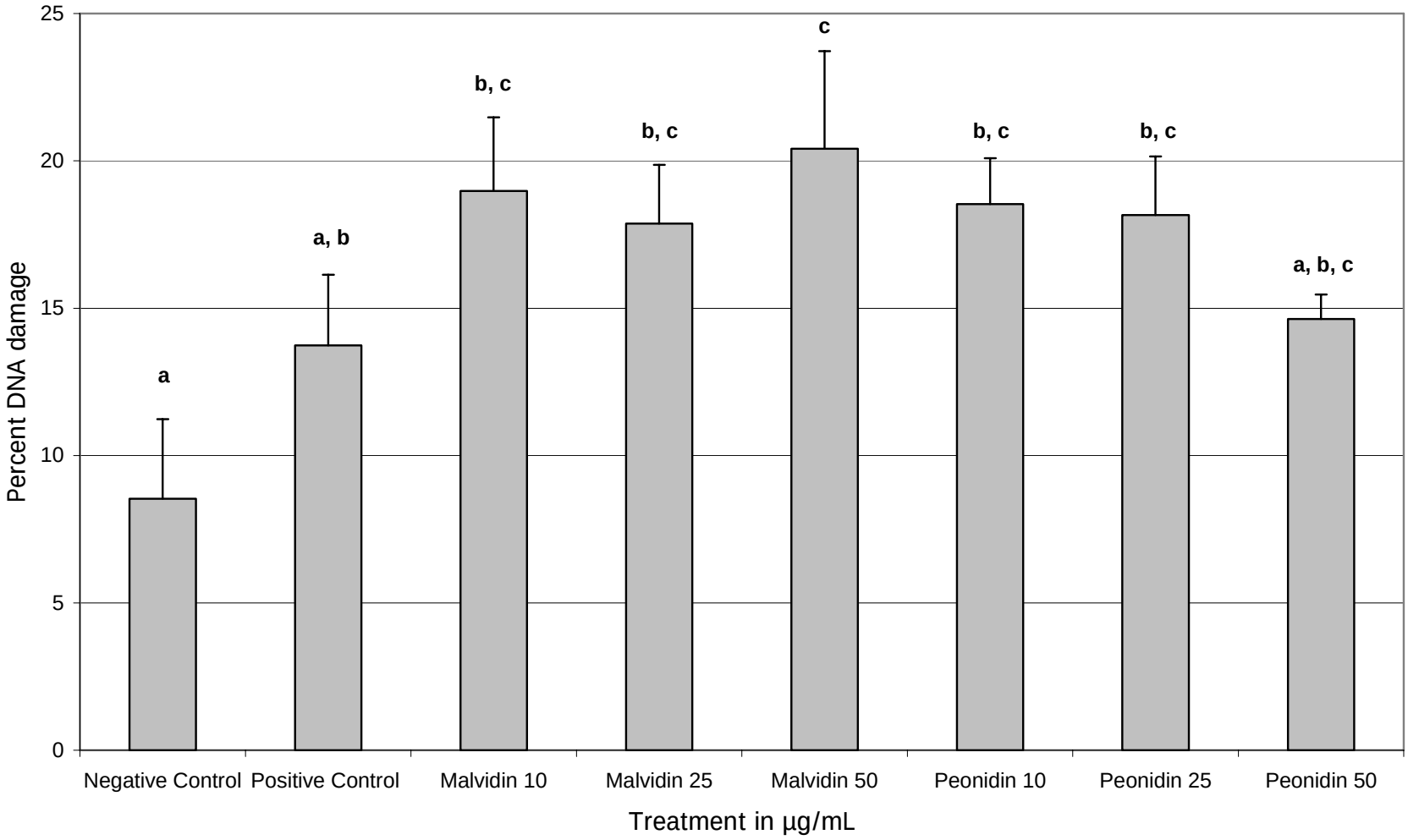
TABLE 4 DNA Damage in H_2O_2 exposed HT-29 cells treated with malvidin and peonidin at concentrations of 1-50 µg/mL [1, 2]

Treatment	DNA Damage	n
Negative Control	18.26 ± 0.22 [a]	2
Positive Control	30.56 ± 7.37 [b, c]	3
Malvidin 1	.	0
Malvidin 5	32.88 ± 2.25 [c]	2
Malvidin 10	20.27 ± 2.69 [a, b]	2
Malvidin 25	34.31 ± 0.64 [c]	2
Malvidin 50	32.48 ± 2.77 [c]	4
Peonidin 1	20.19 ± 2.71 [a]	4
Peonidin 5	23.40 ± 1.38 [a, b]	4
Peonidin 10	25.61 ± 2.76 [a, b, c]	4
Peonidin 25	27.65 ± 1.61 [a, b, c]	4
Peonidin 50	26.35 ± 2.13 [a, b, c]	4

[1] Values are mean ± SE. The negative control treatment received no anthocyanidin and no H_2O_2. Positive control received no anthocyanidin, but did receive H_2O_2. Results with different letters are statistically significant ($p < 0.05$)

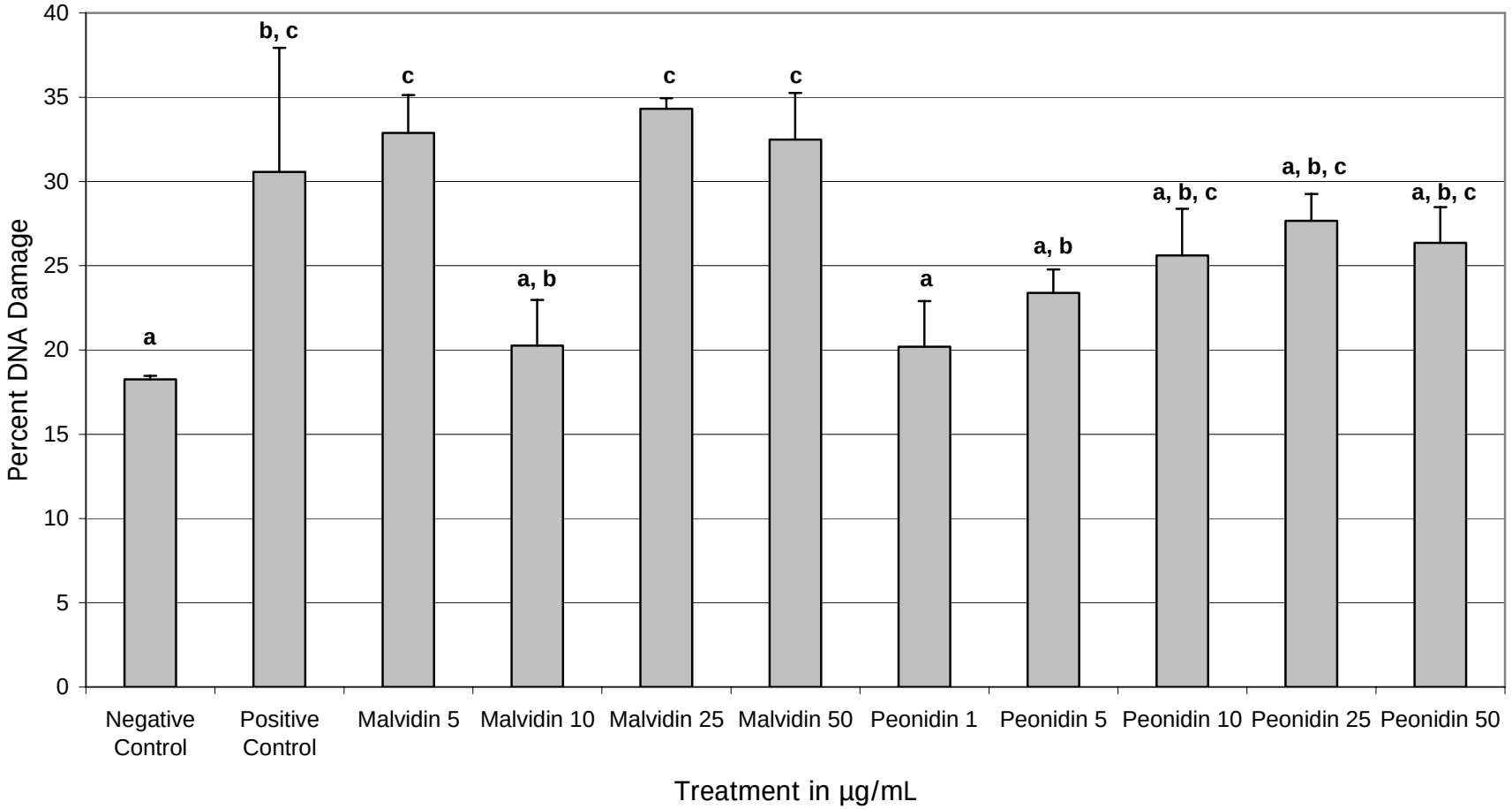
[2] Cells were scored into 5 categories based on tail size. Percent DNA in the tail was calculated as: $(2.5*cells_1 + 12.5*cells_2 + 30*cells_3 + 60*cells_4 + 90*cells_5)/n$ where n=total number of cells. Percent DNA in the tail corresponds to percent DNA damage.

FIGURE 2 DNA Damage in H_2O_2 exposed HT-29 cells treated with malvidin and peonidin [1]



[1]Results with different letters are statistically significant ($p < 0.05$)

FIGURE 3 DNA Damage in H_2O_2 exposed HT-29 cells treated with malvidin and peonidin [1]



[1]Results with different letters are statistically significant ($p < 0.05$)

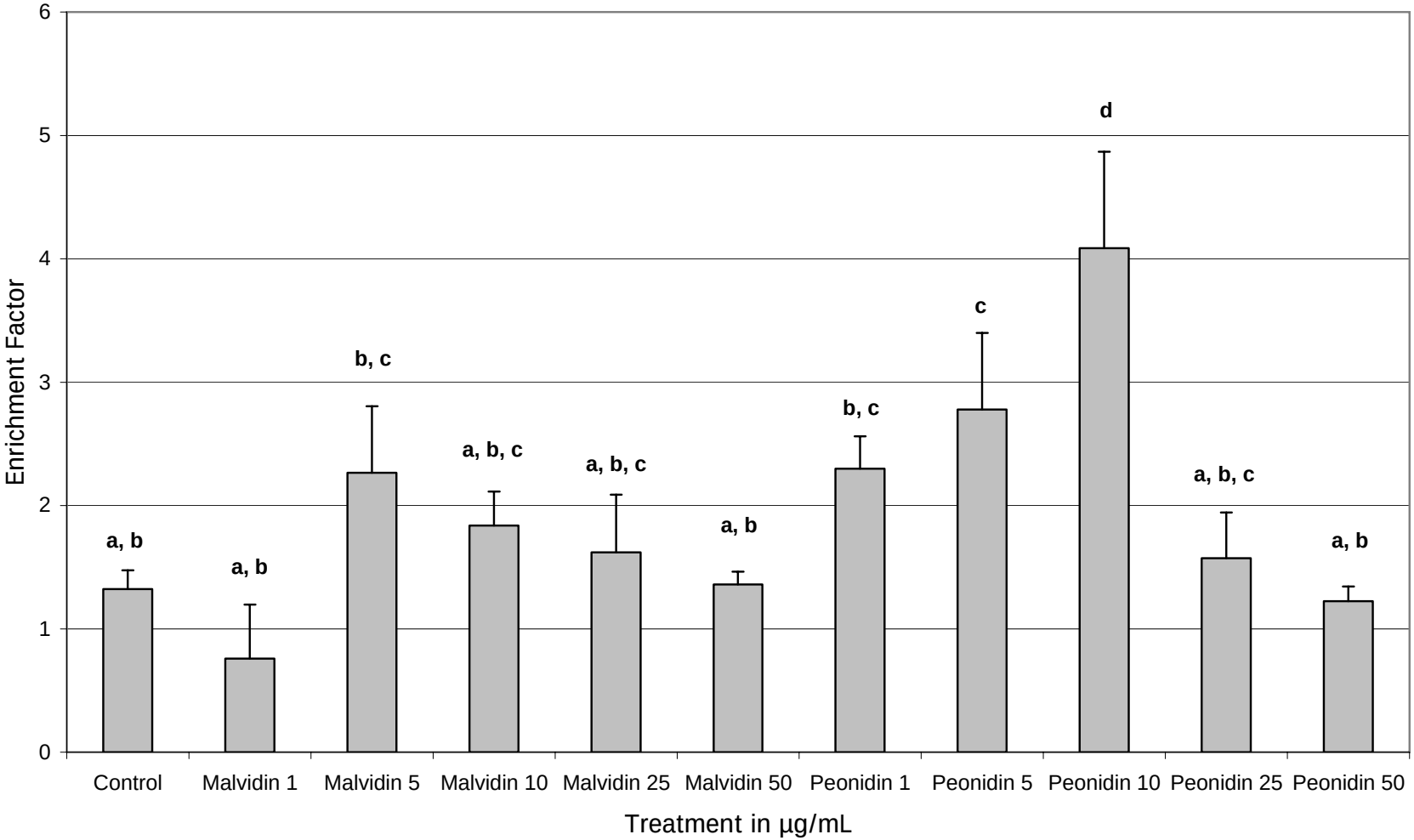
TABLE 5 Apoptosis in H_2O_2 exposed HT-29 cells treated with malvidin and peonidin at concentrations of 1-50 μg/mL[1, 2]

Treatment	Enrichment Factor
Control	1.32 ± 0.15 [a, b]
Malvidin 1	0.76 ± 0.44 [a, b]
Malvidin 5	2.26 ± 0.54 [b, c]
Malvidin 10	1.84 ± 0.28 [a, b, c]
Malvidin 25	1.62 ± 0.47 [a, b, c]
Malvidin 50	1.36 ± 0.10 [a, b]
Peonidin 1	2.30 ± 0.26 [b, c]
Peonidin 5	2.78 ± 0.62 [c]
Peonidin 10	4.09 ± 0.78 [d]
Peonidin 25	1.57 ± 0.37 [a, b, c]
Peonidin 50	1.22 ± 0.12 [a, b]

[1] Values are mean ± SE, n=4. Control received H_2O_2 alone, no anthocyanin treatment. Results with different letters are statistically significant ($p < 0.05$)

[2] Enrichment factor calculated using absorbance: (treatment-background control) / (negative control – background control).

FIGURE 4 Apoptosis in H_2O_2 exposed HT-29 cells treated with malvidin and peonidin[1]



[1] Results with different letters are statistically significant ($p < 0.05$). The effects on apoptosis were dependent on anthocyanidin type ($p < 0.01$) and concentration ($p < 0.001$). There was an interaction between anthocyanidin type and concentration ($p < 0.05$).

TABLE 6 Concentrations of anthocyanidins tested [1, 2]

Malvidin		Peonidin	
µg/mL	µmol/L	µg/mL	µmol/L
1	5.26	1	5.75
5	26.3	5	28.73
10	52.6	10	57.5
25	132	25	143.75
50	263	50	287.5

[1] The molecular weight of malvidin is190, and the molecular weight of peonidin is 174.

[2] Concentrations of 50 µmol/L and above are typically used to achieve cell death and decrease cell proliferation.

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CHAPTER IV

SUMMARY AND CONCLUSIONS

Diet and lifestyle factors play an integral role in the development and prevention of cancer and chronic disease (World Cancer Research Fund / American Institute for Cancer Research, 2007). How this is achieved remains a mystery, as a typical diet may provide more than 25,000 bioactive food constituents, both cancer preventive and carcinogenic (World Cancer Research Fund / American Institute for Cancer Research, 2007). Two dozen potential anticarcinogenic substances have been identified in foods, among them polyphenols (Steinmetz and Potter, 1996). Blueberries are specifically and repeatedly cited for their high antioxidant content and capacity, and potential as cancer chemopreventive agents (Prior and others 1998; Sellapan and others 2002; Kraft and others 2005). Information as to whether blueberry anthocyanin fractions can elicit different responses in different cell types is still lacking, and certainly merits further study as the hope is to discover a differential cancer treatment.

The goal of our study was to determine whether malvidin and peonidin have concentration-dependent effects in human intestinal adenocarcinoma cells stressed with H_2O_2 as measured by DNA damage and apoptosis. We hypothesized that low concentrations would scavenge H_2O_2 and prevent DNA damage; and higher concentrations would induce oxidative stress and increase DNA damage. We further hypothesized that DNA damage would induce apoptosis with variations by concentration of anthocyanidin tested.

The results of our study support our hypothesis that the effects of anthocyanidins are dependent on concentration, though not in the manner anticipated. At low concentrations the anthocyanidin malvidin had no effect DNA damage; and at higher concentrations it may have increased DNA damage. We also hypothesized that low concentrations of anthocyanidins would decrease or have no effect on apoptosis, while higher concentrations would increase apoptosis. To the contrary, lower concentrations of peonidin significantly increased apoptosis, with no increase in apoptosis above 10 μg/mL. The sudden decline in observed effect may be explained however by necrosis; if the concentrations were so high as to cause necrosis, cell death would occur though not via apoptosis. The markers assessed in apoptosis would then not appear in the assay, giving the impression of less cell death.

We cannot conclude that there was an increase in DNA damage with high concentrations of malvidin as the two studies were in conflict. DNA damage was not enhanced by peonidin at any of the concentrations tested, though an antioxidant effect was observed at the lowest concentration of peonidin tested. For apoptosis peonidin, but not malvidin caused significant increases above control. Further, significant relationships were found between anthocyanins and concentrations, as well as an interaction between anthocyanin type and concentration. The results of our study indicate that the anthocyanidins malvidin and peonidin have different effects on oxidative damage in HT-29 colon adenocarcinoma cells. Structural differences between the two may enable them to act by different mechanisms to induce apoptosis or DNA damage.

Future studies should investigate different cancer cell lines and non-tumorigenic cell lines. Several authors have noted the need for studies with cancerous and non-tumorigenic cells receiving the same treatments, to determine whether there are different results in each (Duthie, 2007; Seeram, 2008b). Cancer chemoprevention through diet or anthocyanin supplementation can only be achieved if increases in apoptosis and DNA damage are limited to preneoplastic and initiated cells. If all cells in an organism are similarly affected, then pharmacologic doses can be expected to cause as much harm to healthy cells as to cancerous cells.

Several studies have examined the bioavailability of anthocyanins from the diet, though further research is needed. It remains to be seen what concentrations reach systemic circulation, and what concentration may be needed for effect, as well as how long the organism need be exposed. A factor which may further complicate the effects of bioactive food components in the prevention of cancer is genetics (Seeram, 2008b). Differences in nutrigenomic and nutrigenetic effects exist in areas such as folate metabolism, and may also influence the metabolism and chemopreventive properties of anthocyanins. Ability to absorb anthocyanins may vary by individual and once absorbed the antioxidant and prooxidant properties may be variable. This warrants further study, preferably in clinical trials to assess differences by genotype.

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APPENDICES

APPENDIX A

Anthocyanidin Serial Dilution

NOTES:

- Cover solutions tightly with foil and work with lights off to prevent degradation of compounds.
- Label beakers before starting the dilution series.
- Use caution when opening the extract bottle as contents are vacuum sealed.
- Add DMSO to anthocyanins FIRST- it will not dissolve in media.
- This dilution series results in enough of each treatment for up to 10 samples, increase as necessary.
- If measuring pH, do so before extract treatment OR significantly increase the amount mixed to ensure enough is leftover after treatment.

PROCEDURE:

1) Aseptically transfer 50 mL media into a beaker under the hood
The remaining steps may be done outside the hood

2) Add 100 µL of 10% dimethylsulfoxide (DMSO) to
5 mg extract bottle
and dissolve by turning the bottle gently and pipetting.

Pipette 900 µL media into Eppendorf tube, and add the DMSO-anthocyanidin solution

a) Combine 500 µL 10% DMSO-anthocyanidin solution
49.5 mL fresh media
= 50 µg/mL

3) Combine 5 ml of solution A
5 ml fresh media
= 25 µg/mL

4) Combine 2 ml of solution A
8 ml fresh media
= 10 µg/mL

5) Combine 1 ml of solution A
9 ml fresh media
= 5 µg/mL

6) Combine .2 ml solution A
9.8 ml fresh media
= 1 µg/mL

7) Combine fresh media and 10% DMSO separately, to make media with .1% DMSO to use as control treatment on cells not receiving anthocyanidin

APPENDIX B

Comet Assay with H_2O_2 Treatment for Trevigen kit

NOTES:

- Work with lights off to reduce UV damage to cells!
- PBS needs to be Ca^{2+}, MG^{2+} free!

PROCEDURE:

P*erform steps 1-3 30 minutes prior to the end of the extract treatment.*

1) Prepare lysis solution:

120 ml Lysis solution
12 ml 10% DMSO

Chill in fridge until use.

2) LM Agarose:

Turn on 42°C water bath and boiling water bath.
Boil LM agarose for 5 minutes with the cap loosened using tongs and then place in 42 °C water bath until needed.
(*Place ~3.3 ml in a bottle and put the rest back in the fridge in case of spillage)*

3) Label 1 set of eppendorf tubes for the samples, add 400 µL PBS to each and set aside.
Label comet slides and then put back in slide box until needed.

Warm trypsin in the 37°C water bath for 10 minutes.

4) Harvest Cells:

Remove media containing extracts and trypsinize cells.
(rinse with 0.2 ml trypsin, treat with 0.3 ml, stop reaction with 0.7 ml media)
Use the 1ml pipettes and remember that they fill VERY quickly!

Transfer 100 µL cells to labeled eppendorf tubes with 400 µL PBS.
This dilutes the cells which are roughly at 1 x 10^6 to the desired 1-2 x 10^5cells/ml.

Spin 5 min at 5000rpm (1250 x g)

Wash the cells with PBS without disturbing the pellet; then resuspend cells in 500 µL PBS.

5) Slide Prep:

In an Eppendorf tube, half submerged in water bath, mix 200 µL LMagarose
20 µL cells

Pipette 80ul of mixture onto each of the 2 slide wells and spread with pipette tip
(Note: be careful not to drag tip on slide as it will remove the coating!)

Dry slides flat in fridge for 10 min (or until clear ring around edge is visible)

6) Hydrogen peroxide treatment:

While slides are drying, set up for serial dilution, but don't add H_2O_2 until immediately before treating the slides.
Also fill tray with ice.
Pour lysis solution into a plastic container.

Serial dilution to make 75 uM H_2O_2:
*You may need to double or triple this to accommodate the number of slides

	H_2O_2	PBS	
1	85 ul	915 ul	
2	10 ul	990 ul	
3	20 ul	1980 ul	*Pipette 990 ul twice*

Lay slides on ice.
Pipette 100 µL of 75uM H_2O_2 onto each slide well.
Time the treatment of the slides, for example treat the second slide exactly 10 seconds after the first to ensure that all the slides are treated for exactly 5 min.

Do NOT treat negative control with H_2O_2, use an equivalent amount of PBS.

Tap slides on a paper towel to remove excess H_2O_2 and place in lysis solution.
Remember to continue spacing the slides by 10 seconds.

7) Incubation:
Close lid and incubate the slides in lysis solution for 4 hours in fridge.

8) Electrophoresis prep: (*30 minutes before lysis incubation is complete)*
Prepare denaturing and electrophoresis solution
For 2 L: 24 g NaOH
0.744 g EDTA
About 1.8 L distilled water
Add DI water to 2 L , after NaOH has completely dissolved.

Set up electrophoresis tank in fridge so solution will chill.

When incubation is complete:
9) Line up slides at center of tank. Denature *(incubate)* for 30 minutes.

10) Electrophoresis**:**
Set voltage at 25 V and adjust buffer level to get as close to 300mA as possible (usually around 295 mA)
(Note: Start with buffer just barely covering slides, and add more to increase ampage, take away to increase voltage.)

Run for 30 minutes.

Tap excess buffer off slides and dip in 70% alcohol for 5 minutes.
Air dry slides over night, then store at room temperature (RT) with desiccant in the dark.

SILVER STAINING PROCEDURE

1) Fixation

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

Per sample, 30 ul DI water
50 ul methanol
10 ul glacial acetic acid
10 ul fixation additive, 10X

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

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

Rinse slides with DI water for 30 min.

2) Staining

Solution prep

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

Mix by tapping tube

50 ul Reagent 4

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

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

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

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

SAMPLE PREP:
For tissue:
Follow a homogenization procedure to isolate cells.

For frozen cells:
Thaw by submerging vial in 37°C water bath until all ice is gone
(Transfer to ice cold media, centrifuge 10 min at 2000rpm (200xg))
Resuspend in cold PBS.

CRYOPRESERVATION:
Harvest cells
Resuspend in medium with 10% DMSO.
Count and pipette aliquots of $2x10^6$ cells per eppendorf tube. (Make sure there is room for expansion.)
Put tubes in Styrofoam container and place in -80°C freezer (to freeze at a rate of -1°C)

APPENDIX C

DNA Fragmentation using ELISA Cell Death Detection Kit

NOTES:
Modified for substitution of microplate for eppendorf tubes for the lysis procedure

PROCEDURE:

Prep
**Be sure to get plate shaker, multipipettor and double distilled water from Grider lab

1) Aseptically transfer some PBS (about 25 ml) to a beaker.
2) Label one set of eppendorf tubes for samples. Add 90 ul PBS to tubes.
Be sure to add straight down so that no drops get on the sides of the tubes.

Harvesting cells

1) Gently detach cells in wells using a spatula.
Be very careful not to splash media!

2) Add 10 uL cells to its respective eppendorf tube.
We are assuming the cell concentration to be $1x10^6$ and need it to be $1x10^5$ so are diluting it by a factor of 10

3) Repeat until all wells have been transferred.

4) Centrifuge the tubes 10 minutes at 2700 rpm (200 x g).

5) Carefully remove supernatant.

Lysis

1) Resuspend pellet in 200 µL lysis buffer (bottle 5).
Incubate 30 min. at 15-25 ^oC.

2) During incubation, prepare elisa solutions.
If kit is new, prepare the anti-histone biotin, anti-DNA POD (peroxidase), positive control, ABTS.

If kit is not new, then only make ABTS.

Bottle #	Content	Preparation	For use in	Storage and stability
1	Anti-histone biotin	add 450ul double distilled water, mix thoroughly	Immunoreagent	at 2-8 C (fridge) for 2 mo.
2	Anti-DNA POD	add 450ul double distilled water, mix thoroughly	Immunoreagent	Fridge for 2 mo.
3	Positive control	add 450 ul double distilled water, mix thoroughly	ELISA	Fridge for 2 mo.
7	ABTS	dissolve ***1***,2 or 3 tablets in ***5***,10, or 15 ml Subtrate Buffer (bottle 6) depending on the number of samples tested	ELISA	1 month, Store protected from light! (wrap bottle in foil) Allow to come to RT before use.

3) Centrifuge lysate at 2700 rpm for 10 min (200 x g).
During centrifugation, mix up immunoreagent.

(1/20 vol. Anti-DNA-POD, 1/20 Anti-histone-biotin, 18/20 incubation buffer)

Bottle #	# of tests	10	20	***40***	50
4	incubation buffer	720 ul	1440 ul	***2880 ul***	3600 ul
1	Anti-Histone buffer	40 ul	80 ul	***160 ul***	200 ul
2	Anti-DNA-POD	40 ul	80 ul	***160 ul***	200 ul
	final volume	800 ul	1600 ul	***3200ul***	4000 ul

ELISA

*PIPETTE DIRECTLY INTO THE CENTER OF MP DUE BC OF LOW VOLUMES *
(*be careful to not touch pipette tip to bottom or sides, as this will remove the coating*)

1) Transfer 20 µL from supernatant (which is the cytoplasmic fraction) carefully into the streptavidin coated microplate for analysis. DON'T DISTURB THE PELLET!!!
Also transfer 20 µL from positive control (bottle 3), negative control (untreated cells), background control (incubation buffer, bottle 4) into microplate.

2) Add 80 µL of Immunoreagent. (*using multipipettor)*
Cover MP with adhesive cover foil.
Incubate plate on shaker under gently shaking (300 rpm) for 2 hours at 15-25 ^{o}C.

3) After 1.5 hour, remove ABTS from fridge so it can warm to RT. Turn on 37^{o}C water bath to warm up.

4) After the 2 hour incubation period is complete, remove solution thoroughly by tapping. (*First invert the plate over the sink or a beaker. Take care to not dislodge any of the well strips!!!! Then gently tap the plate on a small stack of paper towels with a kimwipe on top until there is no liquid transferred.*)
Rinse each well 3x with 250-300 µL Incubation buffer (bottle 4)
Remove the solution carefully for each rinse, especially the final rinse.

5) Pipette each well with 100 µL ABTS soln.
Incubate on the plate shaker at 250 rpm until color development is sufficient –about 12-14 minutes. (*any longer- and the positive control color develops too deeply for there MP reader to measure.*)

6) Place ABTS stop solution into the water bath to warm immediately after placing the MP on the shaker, until solute goes into solution.

7) Pipette 100 µL ABTS stop solution into each well.

8) Measure at 405 nm against ABTS solution and 100 µL stop solution as a blank (ref wavelength 490 nm)