DELPHINIDIN ALTERS HYDROGEN PEROXIDE-INDUCED CYTOTOXICITY AND APOPTOSIS IN HUMAN COLON ADENOCARCINOMA CELLS

by

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(Under the Direction of Joan G. Fischer)

ABSTRACT

Delphinidin has been shown to induce apoptosis in cancer cells at high concentrations while protecting normal cells at lower concentrations. The objective of this research was to determine the effect of low concentrations (1-25 μmol /L) of delphinidin on cell viability and apoptosis in hydrogen peroxide (H_2O_2)-treated HT-29 human adenocarcinoma cells. Cells were pretreated with 1-25 μmol /L delphinidin before being treated with H_2O_2. Delphinidin at 1-10 μmol/L increased ($p< 0.05$) cell viability of H_2O_2 -treated cells. Pretreating cells with delphinidin had a biphasic effect on induced apoptosis assessed with caspase-3 activity. Lower concentrations (1-5 μmol/L) significantly ($p< 0.05$) decreased caspase-3 activity induced by H_2O_2 and a higher concentration (25 μmol/L) increased ($p<0.05$) activity of this enzyme compared to H_2O_2 alone. These results suggest that delphinidin has both antioxidant and pro-oxidant properties. Antioxidant properties of delphinidin at lower concentrations may inhibit induced apoptosis in colon cancer cells.

INDEX WORDS: Anthocyanins, Delphinidin, Apoptosis, Antioxidant, HT-29 cells, Colon cancer, Hydrogen Peroxide

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CHAPTER I

INTRODUCTION

Cancer is the second leading cause of death in United States (CDC, 2010). Colorectal cancer is the third most common fatal cancer. It is estimated that in 2009 colorectal cancer claimed nearly 50,000 lives (ACS, 2010a). A number of modifiable life-style factors such as healthy body weight, adequate physical activity, and healthy diet may help to reduce the risk of developing this type of cancer. Diets low in red and processed meats and high in whole grains and fruits and vegetables have been associated with decreased risk for colon cancer (ACS, 2010b). Some studies have found a more specific inverse association between colon cancer risk and fruit intake. Besides being rich in the antioxidant vitamin C, fruits are also a rich source of non-nutritive bioactive compounds such as polyphenols which may have protective properties against cancer initiation and development (WCRF/AICR, 2007).

Cancer cells exhibit six characteristic hallmarks which allow the damaged cell to develop into an invasive tumor. These hallmarks include growth signal autonomy, evasion of growth inhibitory signals, evasion of apoptosis, unlimited replication, sustained angiogenesis, and invasion and metastasis (WCRF/AICR, 2007; Hanahan and Weinberg, 2000). Polyphenols have a number of bioactive functions which may play a critical role modulating these processes. These bioactive functions include acting as antioxidants, inhibiting the cell cycle, inducing apoptosis, and inhibiting angiogenesis and metastasis (Ramos, 2008). One of the bioactive functions which has gained significant attention is their ability to induce apoptosis, or programmed cell death (Khan et al., 2008). The ability of cancer cells to evade apoptosis allows the damaged cells to

grow, divide, and develop into an invasive tumor. The ability of polyphenols to induce apoptosis may help to interfere with cancer progression.

Polyphenol flavonoids, however, have biphasic, opposing effects on most of these cellular processes that are dependent on flavonoid concentration. Wätjen et al. (2005) found that the flavonoids quercetin and fisetin can induce apoptosis at high concentrations while inhibiting induced apoptosis at lower concentrations. In previous studies in our lab, anthocyanins inhibited cell proliferation and induced apoptosis in human colon cancer HT-29 cells (Yi et al., 2005; Srivastava et al., 2007). Similar effect of anthocyanins on apoptosis at relatively high concentrations was also observed in other studies (Hafeez et al.,2008; Hou et al.,2005; Lazzé et al., 2005; Yeh et al., 2005; Yun et al., 2009). At lower concentrations, however, anthocyanins have protected cells from apoptosis induced by radiation or oxidative agents (Afaq et al., 2007; Elisa et al., 2008; Tsoyi et al., Ye et al., 2010). This biphasic effect may be attributed to the pro-oxidant and antioxidant properties of anthocyanins depending on concentration. As pro-oxidants, anthocyanins can increase production of reactive oxidative species (ROS) to the critical levels needed to trigger apoptosis and as antioxidants they decrease production of ROS, thus inhibiting apoptosis (Loo, 2003).

The goal of our study was to determine the effect of low concentrations of the highly bioactive anthocyanin, delphinidin, on hydrogen peroxide (H_2O_2)-induced cytotoxicity and apoptosis in HT-29 cells. Our hypothesis was that pre-treating HT-29 cells with 1-25 μmol delphinidin would significant reduce H_2O_2-induced cytotoxicity and apoptosis. We found that pretreating cells with 1-10 μmol/L delphinidin significantly increased cell viability when cells were exposed to H_2O_2. Pre-incubation with 25 μmol/L delphinidin did not have a significant effect on viability of H_2O_2- treated cells. We also found that delphinidin at concentrations of 1-

25 µmol/L had biphasic effect on H_2O_2 –induced apoptosis, determined by measuring caspase-3 activity. A significant decrease in caspase-3 activity was observed in groups pre-incubated with 1-5 µmol/L while a significant increase was observed at 25 µmol/L. These results suggest that delphinidin has both antioxidant and pro-oxidant properties in a fairly narrow concentration range of 1-25 µmol/L.

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CHAPTER II

LITERATURE REVIEW

Colon Cancer and Diet

Cancer is the second leading cause of death in United States (CDC, 2010). Colorectal cancer is the third most common fatal cancer in both men and women (ACS, 2010b). It is estimated that in 2009 there were 49,920 colorectal cancer-related deaths and 146,970 new cases of colon and rectal cancer (ACS, 2010d). There are a number of modifiable and non-modifiable risk factors associated with colorectal cancer. Non-modifiable factors include age, ethnicity, personal history of colorectal polyps or colorectal cancer, inflammatory bowel disease, and family history of colorectal cancer. Modifiable risk factors include life-style factors such as diet high in red and processed meats, inadequate physical activity, obesity, smoking, and excessive use of alcohol (ACS, 2010e). In many cases colorectal cancer can be prevented by regular screening for colorectal polyps and by managing modifiable risk factors. A diet with low in red and processed meats and high in whole grains and fruits and vegetables may help to prevent development of colorectal cancer (ACS, 2010a).

A large European Prospective Investigation into Cancer and Nutrition (EPIC) study with 452,755 subjects from 10 European countries found an inverse association between fruit and vegetable intake and colon cancer (van Duijnhoven et al., 2009). Another group analyzed 14 cohort studies which examined fruits and vegetable intake and colon cancer risk and found that this association was strongest for distal colon cancer (Koushik et al., 2007). In a review of twenty cohort studies by the World Cancer Research Fund/ American Institute for Cancer

Research (2007) thirteen of the studies showed an inverse association between fruit intake and colon cancer. Fruit are not only high in the antioxidant vitamin C but also are high in non-nutritive phytochemicals such as polyphenols, which are not only potent antioxidants but may also have other bioactive functions.

Chemopreventive properties of these non-nutritive compounds and their effect on cancer in cell and animal models are a thriving area of research. Chemoprevention refers to ability of naturally occurring or pharmacological compounds to suppress, arrest, or reverse carcinogenesis at early stages of development (Russo, 2007). Naturally occurring polyphenols found in fruits and vegetables have chemopreventive properties which may interfere with cancer development (Ramos et al., 2008).

Cancer

Cancer is a group of the diseases characterized by uncontrolled cell growth as a result of mutations in genes that tightly regulate the process of cell growth, division and death. Mutations in these critical genes are due to accumulation of DNA damage generated by both endogenous and exogenous factors. Endogenous factors include inherited genetic mutations, errors in DNA replication, oxidative stress, chronic inflammation and hormonal exposure. Exogenous factors include tobacco use, infectious agents, radiation, environmental chemicals, medication and agents in food. The latter three groups of compounds can contribute to DNA damage following metabolism by phase I and phase II enzymes. The initial step, carried out by the Cytochrome P450 (CYP) family of phase I enzymes, may activate these compounds, forming reactive carcinogenic metabolites. Phase II enzymes such as glutathione-S-transferases (GTSs) and NAD(P)H:quinine oxidoreductase (NQO) can deactivate carcinogens, facilitating their excretion via bile or urine (WCRF/AICR, 2007). Reactive species (RS) can attack DNA molecules leading

to DNA strand breaks, removal of bases and formation of DNA adducts which can lead to the misreading of DNA bases during replication. This damage is particularly dangerous when it leads to mutations in genes which code for proteins responsible for stimulating DNA repair, blocking cell proliferation of damaged cells, and promoting apoptosis. Mutations in tumor suppressor genes which regulate cell proliferation and apoptosis, such as p53, are very common in human cancers (King and Robins, 2006).

In order to prevent the replication of a cell with damaged DNA, cells have a number of regulated checkpoints throughout the cell cycle. The first checkpoint is found in the first gap phase (G1) during which the cell prepares for DNA synthesis (S) phase. If DNA damage is detected during this phase, protein p53 blocks the progression of the cell cycle until DNA can be repaired, arresting the cell in the G1 or G1/S phase. If the cell is able to bypass the first checkpoint or if damage occurs during the second gap phase (G2) between DNA synthesis and mitosis (M), another checkpoint which exists at that phase can lead to G2/M cell cycle arrest. The final checkpoint is the spindle assembly checkpoint during mitosis (King and Robins, 2006). It has been suggested that the G2/M cell cycle arrest can occur in presence or absence of p53. A number of regulatory proteins are involved in induction of cell cycle arrest. These include cyclin-dependent kinases (CDK) which activate progression of the cell cycle, their activators cyclins, and CDK inhibitors, which include p21 and p27. Protein p53 induces cell arrest by stimulating transcription of CDK inhibitor genes. Mutations in these genes or inactivating damage to these proteins can inhibit the induction of cell cycle arrest. In case of severe DNA damage, p53 induces apoptosis by stimulating transcription of pro-apoptotic protein Bax (Vermeulen et al., 2003.)

A single cell that does not respond to these regulatory factors but continues to proliferate and can develop into invasive cancerous tumors. Characteristics of these cells include the six hallmarks of cancer, which are growth signal autonomy, evasion of growth inhibitory signals, evasion of apoptosis, unlimited replication, sustained angiogenesis, and invasion and metastasis (WCRF/AICR, 2007; Hanahan and Weinberg, 2000). Cancerous cells can generate their own growth signals and are not responsive to growth inhibitory signals. They are able to survive conditions which trigger apoptosis in normal cells. Also, unlike normal cells which can only divide 60-70 time before undergoing apoptosis, cancer cells can divide endlessly. As cancer progresses it develops the ability to induce angiogenesis, or growth of new blood vessels, to meet the nutrient needs by up-regulating angiogenic factors such as vascular endothelial growth factor (VEGF) and hypoxia-induced transcription factors (HIFs). When the tumor reaches the membrane encapsulating the organ, cancerous cells can secrete enzymes, such as metalloproteinases (MMPs), which digest the membrane and allow cancer cells to enter into the blood and lymphatic system. From there they can spread to other parts of the body.

Cancer development is often described as three stages: initiation, promotion, and progression. The initiation phase includes the initial damage or transformation of the cells; in the promotion phase these damaged cells escape the checkpoint mechanisms and continue to grow and divide; in the progression phase the tumor continues to grow and invade surrounding tissue. The final stage of progression phase is metastasis, or spreading of cancer to other organs in the body (WCRF/AICR, 2007; King and Robins, 2006).

Conventional cancer treatment includes surgery, chemotherapy, immunotherapy, and radiation therapy. Sometimes combinations of these are used (ACS, 2010c). Both chemotherapy and radiation kill rapidly dividing cells, often by inducing apoptosis. Along with killing the

rapidly dividing cancer cells, these treatments also damage the normal cells that undergo frequent cell division, resulting in many unpleasant side effects (Halliwell and Gutteridge, 1999). Therefore a thriving research area involves the search for chemotherapy treatments that would kill cancer cells without affecting the healthy cells. There is particular emphasis on inducing this cell death via apoptosis, which is able to illuminate dead cells without affecting the surrounding cells. Plant compounds such as polyphenols have shown to be promising agents (Khan et al., 2008).

Oxidative Stress

Oxidative stress is a condition of imbalance between the production of reactive species (RS) and the antioxidant defense system. Oxidative stress is known to play a role in a number of diseases including cardiovascular disease, diabetes, Alzheimer's disease, Parkinson's disease and cancer (Halliwell and Gutteridge, 1999). Some of the molecules which contribute to oxidative stress, such as reactive oxygen species (ROS), are also secondary messengers in cell signaling pathways and may be involved in a number of normal cell functions (Poli et al., 2004). Over expression of these molecules, resulting in oxidative stress, can cause damage to DNA, proteins, and lipids. Oxidative damage to these compounds can result in DNA strand breaks, mutations in genes coding for regulatory proteins, inactivation of gene repair and regulatory proteins, and alterations in integrity of the cell membrane. This can lead to development of cancer. Oxidative stress plays an important role in not only the initiation phase of cancer but also in the promotion and progression phases (Halliwell, 2007). High levels of free radicals are toxic to the cells and induce cell death, apoptosis and, at even higher levels, necrosis (Halliwell and Gutteridge, 1999).

These reactive species are generated during normal redox reactions of cellular respiration but can also be generated by inflammation, radiation, ionization, or by phase I enzymes during

drug metabolism (Poli et al., 2004). Some of the various forms of RS included reactive nitrogen species (RNS) such as nitric oxide (NO) and the reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$) and the hydroxyl radical ($OH^{\bullet}$). These compounds interact with other molecules in a chain reaction to form free radicals, or highly reactive molecules that containing one or more unpaired electrons (Halliwell and Gutteridge, 1999). Of the ROS, hydroxyl radical ($OH^{\bullet}$) is more reactive than H_2O_2 and $O_2^{\bullet -}$. However, the latter two species can react with transition metals or each other to produce the more reactive $OH^{\bullet}$ (**Table 2.1**) (Poli et al., 2004). Hydrogen peroxide (H_2O_2) is often used in cellular models to induce oxidative stress because it can readily cross the cell membrane and induce intracellular oxidative stress. Some of the commonly used biomarkers to test for oxidative stress are the presence of DNA adducts such as 8-hydroxy-2'-deoxyguanosine (8OHdG), DNA strand breaks measured with the comet assay, and lipid peroxidation (LPO) (Halliwell and Whiteman, 2004).

In order to attenuate the damaging effect of oxidative stress in the cells, intracellular levels of RS are controlled by a number of antioxidant defense mechanisms. This antioxidant defense system includes a number of families of antioxidant enzymes (superoxide dismutases (SOD), catalases, and the glutathione peroxidase family (GPx)), detoxifying phase II enzymes, and a number of dietary antioxidants, such as vitamins C and E, carotenoids and plant phenols (Halliwell and Gutteridge, 1999). Dietary antioxidants such as plant phenols can not only directly scavenge the RS by donating electrons to the free radicals but also can up-regulate the expression of antioxidant enzymes and detoxifying enzymes by activating the antioxidant response element (ARE). These same dietary compounds, however, can also act as pro-oxidants, increasing the production of ROSs. Thus they can play an important role regulating levels of RS in cells (WCRF/AICR, 2007). Oxidative stress occurs when the increased production of RS

overwhelms the antioxidant defense system or when this defense system is weakened, due to a decrease in activity of antioxidant defense enzymes or depletion of dietary antioxidants (Halliwell and Gutteridge, 1999).

While ROS are known to cause DNA damage and protein damage, they may also have an important function in cell signaling. It is believed that ROS may act as second messengers in a number of signaling pathways and have a number of normal physiological functions when present in controlled amounts (Halliwell and Gutteridge, 1999). These physiological functions vary depending on the levels of oxidative stress. For example, low levels of ROS stimulate cell proliferation while higher levels activate p53 to induce cell cycle arrest and allow time for DNA repair. Once ROS levels reach a certain threshold they are able to trigger cascade of events leading to apoptosis. Oxidative stress above these threshold levels can shut down the apoptotic process, by inhibiting p53 and oxidizing caspases involved in apoptotic pathway, leading to survival of damaged cells or necrosis. Low to moderate levels of ROS may also activate the NF-kB signaling pathway that inhibits apoptosis. Higher levels of RS may interfere with activation of this pathway. The degrees of oxidative stress which trigger these contradictory events are cell-type-specific (Halliwell, 2007).

Oxidative stress may play an important role in promotion and progression stages of cancer. Cancer cells produce a higher amount of H_2O_2 compared to normal cells and this elevated level of oxidative stress enhances the vitality and proliferation of cancer cells (Loo, 2003). Reactive species also can play a role in progression of cancer by stimulating production of vascular endothelial growth factor (VEGF) ,which promotes angiogenesis, and activating matrix metalloproteinases (MMPs), which are able to digest organ membrane, allowing cancerous cells to enter the blood and lymphatic system and spread to other parts of the body (Halliwell, 2007).

The already increased level of oxidative stress in cancer cells make them more susceptible to induced apoptosis. A number of anticancer drugs and bioactive dietary compounds are able to cause cell cycle arrest and induce apoptosis by increasing production of ROS (Loo, 2003). The presence of dietary antioxidants can delay or block this pro-apoptotic effect, favoring the survival of cancer cells (WRCF/AICR, 2007). Another study, however, showed that increased levels of RS $O_2^{\bullet -}$ due to suppressed activity of antioxidant enzyme Cu/Zn SOD inhibited anti-cancer drug induced apoptosis in human melanoma M14 cells. Over expression of this enzyme which lowered $O_2^{\bullet -}$ levels increased cell sensitivity to drug induced apoptosis (Pervaiz et al., 1999). The suggested explanation for this effect was that increased levels of RS may have oxidized and inactivated pro-apoptotic proteins or that RSs activated the IPK3/AKT signaling pathway which inhibits apoptosis (Halliwell, 2007).

As previously mentioned, a number of dietary compounds play an important role in managing levels of RS and therefore modulate the intracellular activity of these compounds. On one hand, as antioxidants, they can protect against RS induced damage leading to formation of new cancerous cells and may be able to hinder progression of tumors by decreasing the stimulatory effect of ROS on angiogenesis and metastasis. As pro-oxidants, these compounds can induce cell cycle arrest and apoptosis. However, in some cells, the same antioxidant properties of these compounds that may help in preventing cancer development may potentially promote cancer development by inhibiting RS induced apoptosis. In fact the same dietary compounds that can induce apoptosis have been shown to protect against apoptosis, suggesting that they have both antioxidant and pro-oxidant properties (WRCF/AICR, 2007). These functions are cell-type-specific and may also be dose specific. Therefore more research is needed to determine the function of these compounds in different cancer cells at different concentrations.

Apoptosis

Apoptosis, or programmed cell death, is a mechanism used by multicellular organisms to maintain tissue integrity by eliminating redundant or potentially harmful cells (Sun et al., 2004). Unlike necrosis, during which the content of the dying cells is released into the surrounding area triggering an inflammatory response, apoptosis is a controlled process that eliminates damaged cells without affecting the adjacent cells. During apoptosis the cell shrinks and is fragmented into membrane bound apoptotic bodies which are phagocytized by the adjacent cells without inducing inflammatory response (Halliwell and Gutteridge, 1999). Apoptosis has been the target of both pharmacological and dietary anticancer therapy (Hu and Kavanagh, 2003; Sun et al., 2004; Khan et al., 2008).

Apoptotic processes may be activated by two non-exclusive pathways: intrinsic (mitochondrial) pathway or extrinsic (death-receptor) pathway. The intrinsic apoptotic pathway is triggered by intracellular signals such as DNA damage, disruption of the cell cycle, hypoxia, ROS, and/or physical or chemical insult. These signals activate the tumor suppressor protein p53 which acts as a transcription activator of pro-apoptotic proteins and can also directly increase mitochondrial permeability (WCRF/AICR, 2007). Mitochondrial integrity is regulated by pro-apoptotic (Bax, Bak) and anti-apoptotic (Bcl-2, Bcl-xL) proteins of the B-cell lymphoma 2 (Bcl-2) family. When the ratio between these proteins favors pro-apoptosis, the mitochondrial membrane is weakened and cytochrome *c* is released into the cytosol (Sun et al., 2004). Cytosolic cytochrome *c* activates the apoptosome complex that interacts and activates initiator caspase-9. Activated caspase-9 then activates effector caspases, such as caspase-3, which leads to a cascade of irreversible events that end in apoptosis. Once activated, caspase-3 cleaves DNA repair enzyme poly(ADP-ribose) polymerase (PARP), deactivating it, and activates

endonucleases responsible for DNA fragmentation. In the extrinsic pathway, apoptosis is triggered by binding of a ligand to the death receptors on the cell membrane, which activates the initiator caspase-8. Activated caspase-8 may directly activate the effector caspase-3 or in some cases it may recruit the involvement of the intrinsic pathway to amplify the apoptotic signal (Ward et al., 2008).

Both pathways involve activation of caspase-3 enzyme; therefore activity of this enzyme is often used in cell and tissue models to determine if cells are undergoing apoptosis. Other biomarkers of apoptosis include activation of other initiator and effector caspases (-2, -7, -8, and -9), cytosolic cytochrome c, nucleosomal DNA fragmentation, increase in pro-apoptotic proteins Bax or Bak, decrease in anti-apoptotic proteins Bcl-2 or Bcl-xL, and cleaved PARP (Ward et al., 2008). Morphological characteristics of apoptotic cells include membrane blebbing, shrinking of cell and nuclear volume, chromatin condensation and nuclear DNA fragmentation (Khan et al., 2008).

One of the hallmarks of a cancer cell is its ability to evade apoptosis, allowing damaged cells to continue to grow and divide (WCRF/AICR, 2007, Hanahan and Weinberg, 2000). This may be due to mutations in genes or inactivation of proteins that regulate apoptosis, such as p53 and pro-apoptotic proteins activated by p53. Other key proteins which determine if the cell will undergo apoptosis are the Bcl-2 family of proteins that regulate the integrity of mitochondrial membrane. Decreased expression of pro-apoptotic proteins Bax and Bak, activated by p53 pathway, and over expression of anti-apoptotic Bcl-2 or Bcl-xL desensitize the cells to apoptotic signals. Bcl-2 family proteins may be altered independently of p53, hence the shift in Bax/Bcl-2 ratio to favor apoptosis is one of the targets in developing chemotherapy drugs (Evan and Vousden, 2008). The anticancer drugs' mechanism of inducing apoptosis often involves

inducing intracellular production of ROS (Loo, 2003). Studies have also shown that a number of dietary factors can induce apoptosis by reactivating the suppressed p53 pathway and altering the Bax/Bcl-2 ratio to favor apoptosis (Khan et al., 2008). In a number of cell systems, oxidative stress is associated with the Bax/Bcl-2 ratio. Many of these dietary factors are known to induce oxidative stress thus inducing apoptosis (Sun et al, 2004). As previously mentioned, dietary antioxidants can attenuate the production of ROS thus inhibit ROS induced apoptosis and favor the survival of cancer cells.

Polyphenols

Scientific inquiry to understand the mechanism behind the protective effect of fruits and vegetables against a number of chronic diseases has led to a discovery of a number of non-nutritive bioactive compounds in plants, or phytochemicals. The general structure of these compounds consists of several hydroxyl groups on aromatic rings. They are classified as phenolic acids, flavonoids, stilbenes, or ligans based on the number of phenolic rings that they contain and how those rings are connected. Flavoniods are the largest subgroup of polyphenols and can also be divided into six additional subgroups, flavonols, flavones, isoflavones, flavanones, anthocyanins, and favonols, based on variations in the heterocyclic ring. These compounds may also be associated with different sugars, organic acids, and can form complexes, which can further diversify their bioactivity. Some of these polyphenols can be found in all plant products, such as fruits, vegetables, cereals, legumes, nuts and teas. Others are specific to particular foods, such as flavanones which are only found in citrus fruit. Most fruits contain mixtures of polyphenols. The amount of polyphenols in plants varies, depending on growing conditions, storage, industrial processing and cooking methods (Manach et al., 2004). In plants, polyphenols are involved in growth and reproduction as well as protection against photosynthetic

stress, ROS, wounds, and herbivores. They are also responsible for the plants' pigmentation and flavor (Ross and Kasum, 2002; Yang et al., 2001). Antioxidant properties and other biological functions of these compounds may play an important preventive role in a number of chronic diseases associated with oxidative stress, such as cardiovascular and neurodegenerative diseases and cancer (Ross and Kasum, 2002; Issa et al., 2006).

The hydroxyl groups attached to the aromatic rings of these compounds create an electron-rich environment able to neutralize ROS, which makes them powerful antioxidants (Issa et al., 2006). The antioxidant and other bioactive functions (see **Table 2.2**) of polyphenols have the potential to make them a very effective chemopreventive agent during all three stages of cancer. As antioxidants and inducers of detoxifying enzymes, they decrease oxidative stress and prevent DNA damage that can lead to initiation of cancer. If DNA damage is already present, polyphenols are able to inhibit proliferation of damaged cells by inducing cell cycle arrest and apoptosis. Polyphenols have even been shown to inhibit angiogenesis needed for continued tumor growth and metastasis of cancerous cells to other parts of the body. Another important characteristic of these compounds is their seeming ability to differentiate between cancerous and normal cells. Cancer cells are more sensitive to polyphenol induced cell cycle arrest and apoptosis compared to normal cells (Ramos, 2008).

The role of polyphenols as potent inducers of apoptosis in cancer cells has been of particular interest (Khan et al., 2008). Polyphenols may have a number of biological functions associated with the apoptotic process. These functions include increasing oxidative stress, up-regulating p53, modulating the Bax/Bcl-2 ratio to favor apoptosis, and inhibiting activation of signaling pathways, such as NF-kB, which inhibit apoptosis (Ramos, 2008). Wenzel et al. (2000) found that a flavone was even more effective than camptothecin, a compound used in

chemotherapy, in inducing apoptosis in human colon carcinoma cell line HT-29. The flavone increased the mRNA of p21 and Bak while decreasing mRNA levels NF-kB, CDK, and Bcl-xL. Unaltered levels of p53 showed that this pro-apoptotic effect was independent of this often mutated tumor suppressor gene. Also, unlike camptothecin, the flavone had no inhibitory effect on cell growth of nontransformed mouse colonocytes, demonstrating specificity for cancerous cells.

Although the findings above show that polyphenols may be very effective at eradicating some cancer cells, their biphasic effect on cell proliferation and apoptosis is of some concern. Because of their potent antioxidant properties, polyphenols can inhibit induced apoptosis by decreasing production of ROS and modulating Bax/Bcl-2 ratio to favor survival. Choi et al. (2003) reported that some of the flavonoids, (-) epigallocatechin gallate (EGCG) and quercetin, inhibited H_2O_2-induced apoptosis in endothelial cells by decreasing oxidative stress, up-regulating expression of Bcl-2, and down-regulating expression of Bax. The biphasic effect of polyphenols has also been observed in a number of cancer cell lines. Van der Woude et al. (2003) found that while higher concentrations of quercetin decreased cell proliferation in colon carcinoma HCT-116 and HT-29 cells and mammary adenocarcinoma cell line MCF-7, low concentrations actually slightly but significantly increased proliferation of these cell lines. Similar biphasic effects of flavonoids have been observed in the apoptosis process. Quercetin and fisetin at lower concentrations protected rat hepatoma cells from induced apoptosis while inducing apoptosis at higher concentrations. Some overlap in protective and pro-apoptotic dose ranges was observed (Wätjen et al., 2005). Not all flavonoids have a protective effect against induced apoptosis. This effect is most likely structure dependent. Flavonoids that had the greatest antioxidant activity showed the greatest protective effect against apoptosis (Choi et al., 2003).

Their biological activities in cells also depend on cellular uptake (Wätjen et al., 2005). These findings and the fact that many of the pathways involved in apoptosis can be modulated by altering levels of oxidative stress suggest that the ability of phytochemicals to induce or inhibit apoptosis may be related to their pro- and antioxidant properties. Some studies, however, found that their role in apoptosis process was independent of these properties (Wätjen et al., 2005; Russo, 2007). They also suggest that the type of bioactive function exerted by the compounds depends on their functional structure and dose. It should be noted that pro-apoptotic dose levels are at pharmaceutical level and cannot be attained through diet.

When compared to chemotherapeutic drugs, polyphenols may be less toxic to healthy cells at pro-apoptotic doses, but they are still toxic at higher concentrations. As pro-oxidants, at very high concentrations, flavonoids increase formation of RS which can lead to DNA damage and mutations. Furthermore they can impair the activities of antioxidant enzymes which would further increase DNA damage. They may also have other adverse biological effects, such as interfering with thyroid hormone production (Skibola and Smith, 2000). Clinical trials looking at both effectiveness and safety of isolated polyphenols are limited. A small phase I clinical trial by Ferry et al. (1996) was done to study the tumor suppressing effect of quercetin in patients with different types of tumors which were not responding to other treatments. Their findings showed that quercetin was not as effective in inhibiting tumor activity as conventional radiology. Also, increasing doses of quercetin led to a number of adverse reactions, including cardiovascular and renal toxicity. This means that before flavonoids can be used as chemopreventive agents in humans more research needs to be done to determine the effects of polyphenolic compounds at different concentrations, particularly concentrations that are higher than those which can be achieved through diet.

Anthocyanins

Anthocyanins are the red, blue and purple pigmented flavonoids. They can exist in both colored and uncolored form depending on the pH of the environment. Fruit is the main source of anthocyanins in human diet but they can also be found in certain grains and leafy and root vegetables, such as eggplants, cabbage, beans, onions, and radish (Manach et al., 2004). The amounts of anthocyanins found in selected commonly eaten foods in United States are listed in **Table 2.3**. Estimated amount of anthocyanins in a typical American diet based on food intake data from NHANES 2001-2002 is 12.5 mg/day/person (Wu et al., 2006). The actual dietary intake of anthocyanins can vary greatly depending on the diet and the amount of anthocyanins in food. As with other polyphenols, anthocyanin content in food can vary depending on growing conditions, storage and processing and preparation methods, making it even more difficult to estimate dietary intakes (Manach et al., 2004).

Naturally, anthocyanins are found in the glycosylated form, with a sugar(s) attached to their three ring phenolic structure. The attached sugar(s) can be glucose, galactose, rhamnose, xylose, or arabinose. Aglycones are referred to as anthocyanidins and are rarely found in this form in plants. The six most common anthocyanidins are cyanidin, delphinidin, pelargonidin, malvidin, petunidin, and peonidin (**Figure 2.1**). The structural differences are due to the number and position of hydroxyl and methoxyl groups at the 3' and 5' positions of the B-ring (Wang and Stoner, 2008). These structural differences and the type and number of sugars that are attached to skeletal structure effect both the bioavailability and the bioactive functions of different anthocyanins (Prior and Wu, 2006).

Bioavailability of anthocyanins, or the amount of ingested anthocyanins that is absorbed, is important in determining their biological effect. Unlike other flavonoids, bioavailability of

anthocyanins, based on total concentrations in plasma, is very low. Maximum plasma levels ranges are 1-100 nmol/L. They are, however, absorbed in intact glycoside form (Prior and Wu, 2006). Anthocyanins can be found in plasma quickly after ingestion, which suggests that they are absorbed in the stomach. It is believed that the acidic environment in the stomach keeps anthocyanins in a stable cation form, preventing them from being metabolized in the stomach. The fact that so little of ingested anthocyanins are absorbed also suggests that the majority of anthocyanins remain in the digestive tract and can be found in higher concentrations in the gut. It is here that most of the digestion occurs, primarily carried out by the microflora populations in the colon. How quickly these compounds are broken down in the colon seems to depend on the type and number of sugars attached. Aglycones are metabolized more rapidly than glycosides (McGhie and Walton, 2007). Also, glucosides are broken down faster than galactosides and arabinosides and xylosides are most stable (Prior and Wu, 2006).

Differences in anthocyanin structures also affect their bioactive properties. In as study by Kähköhen and Heinonen (2003), delphinidin exhibited the highest radical scavenging activity, followed by cyanidin and peonidin. This increased antioxidant potency of delphinidin may be attributed to the hydroxyl groups at both 3' and 5' positions. Activity of monoglucosides of delphinidin, cyanidin, and malvidin were comparable to that of aglycones. Galactosides had decreased activity. A study by Renis et al. (2008) which compared activities of the aglycone cyanidin (Cy) and cyanidin-3-O-ß glucopyranoside (Cy-3G) had similar findings. The aglycone cyanidin showed more potent antioxidant and pro-oxidant properties at lower concentrations than Cy-3G. Although Cy-3G was not very effective at decreasing production of ROS, it was more effective in preventing H_2O_2-induced DNA damage than Cy. These finding suggest that

antioxidant effects of aglycones and glycosides involve different mechanisms. Antioxidant functions of aglycones seem to be ROS dependent.

Chemopreventive functions of anthocyanins are same as those of other polyphenols (see Table 2.2). In short, these functions include antioxidant activity, inhibiting cell proliferation, inducing apoptosis, and inhibiting angiogenesis and metastasis (Wang and Stoner, 2008). However, previous studies in our lab showed that compared to other polyphenols, such as phenolic acids, flavonols, tannins, and procyaninidins, anthocyanins were more effective at inhibiting cell proliferation in HT-29 and Caco-2 colon cancer cells (Yi et al., 2005). This suggests that anthocyanins may be more potent chemopreventive agents in these cell lines. As with other polyphenols, anthocyanins have a biphasic effect on these critical events during cancer development. For example, anthocyanins are also able to increase cell viability and inhibit apoptosis in cells treated with radiation or oxidizing agents (Afaq et al., 2007; Elisa et al., 2008; Tsoyi et al., Ye et al., 2010). **Table 2.4** gives a summary of effects of anthocyanins on cell viability and apoptosis at different concentrations.

Studies looking at chemopreventive preventive effects of anthocyanins in cell culture showed that both anthocyanin rich fractions and individual anthocyanins are potent inducers of apoptosis. Previous studies in our lab showed that anthocyanin-rich fractions from blueberries at 50-150 μg/mL induced apoptosis in human colon cancer HT-29 cells in a dose dependent manner (Srivastava et al., 2007). Others have found a similar effect of individual anthocyanins on different cancer cell lines (Hafeez et al., 2008; Hou et al., 2005; Lazzé et al., 2005; Yeh et al., 2005; Yun et al., 2009). In an in *vivo* study by Hafeez et al. (2008), delphinidin injections of 2 mg/animal significantly decreased tumor growth and shifted Bax/Bcl-2 ratio to favor apoptosis in human prostate cancer PC3 cells in xenografted mice. The ability of anthocyanins to induce

apoptosis depends on the cell line and anthocyanin structure. A number of studies have suggested that some cancer cell lines are more sensitive to anthocyanin-induced apoptosis than others (Yeh et al., 2005; Hafeez et al., 2008; Lazzé et al., 2004). Studies looking at the effects of different anthocyanins in various cell lines found that delphinidin was the most potent inducer of apoptosis in cancer cells sensitive to anthocyanins but that it had no significant effect on normal cells at the same concentrations (Yeh et al., 2005; Lazzé et al., 2004).

As with other polyphenols, the effect of anthocyanins on apoptosis is biphasic. Anthocyanins are also very effective at inhibiting apoptosis induced by oxidative stress (Afaq et al., 2007; Elisa et al., 2008; Shih et al., 2007; Tsoyi et al., 2008; Ye et al., 2010). Two in vivo studies found that topical application of black soybean seed coat anthocyanin fractions and delphinidin inhibited apoptosis induced by ultraviolet B radiation (Tsoyi et al., 2008; Afaq et al., 2007). In all five of the above mentioned studies, anthocyanins also decreased the levels of induced oxidative stress, suggesting that their ability to inhibit apoptosis may be closely related to their antioxidant properties.

This biphasic effect of anthocyanins does not only depend on cell line and functional structure of the anthocyanin, but also on the concentration studied. Ye at al. (2010) found that purple sweet potato anthocyanin fractions had protective effect against induced apoptosis and cytotoxicity at 5-20 μg/mL but had cytotoxic effect at higher concentrations of 50-200 μg/mL. Similarly a previous study in our lab showed peonidin significantly increased ($p<0.05$) H_2O_2-induced apoptosis in human colon cancer HT-29 cells at ~ 30-58 μmol/mL while significantly decreasing H_2O_2-induced DNA damage at 1 μmol/mL. This suggests that at lower concentrations anthocyanins may act as antioxidants also in cancer cells and potentially inhibit apoptosis associated with increased oxidative stress.

Anthocyanin concentrations required to trigger a particular biological event vary depending on the cell line. For example, delphinidin decreased cell viability to 50% compared to control at 10.8 μmol/mL in human hepatoma $HepG_2$ cells while concentration of 210.3 μmol/mL was needed to achieve the same effect in Hep3B cells (Yeh et al., 2005). The ability of delphinidin to decrease cell viability at concentrations as low as 10.8 μmol/mL and inhibit H_2O_2-induced apoptosis at concentrations as high as 50 μmol/mL (Shih et al., 2007) suggest that it is very important to determine the effect of different concentrations of delphinidin in each particular cell line.

Rationale

Anthocyanins have various chemopreventive functions which may be beneficial in preventing development of cancer (Wang and Stoner, 2008). Other flavonoid compounds, however, have a biphasic effects on these chemopreventive functions at different concentrations, resulting in opposite outcomes in the same cell lines (Wätjen et al., 2009). Although anthocyanins induce apoptosis in cancer cells (Srivastava et al., 2007; Hafeez et al., 2008; Hou et al., 2005; Lazzé et al., 2005; Yeh et al., 2005; Yun et al., 2009), at lower concentrations they inhibit apoptosis (Afaq et al., 2007; Elisa et al., 2008; Tsoyi et al., Ye et al., 2010). Inhibition of apoptosis in cancer cells may have detrimental consequences in cancer progression and development, therefore it is important to determine if lower concentrations of anthocyanins would protect cancer cells from induced apoptosis. Delphinidin has been shown to be both a potent inducer (Hafeez et al., 2008; Yeh et al., 2005; Yun et al., 2009) and inhibitor (Afaq et al., 2007; Shih et al., 2007) of apoptosis and therefore was used in our study.

Objectives

- To investigate the effect of low concentrations (1-25 μmol) of delphinidin on H_2O_2-induced cytotoxicity in HT-29 cells.
- To investigate the effect of low concentrations (1-25 μmol) of delphinidin on H_2O_2-induced DNA fragmentation and caspase-3 activity HT-29 cells stressed with H_2O_2.

Hypothesis

Pretreating cells with 1-25 μmol/L delphinidin will increase cell viability and decrease DNA fragmentation and caspase-3 activity in H_2O_2-treated HT-29 cells compared to the H_2O_2 only group.

TABLE 2.1: Fenton and Haber-Weiss reactions

Fenton Reaction

$H_2O_2 + Fe^{2+} \rightarrow OH\bullet + OH^- + Fe_{3+}$

Haber-Weiss Reaction

$H_2O_2 + O_2\bullet^- \rightarrow OH\bullet + OH^- + O_2$

(Poli et al. 2004)

TABLE 2.2: Bioactive functions of polyphenols.

Antioxidants
Directly scavenge free radicals
Chelate cations involved in Fenton reaction (production of OH·)
Modulate expression/activity of antioxidant enzymes (SOD, GPx, GR)
Pro-oxidants
Enhance production of ROS (cell and dose dependent)
Effect phase-I and II enzymes
Reduced activity of phase-I enzymes CYP1A1 and CYP2E
Increase activity of phase-II enzymes GST and NQO
Activated ARE, stimulating activity of ARE-mediated phase-II enzymes
Induce cell cycle arrest
Down-regulate cyclins and CDK
Up-regulate CDK inhibitors (p21, p27)
Induce G1 cell cycle arrest
Induce G0/G1 cell cycle arrest
Induce G2/M cell cycle arrest
Induce Apoptosis
Increase production of ROS
Activate both intrinsic and extrinsic apoptotic pathways
Inhibits anti-apoptotic proteins Bcl-2 and Bcl-xL
Up regulates p53 and pro-apoptotic protein Bax and Bak
Induce release of cytochrome c
Activates caspases--3, -9, and -8
Inhibit Apoptosis
Decrease production of ROS
Inhibit induced down-regulation of anti-apoptotic Bcl-2
Inhibit up-regulation of pro-apoptotic Bax
Inhibit induced activation of caspase-3
Antiangiogenic
Down-regulate vascular endothelial growth factor
Down-regulate hypoxia-inducible factor 1α
Effect observed in cancer cells but not normal cells
Inhibits metastasis
Decrease production of ROS
Down-regulate MMP-9 and -2
Modulate signaling pathways
Inhibit NF-kB signaling pathway
Inhibit PI3K/AKT signaling pathway
Anti-inflammatory
Inhibit activity of COX-2

Ramos, 2008; Choi et al., 2002; Wenzel et al., 2000; Woude et al., 2003; Wätjen et al., 2005.

TABLE 2.3: Anthocyanin content in selected common foods[1].

Food	**ACN/100 g[2]**
Cultivated blueberry	387 mg
Red cabbage	322 mg
Blackberry	245 mg
Cranberry	140 mg
Sweet cherry	122 mg
Concord grape	120 mg
Red radish	100 mg
Raspberry	92 mg
Eggplant	86 mg
Red onion	49 mg
Black beans	45 mg
Red grape	27 mg
Strawberry	21 mg
Red delicious apple	12 mg

[1] Data from Wu et al., 2006

[2] 100 g of fresh weight or consumed form.

TABLE 2.4: Biological Effect of Anthocyanins and Anthocyanin Rich Fractions *in vitro* and *vivo* Models.

Reference	Model	Compound	Concentration	Results
Afaq *et al*., 2007	Human immortalized HaCaT keratinocytes	Dp	1-20 µmol/L	↓* cytotoxicity due to UVB irradiation in dose dependent manner
			10 µmol/L	↓ UVB induced oxidative stress
				↓ UVB induced caspase dependent apoptosis
	SKH-1 hairless mice		1 mg/100 µL	↓ UVB induced formation of 8-OHdG in skin cells
		(topical application)	DMSO/ mouse	↓ number of apoptotic cells in skin tissue.
Elisia *et al.*, 2008	Caco-2 colon cancer cells	Purified blackberry anthocyanin extracts	0.8 -100 µg/mL	Had no effect on cell viability.
			3.5-50 µg/mL	↓* AAPH induced cellular oxidation in dose dependent manner
			1.6-25 µg/mL	↓* AAPH induced cytotoxicity, CellTiter-Glo Assay
			100 µg/mL	↓ proportion of cells in AAPH induced sub-G1 phase, apoptotic cells
Hafeez *et al*., 2008	Human prostate cancer cells PC3, LNCaP, C4-2,	Dp	30-180 µmol/L	↓ cell viability of prostate cancer cells in dose dependent manner
				Insignificant effect on viability of prostate epithelial cells
	Prostate cancer PC3 cells		60-180 µmol/L	↑ cell cycle arrest, modulating cell cycle regulatory proteins
			120-180 µmol/L	↑ caspase dependent apoptosis
				↓ activation of NF-κB
	PC3 cells in xenograft mice	(injection)	2 mg/animal	Did not cause weight loss or decrease in food intake
				↓* * tumor growth
				↑ pro-apoptotic Bax protein, ↓ anti-apoptotic Bcl-2 protein in tumor
Hou *et al*., 2005	HL-60 human leukemia Cells	Dp3-Sam	50-125 µmol/L	↓* cell viability in a dose dependent manner
				↑* intracellular ROS production
			50-100 µmol/L	↑ apoptosis (DNA fragmentation) in dose dependent manner
			100 µmol/L	↑ caspase dependent apoptosis
Lazzé et al., 2004	Caco-2 colon cancer cells	Cy, Dp	50-200 µmol/L	No effect on cell viability
		Cy	100-200 µmol/L	No effect on cell cycle
		Dp	200µmol/L	↑ apoptosis in dose
	Uterine carcinoma HeLa S3	Dp	150-200 µmol/L	↓ cell viability (59-50%)
				↑ apoptosis in dose dependent manner
		Cy	150-200 µmol/L	No effect on cell cycle
	Normal fibroblasts NHF	Cy,Dp	50-200 µmol/L	No effect on cell viability
		Cy	150-200 µmol/L	↑ proportion of cells in G1 phase, ↓ proportion of cells in S phase
		Dp	150-200 µmol/L	↓ proportion of cells in G1, ↑ proportion of cells in S phase
				Had no significant effect on apoptosis

Patterson, 2008	Human colon caner HT-29 cells	Mv Pn	50 µg/mL (263 µmol/L) 1 µg/mL (5.75 µmol/L) 5-10 µg/mL (28.73-57.5 µmol/L)	↑ * H2O2 induced DNA damage ↓* H2O2 induced DNA damage ↑ * H2O2 induced apoptosis Peonidin showed greater induction of apoptosis compared to malvidin
Shih et al., 2007	Rat liver Clone 9 cells	Cy, Ku, Dp, Mv Cy and Dp	50 µmol/L 50 µmol/L	No significant effect on cell viability ↑ antioxidant capacity, ↑ activity of antioxidant enzymes ↓* H202 induced cytotoxicity ↓* H202 induced caspase dependent apoptosis
Srivastava et al., 2007	Human colon cancer HT-29 cells	Frozen blueberries anthocyanin fractions	50-150 µg/mL	↑ * caspase-3 dependent apoptosis ↓* activity of detoxifying enzymes of glutathione-S transferase family
Tsoyi et al., 2008	Human immortalized HaCaT keratinocytes	Black soybean seed coat anthocyanins	1-100 µg/mL 10-100 µg/mL	Had no effect on cell viability ↓ ** UVB induced ROS production ↓ ** UVB induced cytotoxicity ↓ * UVB induced caspase dependent apoptosis
	HR-1 Hairless mice	(topical application)	50-100 mg/kg in 100 µL H2O	↓ * the number of cells undergoing UVB induced caspase dependent apoptosis
Ye et al., 2010	Rat PC12 pheochromo-cytoma cells	Purple Sweet Potato Anthocyanins	0.1-20 µg/mL 50-200 µg/mL 5-20 µg/mL	Had no effect on cell viability ↑ * cytotoxicity ↓* Aβ induced cytotoxicity in dose dependent manner ↓* Aβ induced oxidative stress (↓ROS, ↓LPO) in dose dependent manner ↓* Aβ induced caspase dependent apoptosis
Yeh et al., 2005	Human hepatoma HepG2 cells	Cy, Dp, Mv Pg, Pn Dp	10-200 µmol/L 100 µmol/L 50-200 µmol/L 100 µmol/L 100 µmol/L	↓ cell viability (50% cell death at 18.4, 10.8, and 50.4 µmol/L respectively) ↑ DNA fragmentation ↑* production of ROS ↓ cell viability Did not ↑ DNA fragmentation ↑* caspase-3 dependent apoptosis
	Hep3B cells	Cy, Dp, Mv	50-200 µmol/L	↓ cell viability (50% cell death at 168.5, 210.3, and 182.7 µmol/L respectively)
Yun et al., 2009	Human colon cancer HCT116 cells	Dp	60-240 µmol/L 120- 240 µmol/L	↓ cell viability in dose dependent manner ↑ caspase dependent apoptosis ↑ cell cycle arrest ↓ activation of NF-κB

*=p<0.05, **(p<0.01), †= p<0.001.

Abbreviations: Cy, cyanidin; Ku, kuromanin; Dp, delphinidin; Mv, malvidin; Pg, pelargonidin; Pn, peonidin; UVB, untraviolet B; AAPH, 2, 2'-azobis (2-amidinopropane) dihydrochloride; NF-κB, nuclear factor-κB; LPO, lipid peroxidation; PCNA= proliferating cell nuclear antigen, PARP= poly(ADP-ribose) polymerase, Dp3-Sam = Delphinidin 3-sambudioside; ROS, reactive oxygen species; Aβ, β-Amyloid.

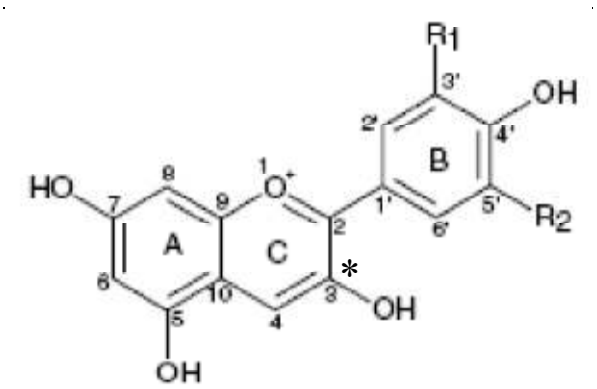

FIGURE 2.1: Common anthocyanidins.

Anthocyanidin	**R_1**	**R_2**
Cyanidin (Cy)	OH	H
Delphinidin (Dp)	OH	OH
Malvidin (Mv)	OCH_3	OCH_3
Peonidin (Pn)	OCH_3	H
Petunidin (Pt)	OCH_3	OH
Pelargonidin (Pg)	H	H

* Sugar moieties in glycosylated anthocyanins are usually attached at this position.

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CHAPTER III

DELPHINIDIN ALTERS HYDROGEN PEROXIDE INDUCED CYTOTOXICITY AND APOPTOSIS IN HUMAN COLON ADENOCARCINOMA CELLS

Abstract

Delphinidin has been shown to induce apoptosis in cancer cells at high concentrations while protecting normal cells at low concentrations of delphinidin. The objective of this research was to determine the effect of lower levels (1-25 μmol /L) of delphinidin on cell viability and apoptosis in hydrogen peroxide stressed HT-29 human adenocarcinoma cells. Cells, incubated at 37ºC, 5% CO2, 95% air, were pre-treated for 4 h with 1, 5, 10, and 25 μmol /L delphinidin before being stressed with 100 μmol/L H_2O_2 for 2 h. Cell viability was determined using the MTT assay 36 h after treatment. Delphinidin had a protective effect against H_2O_2-induced cytotoxicity, significantly ($p< 0.05$) increasing cell viability at 1-10 μmol/L compared to H_2O_2 alone. This suggests an antioxidant effect at low concentrations. Apoptosis was assessed by measuring caspase-3 activity 48 h after treatment. As hypothesized, there was significant ($p<0.05$) decrease in caspase-3 activity in H_2O_2 treated cells but only with 1-5 μmol delphinidin/L, with the lowest activity observed at 1 μmol/L. There was no difference in caspase-3 activity in cells treated only with H_2O_2 and those pretreated with 10 μmol/L delphinidin. An increase in activity ($p<0.05$) was observed at 25 μmol/L. These results support the protective effect of low concentration of delphinidin against H_2O_2-induced cell death, specifically apoptosis, in human colon adenocarcinoma cells.

Introduction

Cancer is the second leading cause of death in United States (CDC, 2010). Colorectal cancer is the third most common fatal cancer. It is estimated that in 2009 colorectal cancer claimed nearly 50,000 lives (ACS, 2010a). A number of modifiable life-style factors such as healthy body weight, adequate physical activity, and healthy diet may help to reduce the risk of developing this type of cancer. Diet low in red and processed meats and high in whole grains and fruits and vegetables has been associated with decreased risk for colon cancer (ACS, 2010b). Some studies have found an inverse association between colon cancer risk and fruit intake. Besides being rich in the antioxidant vitamin C, fruits are also a rich source of non-nutritive bioactive compounds such as polyphenols which may have protective properties against cancer initiation and development (WCRF/AICR, 2007).

Cancer cells exhibit six characteristic hallmarks which allow the damaged cell to develop into an invasive tumor. These hallmarks include growth signal autonomy, evasion of growth inhibitory signals, evasion of apoptosis, unlimited replication, sustained angiogenesis, and invasion and metastasis (WCRF/AICR, 2007; Hanahan and Weinberg, 2000). Polyphenols may play a critical role in modulation of these processes, by serving as antioxidants, inhibiting the cell cycle, inducting apoptosis, and inhibiting angiogenesis and metastasis (Ramos, 2008). One of the bioactive functions which has gained significant attention is their ability to induce apoptosis, or programmed cell death (Khan et al., 2008). The ability of cancer cells to evade apoptosis allows the damaged cells to grow, divide, and develop into an invasive tumor. The ability of polyphenols to induce apoptosis may help to interfere with cancer progression.

Polyphenol flavonoids, however, have biphasic, opposing effects on most of these bioactive functions depending on their concentration. Wätjen et al. (2005) found that flavonoids

quercetin and fisetin can induce apoptosis at higher concentrations while inhibiting induced apoptosis at lower concentrations. Previous studies our lab showed that 50-150 μg/mL blueberry anthocyanin fractions inhibited cell proliferation and induced apoptosis in human colon cancer HT-29 cells (Yi et al., 2005; Srivastava et al., 2007). Similar effects of anthocyanins on apoptosis at relatively high concentrations were also observed in other studies (Hafeez et al.,2008; Hou et al.,2005; Lazzé et al., 2005; Yeh et al., 2005; Yun et al., 2009). At lower concentrations, however, anthocyanins have shown to protect cells from apoptosis induced by radiation or oxidative agents (Afaq et al., 2007; Elisa et al., 2008; Tsoyi et al., Ye et al., 2010). This biphasic effect may be attributed to the pro-oxidant and antioxidant properties of anthocyanins depending on concentration. As pro-oxidants, anthocyanins can increase production of reactive oxidative species (ROS) to reach the critical levels need to trigger apoptosis and as antioxidant they decrease production of ROS thus inhibiting apoptosis (Loo, 2003).

The objectives of our study were to determine the effect of low concentrations (1-25 μmol) of a highly bioactive anthocyanin, delphinidin, on hydrogen peroxide (H_2O_2)-induced cytotoxicity and apoptosis, determined by DNA fragmentation and caspase-3 activity, in human colon cancer HT-29 cells. Our hypothesis was that pretreating HT-29 cells with 1-25 μmol delphinidin would significant reduce H_2O_2-induced cytotoxicity, DNA fragmentation, and caspase-3 activity.

Methods

Cell Cultures and Treatment: Human colorectal adenocarcinoma HT-29 cells were purchased from ATCC (Manassas, Virginian). Cells were cultured in 75 cm^2 flasks using ATCC McCoy's medium with 10% fetal bovine serum at 37 ºC with 5% CO_2 in a humidified atmosphere. The

media was changed every 2-3 days and cells were passaged when 60-80% confluent (Patterson, 2008).

For treatments, cells were seeded at a concentration of 1 x 10^5 cells/mL and incubated for 24 hours. This cell concentration was used for all treatments based on previous studies that showed that hydrogen peroxide-induced cell death in HT-29 cells was dependent on cell concentration in addition to hydrogen peroxide (H_2O_2) concentration (Salh et al., 2000). After 24 hours, treatment groups were incubated for 4 hours in 0.01% DMSO media containing 1, 5, 10, and 25 μM of delphinidin chloride, purchased from Chromadex (See **Table 3.1** for μg/mL equivalents). The 4 hour incubation time was selected based on research by Youdim and Joseph (2000) that showed that maximum cell anthocyanin incorporation was achieved after 4 hours and longer incubation periods did not increase incorporation. Control and hydrogen peroxide (H_2O_2) only groups were incubated with 0.01% DMSO. Following the 4 hour incubation, all the groups with exception of the control group were incubated for 2 hours in media containing 100 μM of H_2O_2. A concentration of 100 μmol/L of H_2O_2 was used to induce apoptosis based on our preliminary studies that showed a significant decrease in cell viability compared to control (**Figure 3.1**) Cells were then incubated in fresh media for various time durations depending on the assay.

Viability: The MTT Cell Proliferation Assay was purchased from ATCC and was used to assess cell viability following incubation with delphinidin chloride and H_2O_2. In metabolically active cells, yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltretrazolium bromide) is reduced by dehydrogenase enzymes to produce NADH and NADPH. The result is purple formazan that can be measured by spectrophotometric means and is accepted as a reliable way to measure cell viability and proliferation. Cells were seeded in 96-well plate (n=8 per treatment

group) with total volume of 0.1 mL/well and cell number of 1 x 10^4 cells/well. Following treatment, cells were incubated for 36 hours before performing the assay (ATCC).

The assay was performed following instructions provided with the kit. Briefly, 10 µL of MTT Reagent was added to each well and the plate was returned to the incubator for another 2 hours, or until purple precipitate was clearly visible under microscope. 100 µL of detergent reagent was added to all wells and the plate was gently swirled. The plate was covered tightly with foil to protect it from the light and was left at room temperature over night. Cell viability was measured at 570-655 nm with a microplate reader and was reported as percent compared to the control (ATCC).

DNA Fragmentation: DNA fragmentation was tested using the Cell Death Detection enzyme-linked immunosorbant assay (ELISA) Plus kit (Roche). During the apoptotic process, activated endogenous endonucleases cleave double stranded DNA generating mono- and oligonucleasomes. This assay is based on a quantitative sandwich-enzyme-immunoassay-principle and uses mouse antibodies to determine the amount of mono- and oligonucleosomes in the supernatant of lysed, centrifuged cells (Roche).

Cells were seeded in twelve-well plates with total volume of 4 mL/well. Cells were incubated for 48 hours after the treatment and then harvested via trypsinizations and combined with reserved media from that well. Cells were then centrifuged for 5 minutes at 100 x g, old media was removed and cells were resuspended in 1 mL phosphate buffered saline (PBS). Cell counts were performed and 1 mL solutions 1 x 10^5 cells/mL obtained for each treatment group. Samples were centrifuged for 10 minutes at 200 x g in the eppendorf centrifuge. Supernatant was removed and the pellet was re-suspended in 200 µL lysis buffer provided by the kit and

incubated for another 30 min at 15-25 ºC. After incubation, cells were centrifuged again at 200 x g for 10 minutes (Patterson, 2008).

Without disturbing the cell pellet, 20 µL of supernatant (n=3 per treatment group) was then transferred from the Eppendorf tubes to the streptavidin coated microplate provided in the kit for analysis. Positive control containing DNA-Histone-Complex was provided by the kit. Untreated cells and buffer were used as negative and background control respectively. Care was taken to add samples without touching the bottom or sides of the microplate wells to not disturb the coating. 80 µL of immunoreagent solution containing anti-histone-biotin and anti-DNA-PODmade according to instruction in the kit was added to each well and the microplate was covered with adhesive cover foil and incubated for 2 hours at 15-25 ºC on a plate shaker set to 250 rpm (Patterson, 2008).

After incubation wells were washed three times with 250 µL incubation buffer and all liquid was removed. All wells were then treated with 100 µL of 2,2´-azino-di-[ethylbenzthiazoline sulfonic acid] (ABTS) solution prepared as indicated by the kit. Samples were returned back to the plate shaker for approximately 5 minutes to allow color to develop and then 100 µL of ABTS stop solution was added to each well. The plate was read by the absorbance microplate reader, ELx800^{TM} (Biot-Tek). Results were reported expressed as enrichment factor and calculated as (treatment-background control) / (negative control-background control) (Roche).

Caspase-3 Activity: Caspase-3 activity was assessed using the Caspase-3 Colorimetric Activity Assay Kit (Chemicon International, Inc). Caspase-3 is able to recognize and cleave a substrate containing the DEVD (Asp-Glu-Val-Asp) motif. The assay measures the activity of caspase-3

enzyme by measuring the quantity of free chromophore *p*-nitroaniline (*p*NA) after it has been cleaved from labeled substrate DEVD-*p*NA (Chemicon, David/Biocompare).

Cells were seeded in 75 cm^2 flasks. The flasks were incubated for 48 hours after the treatments and cells were harvested via trypsinization, reserving the old media and adding it back to flasks. Cells were centrifuged at 100 x g for 6 minutes, media was removed, and cells were resuspended in 1-2 mL of PBS, depending on concentration of cells. Cells were then counted and 1 mL of 1.5 x 10^6 cells/mL was obtained for each group. This 1 mL was then centrifuged for 10 minutes at 1500 rmp in Eppendorf centrifuge. Supernatant was removed and the cell pellet was resuspended in 300 µL chilled 1 X cell lyses buffer provided in the kit. Solutions were incubated on ice for 10 minutes and then centrifuged for another 5 minutes at 10,000 x g (Chemicon International, Inc).

In a 96-well plate, 20 µL of 5X assay buffer and 10 µL of defrosted caspase-3 substrate, provided in the kit, were added to wells (n=3 per treatment group). 70 µL of the supernatant from the samples was then added to those wells. Samples from untreated group were used as negative control, H_2O_2 only group as positive control and assay buffer plus caspase-3 substrate as background. The plate was incubated for 2 hours at 37ºC. The wells were read at 405 nm using absorbance microplate reader, ELx800^{TM} (Biot-Tek) (Chemicon International, Inc.). References were pNA concentration of 50, 25, 10 and 5 µM. The caspase-3 activity was reported as fold increase compared to negative control calculated as (treatment-background control)/(negative control- background control).

Statistical Analysis: Statistical analysis was done with the Statistical Analysis Software (SAS Version 9.13). Means, SEM and analysis of variance were determined and Fisher's least

significant difference test used for post-hoc test. A value of $p < 0.05$ was considered to be statistically significant.

Results

Viability: The effects of pre-incubating HT-29 cells with 1-25 μmol/L delphinidin before treatment with 100 μmol/L H_2O_2 are shown in **Table 3.2** and **Figure 3.2**. A significant increase ($p<0.05$) in cell viability of H_2O_2 treated cells group was observed in groups pre-incubated with 1-10 μmol/L delphinidin compared to cells treated with H_2O_2 alone. Pre-incubation with 1-10 μmol/L delphinidin increased viability to 42 - 48%, compared to 36% in the H_2O_2 alone group. Pre-incubation with 25 μmol/L delphinidin did not have a significant effect on viability of H_2O_2-treated cells. These results suggest an antioxidant effect of delphinidin at lower concentrations, protecting cells from induced oxidative stress.

DNA fragmentation: No DNA fragmentation was detected at 48 hours after the H_2O_2 treatment The assay used measures the amount of fragmented DNA in the supernatant of lysed, centrifuged cells. DNA fragmentation occurs during the final stages of apoptotic process. Cytosolic cytochrome *c* released from the mitochondria triggers cascade of events which lead to activation of caspase-3 enzyme. Caspase-3 then activates endonucleases responsible for DNA fragmentation (Ward et al., 2008). Previously in our lab we were able to detect DNA fragmentation at 24 and 40 hours after incubation. The fact that caspase-3 activity was detected at 48 hours after incubation suggests that longer incubation time may be necessary to observe DNA fragmentation.

Caspase-3 activity: Caspase-3 activity is often used as a biomarker to determine the presence of apoptosis. The effects of 1-25 μmol/L delphinidin on caspase-3 activity in H_2O_2 treated cells were concentration dependent and are shown in **Table 3.3** and **Figure 3.3**. A significant decrease

($p<0.05$) in caspase-3 activity was observed in groups pre-incubated with 1-5 μmol/L delphinidin. The greatest effect was observed in the 1 μmol/L delphinidin group with a 2.85 fold increase above control compared to a 6.81 fold increase in the H_2O_2 only group. These results again suggest an antioxidant effect of delphinidin at lower concentrations, thus preventing apoptosis. There was no significant effect of delphinidin on caspase-3 activity at 10 μmol/L concentration. At 25 μmol/L, however, delphinidin significantly increased ($p<0.05$) caspase-3 activity compared to H_2O_2 only group. This finding suggests a possible pro-oxidant effect of delphinidin at slightly higher concentrations, resulting in increased apoptosis. A second experiment confirmed the significant decreases ($p<0.05$) in caspase-3 activity at 1-5 μmol/L and significant increase ($p<0.05$) in caspase-3 activity at 25 μmol/L. In the second experiment a significant decrease ($p<0.05$) in caspase-3 activity was also observed at 10 μmol/L.

Discussion

Flavonoids, including anthocyanins, are known to have chemopreventive properties in cell models. These chemopreventive properties include decreasing oxidative damage, inhibiting proliferation, inducing apoptosis, and inhibiting angiogenesis and metastasis. All of these properties may be associated with oxidative stress and can be attributed, in part, to the antioxidant and pro-oxidant properties of flavonoids (Ramos, 2008). Although oxidative stress is commonly associated with damage to DNA and other cellular components in the initiation stage of cancer, it also plays an important role in cancer progression. Moderate levels of oxidative stress can activate signaling pathways to inhibit cell proliferation and induce apoptosis, thus eliminating the damaged cells (Halliwell, 2007). As pro-oxidants, flavonoids can increase oxidative levels in cancer cells to reach the threshold need to induce apoptosis (Ramos, 2008)

However, by decreasing oxidative stress they may save cancer cells from apoptosis induced by oxidative stress. Therefore, although these antioxidant properties are beneficial in preventing oxidative damage in healthy cells, they can have detrimental consequences in cancer cells (Russo, 2007).

The aim of our study was to determine if lower concentrations of the anthocyanidin delphinidin (1-25 μmol/L) than typically tested in inducing apoptosis would exhibit a protective effect against induced apoptosis in human colon adenocarcinoma HT-29 cells. This protective effect of delphinidin against induced apoptosis was observed in other cell models (Afaq et al., 2007; Shih et al., 2007). Anthocyanidins cyanidin and delphinidin have been found to be two of the most bioactive anthocyanins in both inducing (Lazzé et al.,2004; Yeh et al., 2005) and inhibiting apoptosis (Shih et al., 2007). Some studies have shown delphinidin to be slightly even more bioactive than cyanidin in inducing apoptosis in some cell lines (Lazzé et al.,2004; Yeh et al., 2005). Delphinidin was also found to have the highest radical scavenging activity compared to other anthocyanidins, mostly likely due to hydroxyl groups at both 3' and 5' positions (Kähköhen and Heinonen, 2003). This suggests that delphinidin's potent ability to induce or inhibit apoptosis may be associated with its strong pro-oxidant and antioxidant activity. Therefore, it is important to determine if lower concentrations of delphinidin would exhibit antioxidant activity in colon cancer cells, protecting them H_2O_2-induced oxidative stress.

First, we wanted to determine if pretreating cells with 1-25 μmol/mL of delphinidin would increase cell viability of HT-29 cells treated with 100 μmol/mL H_2O_2. Our findings showed that delphinidin at 1-10 μmol/mL (0.306-3.06 μg/mL) had a protective effect against H_2O_2-induced cytotoxicity, significantly increasing cell viability ($p<0.05$). These results were similar to those in our previous study, where we found that peonidin at the lowest concentration

of 1 μg/mL decreased H_2O_2-induced DNA damage in HT-29 cells ($p<0.05$) (Patterson, 2008). A similar protective effect of delphinidin was observed by Afaq et al. (2007) who found that pretreating human immortalized HaCaT keratinocytes with 1-20 μmol/mL delphinidin significantly ($p<0.05$) decreased UVB induced oxidative stress and cytotoxicity. Unlike our findings, Afaq et al. found the greatest protective effect at 10 μmol/mL concentration. This may be because HT-29 cells are more sensitive to antioxidant properties of delphinidin. We did not find a protective effect at 25 μmol/L. Shih et al. (2007), however, found that pre-treating rat liver Clone 9 cells with 50 μmol/mL delphinidin significantly increased ($p<0.05$) cell viability compared to a H_2O_2 alone group. It may be again that the concentration effects are dependent on cell type.

In this study delphinidin exhibited a protective effect against H_2O_2-induced decrease in cell viability at lower concentrations of 0.306- 3.06 μg/mL. In comparison, anthocyanin fractions from black soybean seed coat (Tsoyi et al., 2008) and purple sweet potato (Ye et al., 2010) did not show a protective effect at these concentrations against induced cytotoxicity and apoptosis in HaCaT keratinocytes and PC12 pheochromocytoma cells. In these studies a protective effect was observed at 10-100 μg/mL and 5-20 μg/mL from black soybean seed coat and purple sweet potato, respectively. Blackberry anthocyanin extracts, however, did have a protective effect at 1.6 μg/mL but not at 0.8 μg/mL in colon Caco-2 cells (Elisa et al., 2008). A possible explanation for these differences, besides cell type differences, is that these fractions have a mixture of anthocyanins, some of which may be less bioactive or have synergistic effects. Also, most of anthocyanins found in the natural form are glycosylated which may decrease their antioxidant properties depending on the type of sugar that is attached (Kähköhen and Heinonen, 2003).

To determine if the increase in cell viability was associated with an inhibition of apoptosis, we looked at caspase-3 activity in anthocyanin treated cells following H_2O_2 exposure. The caspase-3 enzyme is responsible for activating DNA cleaving enzymes in both intrinsic and extrinsic apoptosis pathways and is often used to affirm apoptosis. We observed a biphasic effect of delphinidin on caspase-3 activity even at a relatively narrow concentration range of 1-25 µmol/mL. We found that lower concentrations of delphinidin, 1-5 µmol/mL (0.306-1.53 µg/mL) significantly decreased H_2O_2 induced caspase-3 activity while 25 µmol/mL (7.66 µg/mL) significantly increased activity of this enzyme in H_2O_2 treated cells. These results were similar to those from our previous study, in which we found that anthocyanidin peonidin at 1 µg/mL decreased ($p<0.05$) H_2O_2-induced DNA damage while significantly increasing H_2O_2-induced apoptosis at 10 µg/mL (Patterson, 2008). Wätjen et al. (2005) found similar biphasic effects of the flavonoids quercetin and fisetin in a H4IIE rat hepatoma cell model. Quercetin and fisetin decreased cell viability to 50% compared to control at concentrations of 35 ± 4 µmol/mL and 48 ± 3 µmol/mL respectively. At 25 µmol/mL, both of these flavonoids reduced H_2O_2- induced caspase-3 activity by about 50%.

Not all studies on anthocyanin fractions support a biphasic pattern of effect on cell viability and apoptosis. Similar to our findings, Ye et al. (2005) also found that higher concentrations of 50-200 µg/mL purple sweet potato anthocyanins increased ($p<0.05$) cytotoxicity in PC12 pheochromocytoma cells while exhibiting protective effects at lower concentrations. In contrast, blackberry anthocyanin fractions increased cell viability at concentrations of 1.6 to 100 µg/mL in Caco-2 cells (Elisa et al., 2008). Similarly, black soybean seed coat anthocyanins inhibited apoptosis at 10 - 100 µg/mL in HaCaT keratinocytes, with the greatest inhibitory effect observed at 100 µg/mL (Tsoyi et al., 2008). Previous studies in our lab,

however, showed that 50-150 µg/mL of anthocyanin fractions from frozen blueberries induced apoptosis in HT-29 cells (Srivastava et al., 2007). These studies suggest that mixtures of anthocyanins and possibly other polyphenols in these fractions may have wide ranges of protective concentrations and that there may be an overlap in protective and cytotoxic concentration ranges depending on the source of anthocyanins.

Some have examined the effects of anthocyanins on inhibition of apoptosis in *vivo*. Both Afaq et al. (2007) and Tsoyi et al. (2008) found that topical application of delphinidin or black soybean seed coat anthocyanins inhibited UVB radiation induced apoptosis in skin tissue of SKH-1 and HR-1 hairless mice. These findings show that the protective effect of anthocyanins found in *vitro* models is also seen in *vivo*. Although this protective effect in healthy cells against UVB radiation is beneficial, inhibition of apoptosis in cancer cells can have detrimental results. Our findings suggest that a lower concentration of delphinidin can inhibit induced apoptosis, allowing cancer cells to continue to grow and proliferate.

It is estimated that typical American diet contains about 12.5 mg of anthocyanins (Wu at al., 2006). Bioavailability of anthocyanins is even lower than other flavonoids, suggesting that anthocyanins remain in the gut and may have an important chemoprotective function in the gastrointestinal tract. In the gut, however, anthocyanins are quickly metabolized by the microflora, making it hard to predict the concentrations found in colon (McGhie and Walton, 2007). It is possible that the concentrations of 0.31 –7.66 µg/mL used in this study and the protective concentrations of anthocyanin fractions of up to 100 µg/mL observed in other studies may be achieved in the gut through diet. Additionally, it is difficult to predict the concentration of anthocyanins that would reach the colon in an unmetabolized form when administered at pro-

apoptotic concentrations. Therefore there is a need for additional studies to determine the effect of anthocyanins on apoptosis in *vivo* before they can be used as chemopreventive agents.

There are a number of limitations in working with isolated anthocyanidins and cell culture models. In nature, anthocyanins are found in glycosylated form. Depending on the type of sugar attached, glycosylation can alter antioxidant properties of anthocyanins (Kähköhen and Heinonen, 2003). Bioactive properties of aglycones have been shown to be dependent on oxidative stress while glycosides may have bioactive functions independent of oxidative stress (Renis et al., 2008). Also, there may be other regulatory mechanisms in *vivo* that absent in cell culture models. Cell culture models are also exposed to higher concentrations of oxygen compared to in *vivo* environment. This excessive exposure to oxygen increased production of ROS leading to higher levels of oxidative stress than would be found in vivo (Halliwell, 2007). This increase in oxidative stress makes it hard to accurately study the concentrations of antioxidants or pro-oxidants needed to exhibit similar response in *vivo*.

Our findings showed that pretreating cells with 1-10 μmol/L delphinidin significantly increased cell viability of H_2O_2 –stressed cells. Also, 1-5 μmol/L delphinidin significantly decreased caspase-3 activity compared in H_2O_2-treated cells. These results suggest that at lower concentrations delphinidin may have had an antioxidant effect and protected colon HT-29 cells from apoptosis induced by oxidative stress. The ability of delphinidin to inhibit apoptosis in the cancer cell may have detrimental consequences in cancer outcomes. It may be, however, that this protective effect is only affective against apoptosis induced via an intrinsic pathway which is usually associated with increased oxidative stress. Also, the ability of antioxidants to inhibit or facilitate apoptosis may depend on the levels of oxidative stress in cells. Apoptosis may be inhibited by both inadequate and excessive levels of oxidative stress (Halliwell, 2007). Thus, in

cases where apoptosis is inhibited due to excessive oxidative stress, the antioxidant functions of anthocyanins may help to promote apoptosis. More studies are needed to determine the ultimate effect of different concentration of delphinidin on different types of tumors.

TABLE 3. 1: Delphinidin[1] Concentration Conversions.

µmol/L	µg/mL
1	0.306
5	1.53
10	3.06
25	7.66

[1] Molecular weight of delphinidin is 306.27 g/mol.

TABLE 3.2: Cell Viability of Hydrogen Peroxide Treated HT-29 Human Adenocarcinoma Cells Pretreated with Delphinidin[1,5].

Treatment	% Viability
Control[2]	100 ± 0.00^{a}
H_2O_2[3]	35.40 ± 1.84^{d}
1 µmol/L[4]	48.73 ± 2.82^{b}
5 µmol/L	$44.72 \pm 2.22^{b,c}$
10 µmol/L	$42.87 \pm 2.10^{b,c}$
25 µmol/L	$40.78 \pm 1.92^{c,d}$

[1]Mean ± SEM (n=8/group). Expressed as percent viability compared to control group.

[2] Control was not pretreated with delphinidin or H_2O_2.

[3] H_2O_2 group was treated with 100 µmol H_2O_2 without pretreatment with delphinidin.

[4] Remaining groups were pretreated with delphinidin and then treated with H_2O_2.

[5] Treatment means with different letters are significantly different ($p<0.05$).

TABLE 3.3: Caspase-3 Activity in Hydrogen Peroxide Treated HT-29 Human Adenocarcinoma Cells Pretreated with Delphinidin[1].

Treatment	Fold Increase[2]
Control[3]	1.00 ± 0^{e}
H_2O_2[4]	6.81 ± 0.23^{b}
1 μmol/L[5]	2.85 ± 0.08^{d}
5 μmol/L	4.85 ± 0.13^{c}
10 μmol/L	7.35 ± 0.20^{b}
25 μmol/L	8.12 ± 0.17^{a}

[1] Mean ± SEM (n=3/group) . Results with different letters are significantly different ($p<0.05$).

[2] Fold increase compared to the control was calculated as (treatment-background control)/(control- background control).

[3] Control was not pretreated with delphinidin or treated with H_2O_2.

[4] H_2O_2 group was treated with 100 μmol H_2O_2 without pretreatment with delphinidin.

[5] Remaining groups were pretreated with delphinidin and then treated with H_2O_2.

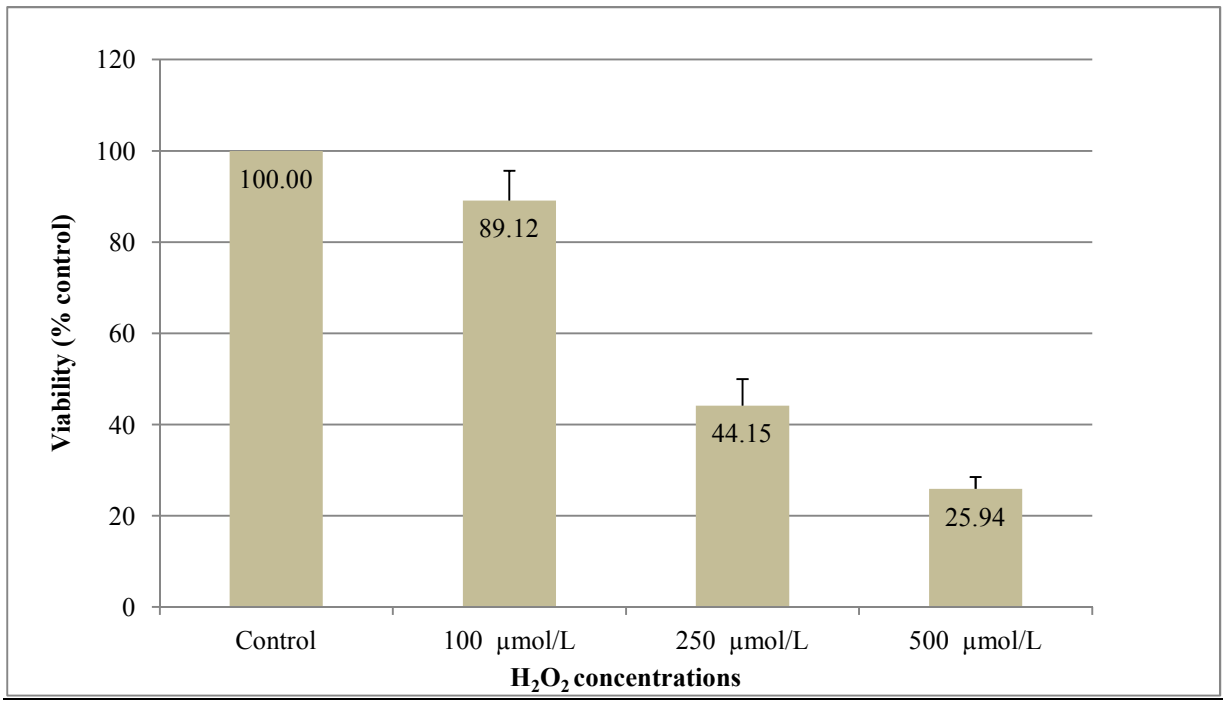

FIGURE 3.1: Cell Viability of HT-29 Cells Treated with Different Concentrations of H_2O_2.

Values are mean ± SEM (n=8/group).

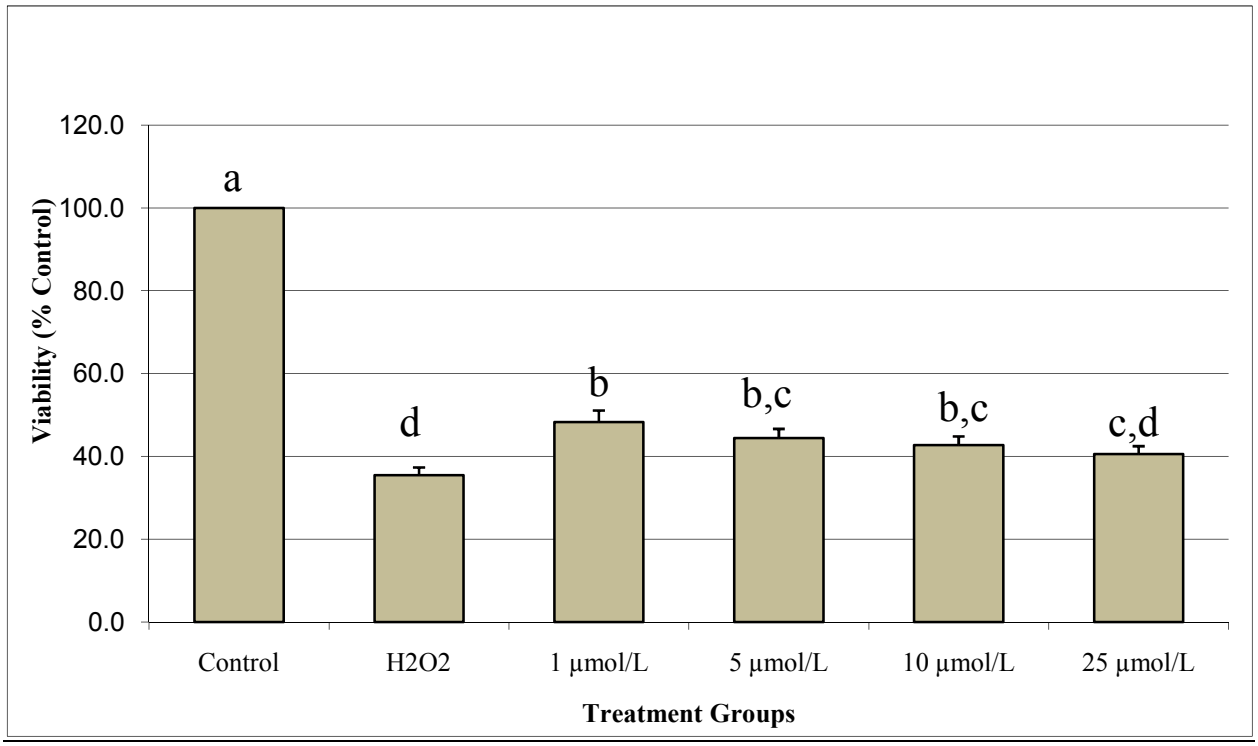

FIGURE 3.2: Cell Viability of Hydrogen Peroxide Treated HT-29 Human Adenocarcinoma Cells Pretreated with Delphinidin[1,2,3,4,5].

[1] Mean ± SEM (n=8/group). Expressed as percent viability compared to control group.
[2] Control was not pretreated with delphinidin or H_2O_2.
[3] H_2O_2 group was treated with 100 µmol H_2O_2 without pretreatment with delphinidin.
[4] Remaining groups were pretreated with delphinidin and then treated with H_2O_2
[5]Treatment means with different letters are significantly different ($p<0.05$).

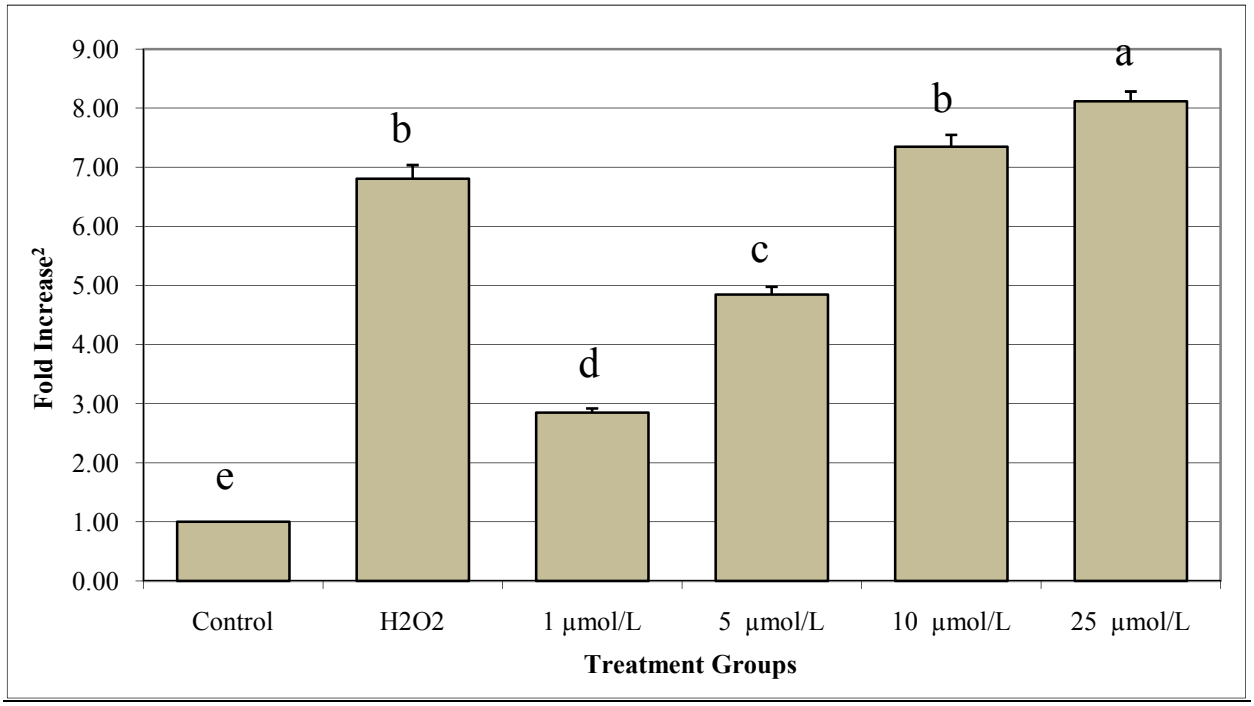

FIGURE 3.3: Caspase-3 Activity in Hydrogen Peroxide Treated HT-29 Human Adenocarcinoma Cells Pretreated with Delphinidin[1,3,4,5].

[1] Mean ± SEM (n= 3/group). Results with different letters are significantly different ($p<0.05$).
[2] Fold increase compared to the control was calculated as (treatment-background control)/(control- background control).
[3] Control was not pretreated with delphinidin or treated with H_2O_2.
[4] H_2O_2 group was treated with 100 µmol H_2O_2 without pre-incubation with delphinidin.
[5] Remaining groups were pretreated with delphinidin and then treated with H_2O_2.

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CHAPTER IV

SUMMARY AND CONCLUSIONS

Summary

Polyphenols, non-nutritive compounds in plants, have bioactive functions which may help to interfere in various stages of cancer development (Ramos et al., 2008). One particular role which has been getting much attention is the ability to induce apoptosis, or programmed cell death, in cancer cells (Khan et al., 2008). Flavonoid anthocyanins have been shown to be potent inducers of apoptosis (Srivastava et al., 2007; Hafeez et al.,2008; Hou et al.,2005; Lazzé et al., 2005; Yeh et al., 2005; Yun et al., 2009). Some studies, however, showed that other flavonoids have biphasic effect on apoptosis in cancer cell models depending on concentration of the flavonoids (Wätjen et al., 2005). Others have also found that lower concentrations of delphinidin inhibited apoptosis induced to radiation or oxidizing agents (Afaq et al., 2007; Elisa et al., 2008; Tsoyi et al., Ye et al., 2010). Therefore it is important to determine the effect of low concentrations of delphinidin on induced apoptosis in cancer cells.

The objective of our study was to determine if low concentrations (1-25 μmol/L) of delphinidin would decrease hydrogen peroxide (H_2O_2) induced cytotoxicity and apoptosis in human colon cancer HT-29 cells. Our results showed that pretreating cells with 1-10 μmol/L delphinidin significantly ($p<0.05$) increased cell viability of H_2O_2 –stressed cells. Also, 1-5 μmol/L delphinidin significantly decreased ($p<0.05$) caspase-3 activity compared in H_2O_2-treated cells. This suggests that the increase in cell viability was most likely due to a decrease in

H_2O_2- induced apoptosis. At 25 µmol/L, delphinidin significantly increased ($p<0.05$) H_2O_2 - induced apoptosis, suggesting that it acts as antioxidant at lower concentrations and as pro-oxidant at slightly higher concentrations. The ability of delphinidin to inhibit apoptosis in the cancer cell at lower concentrations may have detrimental consequences in cancer outcomes and therefore more studies are needed before anthocyanin can be used as a chemotherapeutic agent.

Limitations

There are limitations in working with cell culture models and individual anthocyanidins. The cell culture process, in itself, increases production of ROS due to exposure to much higher oxygen concentrations compared to the concentrations found in the body. Cells grown in this pro-oxidant environment may adapt or evolve to use ROS for signaling pathways not normally used *in vivo* (Halliwell, 2007). Therefore cells in culture may have different sensitivities to antioxidant and pro-oxidant agents and caution should be used when interpreting results at various concentrations. Also, organisms may also have other mechanisms of maintaining homeostasis which may be absent in cell models.

In nature, anthocyanins are primarily found in a glycosylated form instead of as anthocyanidins. Depending on the type of sugar attached, glycosylation can alter antioxidant properties of anthocyanins (Kähköhen and Heinonen, 2003). Some studies have also shown that bioactive functions of aglycones may be more ROS dependent than those of glycosides (Renis et al., 2008). So glycosylated anthocyanins may be less likely to act as antioxidants and protect cells from oxidant-induced apoptosis. Also, the main sources of dietary anthocyanins are fruits. Fruit is also a rich source of antioxidant vitamin C and other polyphenols. A combination of these compounds may have an additive, synergistic, or preventive effect on the bioactive

functions of anthocyanins. Thus, the results of our study may not be the same as results obtained from fruit extracts.

Future Studies

Our findings showed that low levels of delphinidin exhibit antioxidant properties in cancer cells, protecting them from apoptosis induced via increased oxidative stress. However, more research is needed to determine the ultimate effect of these compounds on cancerous tumors in *vivo* models. Future studies should look into the effect of low levels of delphinidin on extrinsic, or receptor mediated, apoptosis pathway, at the effect of glycosylated form of delphinidin on induced apoptosis and the ultimate effect of low levels of delphinidin on tumor progression in *vivo* models.

Our model of induced apoptosis involved intrinsic apoptosis pathway where apoptosis was induced via increase in oxidative stress. Delphinidin is a potent antioxidant and most likely inhibited apoptosis by decreasing oxidative stress. Extrinsic, or receptor mediated, apoptosis does not involve oxidative stress, and therefore delphinidin may not have the same effect on apoptosis induced via extrinsic pathway. Therefore future studies should look into the effect of low levels of delphinidin on apoptosis induced via extrinsic pathway.

Most of anthocyanins obtained from diet are in glycosylated form. Glycosylated anthocyanins may not be as potent antioxidants as aglycones. In order to determine the effect of dietary anthocyanins on induced apoptosis more studies that look at the effect of glycosylated anthocyanins are needed.

Finally, in order to determine the ultimate effect of low levels of delphinidin on progression of cancerous tumors, studies using in vivo models are needed. We found that lower levels delphinidin acted as an antioxidant and inhibited apoptosis by decreasing oxidative stress.

Oxidative stress plays different functions in cancer development process and antioxidants have the potential to modulate these processes. In order to determine the ultimate effect of antioxidants on tumors in vivo studies are needed.

Application

Our findings suggest that at lower concentrations delphinidin acts as an antioxidant and can inhibit apoptosis induced by increased oxidative stress. Our findings, however, are too limited to base nutritional recommendations on. More in vivo studies are needed.

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APPENDIX A

DELPHINIDIN DILUTIONS

Notes:

- Measure out 0.306 g of delphinidin chloride in a small weighing boat.
- Dissolve delphinidin in 0.1 mL of DMSO before adding 0.9 mL of media.
- Starting dilution is 10 mmol/L.
- Add DMSO to media to get concentration 0.1% DMSO.

	1 µmol	**5 µmol**	**10 µmol**	**25 µmol**
Flasks	15 ml	15 ml	15 ml	15 ml
Well plates	4 ml	4 ml	4 ml	4 ml
Total:	19 ml	19 ml	19 ml	19 ml

Delphinidin
Molar mass: 306.27g

1 M 306.27 mg/ ml
10 mM 3.0627 mg/ ml

Number of treatments: 3
Total amount of delphinidin: **9.1881 mg**

Serial Dilutions

1 mM: 0.1 ml 10 mM and 0.9 ml medium = 1 ml
100 µmol: 1 ml 1 mM and 9ml medium = 10 ml

Calculations:

25 µmol:

dilution factor: $\frac{100\ \mu mol/L}{25\ \mu mol/L} = 4$

Final volume: 19 ml

$\frac{19\ ml}{4}$ **= 4.75 ml** plus 14.25 ml medium

10 µmol:

dilution factor: $\frac{100\ \mu mol/L}{10\ \mu mol/L} = 10$

Final volume: 19 ml

$$\frac{19 \text{ ml}}{10} = \textbf{1.9 ml}$$ plus 17.1 ml medium

5 µmol:

dilution factor: $$\frac{\frac{100 \text{ µmol/L}}{5 \text{ µmol/L}}}{} = 20$$

Final volume: 19 ml

$$\frac{19 \text{ ml}}{20} = \textbf{0.95 ml}$$ plus 18.05 ml medium

1 µmol:

dilution factor: $$\frac{\frac{100 \text{ µmol/L}}{1 \text{ µmol/L}}}{} = 100$$

Final volume: 19 ml

$$\frac{19 \text{ ml}}{100} = \textbf{0.19 ml}$$ plus 18.81 ml medium

Total volume of 100 µM needed: 7.79 ml

APPENDIX B

MTT CELL VIABILITY ASSAY

Seeding Wells
1.) Cells were harvested using trypsinozation.
2.) Cells were counted and diluted to 1 x 10 ^5/ mL with total volume of 6 mL.
3.) In a 96-well microplate (MT), 100 uL of media only was pipetted into the first column of wells, total of 8 wells.
4.) 100 uL of the diluted cell solution was then pipetted into the following 6 columns of well (total of 36 wells)
5.) Seeded MT was incubated for 24 hours.

Treatments
1.) First two columns were treated with 100 µL of 0.1% DMSO media and the following four columns with 1, 5, 10, and 25 µmol/L delphinidin for 4 hours.
2.) Treatment media was removed and last 5 columns were treated with 100 µL of 100 µmol/L H_2O_2 for 2 hours, first column had fresh media.
3.) Treatment media was removed and fresh media was added to all the columns.

MTT Assay
1.) After 36 hour incubation, 10 uL of MTT Reagent was added to each well, including the ones containing only media.
2.) MT was returned to the incubator and incubated for another 2 hours, or until presence of intracellular purple precipitate can be observed when looked at under the microscope (up to 4 hours).
3.) Add 100 uL of Detergent Reagent to all the wells and gently swirl.
4.) Cover the MT with foil to keep it dark and leave it at room temperature over night.
5.) Measure absorbance at 570 nm using the microplate reader in the Food Science Lab.
6.) Averages for each treatment were determined and the blank value was subtracted from them. Values were expressed as percentage compared to the control.

APPENDIX C

CELL DEATH DETECTION ELISA ASSAY

Harvesting Cells
1.) Media was removed from the six wells and pipetted into corresponding labeled 5 ml tubes. (NOTE: Do not discard the old media)
2.) Wells were rinsed with 0.3 ml of Trypsin-EDTA (0.25%) and incubated for 6-8 minutes with 0.38 ml of fresh Trypsin-EDTA.
3.) The reserved old media was then added back to the corresponding well.
4.) Detached cells and media from the well was pipetted back into labeled 5 ml tubes.
5.) 5 ml tubes with cells and media were centrifuged for five minutes at 100 x g and supernatant was discarded.
6.) Cell pallet was re-suspended in 1 ml phosphate buffered saline (PBS).

Preparing Culture Supernatant
1.) Count cells in each treatment group.
2.) Cells were diluted with PBS to obtain 1 ml of 1 x 10^5 concentration.
3.) Eppendorf tubes with 1 ml of 1 x 10^5 cells were centrifuged for ten minutes at 200 x g (2700 rpm) in the Eppendorf centrifuge.
4.) Carefully remove supernatant and re-suspend in 200 uL of lysis buffer (bottle 5) provided in the kit.
5.) Incubate for 30 minutes at room temperature (15-25 C) (NOTE: while waiting prepare the Immunoreagent and if using new kit, the ELISA solutions provided in the Kit).
6.) Cells were then centrifuged again for ten minutes at 200 x g in the Eppendorf centrifuge.

Preparing the Working Solutions
1.) Prepare the following solutions by reconstituting them with distilled water.

Preparing the Immunoreagent
1.) Immunoreagent was prepared by combining the following solutions in this ratio: 1/20 Anti-DNA-POD (bottle 2, reconstituted), 1/20 Anti-histone-biotin (bottle 1, reconstituted) and 18/20 Incubation Buffer (bottle 4). Final amount needed depends on the number of samples tested.

ELISA Assay
1.) Transfer 20 uL of supernatant from the lysed, spinned down cells carefully into the streptavidin coated microplate provided in the kit. **Be careful not to disturb the pallet or the bottom of the microplate well.** (Run three samples for each treatment group). Also, transfer 20 uL from positive control (bottle 3) and background control (incubation buffer, bottle 4) into additional wells in the microplate.
2.) Add 80 uL of prepared Immunoreagent to each well. Cover with adhesive cover foil provided in the kit and incubate on shaker (250 rpm) at room temperature for 2 hours.
3.) After 1.5 hours, remove ABTS from fridge to it can warm to room temperature.

4.) When 2 hour incubation period is complete, gently remove the solution using a pipettor. **Be careful not to touch the bottom of the well.** Rinse each well 3 times with 250 uL Incubation buffer, carefully removing the solution each time.
5.) Pipette 100 mL ABTS solution into each well and incubate on the shaker for 5 minutes, or until the green color in the positive control well develops (should still be transparent).
6.) Add 100 mL ABTS to each well.
7.) Measure at 405 nm against ABST solution and 100 uL stop solution as a blank (reference wavelength 490 nm).

APPENDIX D

CASPASE-3 ACTIVITY ASSAY

Seeding of Cells
1.) Cells were harvested via trypsinization and counted.
2.) Cells were seeded in 75 cm^2 flasks at concentration of 1 x 10^5 cells/ mL, with total volume of 15 mL in each flask. Total of 11 flasks were used: one for control group and two for every other treatment.
3.) Flasks were incubated for 24 hours before treatment.

Treatment
1.) Three flasks were treated with 15 mL of 0.1% DMSO media and the following eight flasks with 1, 5, 10, and 25 μmol/L delphinidin for 4 hours.
2.) Treatment media was removed and all flasks except for one of the flasks treated with 0.1% media (control) were treated with 15 mL of 100 μmol/L H_2O_2 for 2 hours. Control flask was incubated with fresh media.
3.) Treatment media was removed and 15 mL of fresh media was added to all the flasks.

Cell Harvesting
1.) Old media was reserved and pipetted into labeled 50 mL Centrifuge tubes. (Two flasks for the same treatment were combined into one tube. Control group consisted of only one flask)
2.) Cells were detached using trypsinization, old media was added back into the flasks to deactivate trypsin, and cells and media were pipetted back into the 50 mL tubes.
3.) Cells in the tubes were centrifuged for minutes at 1000 rpm, in Dr. Grider's lab.
4.) Old media was removed, carefully without disturbing the pallet, and cells were re suspended in 2 mL of PBS for control and 1 mL of PBS for all other treatments.

Caspase-3 Assay
(NOTE: will need a tray with ice)
1.) Cell counts were done and cells were diluted to 1 mL of 1.5 x 10^6 cells/ mL in 1.5 mL Eppendorf tubes.
2.) Cells were centrifuged in eppendorf centrifuge for 10 at 1500 rpm. (NOTE: while waiting defrost 5X Cell Lysis Buffer provided in the kit and dilute it to 1X. Keep it chilled in the ice tray.)
3.) Remove the supernatant and resuspend cells in 300 uL of chilled 1X Cell Lysis Buffer and incubate cells on ice for 10 minutes. (NOTE: while waiting defrost Caspase-3 substrate and pNA Standard and prepare dilution series of pNA).
4.) Centrifuge for another 5 minutes at 10,000 x g (11,000 rpm). Place on ice.
5.) The volumes of solutions given in the kit instructions were used.