IMPACT OF ANTHOCYANINS AND DIETARY CARBOHYDRATES ON INTESTINAL TUMORIGENESIS IN *APC*$^{MIN/+}$ MICE

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ABSTRACT

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Consumption of anthocyanins reduces the development of intestinal tumors in *Apc*$^{Min/+}$ mice, whereas diets rich in sucrose increase intestinal tumor development. The aim of this study was to determine the efficacy of anthocyanins to reduce intestinal tumorigenesis in *Apc*$^{Min/+}$ mice consuming diets rich in sucrose and other dietary carbohydrates. Mice were assigned to six different modified AIN-93G diets, which contained different carbohydrate sources (starch, sucrose, or glucose) and different doses of anthocyanins (0 or 1500 mg/kg) in a factorial design. To enhance tumor development, mice were injected for three consecutive weeks with azoxymethane (8 mg/kg) prior to dietary intervention. Mice consuming sucrose showed the largest colonic adenomas compared to mice consuming anthocyanin-sucrose, anthocyanin-glucose, glucose, and starch ($P<0.05$). These results demonstrate that anthocyanins and different carbohydrates influence intestinal adenoma development in *Apc*$^{Min/+}$ mice; however, additional research is required to determine the mechanisms for the observed effects.

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INTRODUCTION

Approximately one out of two American men and women will develop cancer during their lifetime. The risk of developing colorectal cancer, the second leading cause of cancer related deaths, is approximately five percent (National Cancer Institute, 2013). Nearly one-third of all cancer related deaths have been attributed to inappropriate diet and lack of physical activity (American Cancer Society, 2013). However, colorectal cancer mortality rates have declined over the previous two decades, and this decline has been linked to improved cancer screening and earlier detection methods.

The American Cancer Society recommends limited consumption of red meat, processed meats, calorie-dense foods, refined carbohydrates, and sugar-sweetened beverages; and emphasizes increased consumption of fruits, vegetables, and whole grains. Epidemiological data support these recommendations. For example, people who consumed diets higher in red meat, processed meat, refined grains, and sugar-sweetened beverages had a higher risk of developing certain cancers (American Cancer Society, 2012). In contrast, people who regularly consumed more fruits and

vegetables had a lower risk of developing cancer (Miller et al., 2010).

The dietary components of calorie-dense diets most commonly associated with increased colorectal cancer risk are sucrose and refined carbohydrates. Both have been positively correlated to increased risk in several epidemiological studies (Franceschi et al., 2001; Bostick et al., 1994; Slattery et al., 1997).

Animal experiments also have demonstrated a relationship between sucrose consumption and colorectal tumorigenesis (Stamp et al., 1993; Caderni et al., 1993; Caderni et al., 1997; Poulsen et al, 2001; Kristiansen et al., 1995; Kristiansen et al., 1996; Wang, 2005; Wang et al., 2009).

On the other hand, fruits and vegetables contain a wide variety of phenolic compounds, which possess the potential to act as chemopreventative agents due to their strong antioxidant and anti-inflammatory activities (Blando et al., 2004). For example, tart cherries are rich in phenolic compounds called anthocyanins. Other fruits rich in anthocyanins are blueberries, strawberries, elderberries, and cranberries.

Evidence from cell culture, animal and human studies have demonstrated anthocyanins are absorbed and act as

antioxidants and anti-inflammatory agents *in vitro* and *in vivo*. However, the potential for anthocyanins to inhibit cancer in animals fed diets rich in refined carbohydrates has not yet been determined.

This study was designed to determine the influence of anthocyanins on intestinal adenoma development associated with refined sugar consumption in $Apc^{Min/+}$ mice, a model for human colorectal cancer.

REVIEW OF THE LITERATURE

1. Incidence of Colorectal Cancer

Colorectal cancer is the third most commonly diagnosed cancer, and the second highest cause of cancer mortality in both men and women in the United States (American Cancer Society, 2013). In 2012, the National Cancer Institute estimated 143,460 new incidences per year in the U.S., of which approximately 51,690 will be fatal. The lifetime risk for developing colorectal cancer for American males and females is 4.96 percent (National Cancer Institute, 2013). Risk also increases with age, as 90 percent of all colorectal cancer cases occur in people 50 years of age and older. In addition to age, other risk factors include: family history, genetic conditions, lack of physical activity, alcohol consumption, and tobacco use (Centers for Disease Control and Prevention, 2013). Specific dietary components also have been negatively and positively correlated to risk. Diets high in protein and fat (Potter et al., 1993), and refined carbohydrates (Franceschi et al., 2001; Bostick et al., 1994; Slattery et al., 1997) have been linked to increased colon cancer risk, whereas diets that regularly incorporate vegetables and fruit

reduce cancer risk (Terry et al., 1998; Terry et al., 2001; Miller et al., 2010).

2. Genetic and Molecular Basis of Colon Cancer

Inherited genetic defects have been useful in elucidating molecular mechanisms of carcinogenesis, and at least 15 percent of colon cancers can be linked to inherited genetic diseases. The two primary genetic diseases associated with colorectal cancer are familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). Familial adenomatous polyposis is a dominantly inherited disease affecting approximately 1 in 10,000 people (Jasperson et al., 2010) and accounts for one to three percent of colon cancer incidences (Burt, 2000). Patients with FAP develop hundreds to thousands of adenomas during their 20's and 30's. Although the adenomas are usually benign, the large number of adenomas leads to a higher risk that some will progress into carcinomas (Kinzler and Vogelstein, 1996).

Familial adenomatous polyposis is the result of a mutation in the adenomatous polyposis coli (*Apc*) gene. The *Apc* gene codes for a protein that is important for the Wnt signaling pathway, intercellular adhesion and apoptosis (Gryfe et al., 1997; Fearnhead et al., 2001). Adenomas can

develop when the normal *Apc* allele is lost or mutated. Patients with FAP only have one normal *Apc* allele, and a mutation can result in *Apc* gene inactivation (Burt, 2000). Additionally, mutations or deletions in both copies of *Apc* are commonly present in approximately 80 percent of sporadic colorectal tumors (Markowitz and Bertagnolli, 2009; Fearnhead et al., 2001).

Carcinogenesis is a multistep process that involves genetic or epigenetic alterations of multiple genes, and colorectal cancers commonly have three or more genetic alterations (Barrett, 1993; Calabrese et al., 2005). The process of carcinogenesis involves initiation, promotion, and progression (Pitot et al., 1981). Initiation is the irreversible alteration of a cell and is commonly associated with genetic mutations. Promotion is the clonal expansion of mutated cells into visible benign tumors or preneoplastic cells. Progression is the transition phase when benign tumors or preneoplastic cells become malignant cancers (Barrett, 1993). However, carcinogenesis is not a linear mechanism involving a straightforward series of genetic alterations. Rather, it is a result of multiple alterations that affect tumor suppressor genes, proto-oncogenes, and DNA repair genes (Gryfe et al., 1997). Genes commonly mutated in genetic or sporadic colon cancers are

Apc, Kristen rat sarcoma (*Kras*), deleted in colorectal carcinoma (*Dcc*), and the tumor protein p53 (TP53) (Vogelstein et al., 1988).

The *Apc* gene is a tumor suppressor gene located on the 5q12 chromosome and codes for a protein that is important for the Wnt signaling pathway, intercellular adhesion and apoptosis (Gryfe et al., 1997; Fearnhead et al., 2001). Mutations in the *Apc* gene can result in the loss of the APC protein in the Wnt signaling pathway, resulting in the accumulation of β-catenin in the cytoplasm. Liberated β-catenin translocates to the nucleus and activates genes involved in cellular growth and proliferation such as cyclin D1 and c-myc (Kwong and Dove, 2009).

The *Kras* gene is a proto-oncogene, which encodes a GTPase protein involved in proliferation, apoptosis, cell-cell adhesion, and cell motility. Cells with a mutated *Kras* gene have characteristics such as poor cellular adhesion, increased motility, increased growth, and invasiveness, which are components of tumorigenesis (Pollock et al., 2005).

The *Dcc* gene is located on chromosome 18q and approximately 60 to 80 percent of all colorectal cancers have been shown to lose this gene. It encodes a membrane bound protein, which functions as a tumor suppressor by

signaling cell death via a caspase-dependent cell death pathway (Mehlen and Fearon, 2004). Absence of *Dcc* fosters an environment for enhanced cell survival.

Loss of the TP53 gene on chromosome 17p occurs in approximately 70 percent of colorectal cancers. This gene encodes a nuclear phosoprotein (p53) involved in activating DNA transcription and DNA repair. Proteins produced by p53 activation are involved in cell cycle arrest and include the p21 protein. The ability to arrest the cell cycle and repair DNA damage is lost with mutations to the TP53 gene, and can lead to cells with enhanced proliferation and the ability to escape programed cell death (Gryfe et al., 1997).

Enhanced proliferation, growth motility, invasiveness, survival and immortality are biological capabilities acquired throughout the multistep development of colorectal cancer and are considered to be "hallmarks of cancer" (Hanahan and Weinberg, 2011). The foundation of these hallmarks is genetic instability, which fosters an environment for increased accumulation of genetic mutations. An additional hallmark, inflammation, has been proposed by Colotta et al. (2009) as a key modulator of genetic instability.

3. Inflammatory Mechanism of Colon Cancer

For more than a two hundred years scientists have suspected a link between cancer and inflammation. In the mid-1800's, Rudolf Virchow hypothesized cancers originated at sites of chronic inflammation. Virchow believed that irritants, tissue injury, and inflammation enhanced cell proliferation (Coussens and Werb, 2002). In itself, enhanced cell proliferation does not cause cancer; however, cells subjected to chronic inflammatory microenvironments have been shown to acquire genetic mutations that can lead to unregulated proliferation, survival, angiogenesis, metastasis, and reduced response to therapeutic agents (Colotta et al., 2009).

Inflammation is linked to the multistep carcinogenesis process by acting either as an initiator or promoter of tumors. As an initiator, an inflammatory microenvironment can increase the rate of genetic mutations. Inflammatory cells can also induce DNA damage by producing reactive oxygen species (ROS) or cytokines, such as tumor necrosis factor-α (TNF-α), which stimulate ROS production (Grivennikov et al., 2010).

As a promoter, inflammation activates transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which stimulates initiated

cells to turn on genes that stimulate cell proliferation, invasiveness, angiogenesis, and survival (Grivennikov et al, 2010; Sandur et al., 2009).

A selection of key inflammation regulators in colon cancer include proinflammatory cytokines such as TNF-α and interleukin-6 (IL-6), and the nuclear transcription factor NF-κB. Cyclooxygenase-2 (COX-2), an enzyme responsible for prostaglandin production, is also a key component in inflammatory responses and a target for colorectal cancer treatment.

NF-κB is activated by infectious agents, cytokines, and danger signals secreted by necrotic cells (Karin, 2006). Cell-proliferating genes regulated by NFκB include: TNF-α, IL-6, interleukin-1 (IL-1), and cyclin D1 (Sethi et al., 2008). Activation of NF-κB also promotes cell survival through the activation of genes that negatively regulate apoptosis. A selection of these genes includes B cell lymphoma-2 (*Bcl-2*) and B cell lymphoma extra large (*Bcl-xL*). These proteins suppress the release of cytochrome C from the mitochondria. Cell invasiveness is mediated through NF-κB by the activation of matrix metalloproteinases (MMP), which are also proangiogenic (Sethi et al., 2008). In general, the tumor promoting effect of NF-κB has classically been described to be activated by viruses,

bacterial infections, and proinflammatory cytokines. These agents activate the IκB kinase (IKK) complex, which is composed of three subunits: IκB kinase-α (IKK-α), IκB kinase-β (IKK-β), and IκB kinase-γ (IKK-γ). The activated IKK complex phosphorylates NF-κB inhibitory subunits called IκBs, which liberates NF-κB to translocate to the nucleus for gene transcription (Karen and Greten, 2005).

Tumor necrosis factor-α is a cytokine and a regulator of host defense and inflammation. It is produced by monocytes, macrophages and T-cells to stimulate proliferation, differentiation and inflammation in many different types of cells. Tumor necrosis factor-α is also produced by tumor cells and can act as an autocrine or paracrine agent (Aggarwal et al., 2006). When TNF-α binds to its receptors, TNFR1 or TNFR2, inflammation and cell survival pathways are activated via NF-κB and caspase 8 in colonic epithelial cells (McConnell and Yang, 2009). NF-κB also activates genes for IL-6, inducible nitric oxide synthase, and aldose reductase (AR), which are involved in inflammatory mechanisms. Over expression of COX-2, mediated by TNF-α, is dependent on NF-κB (Tammali et al., 2007). In tumorigenesis, TNF-α is linked to unregulated

proliferation, survival, invasion, and angiogenesis in several types of epithelial cells (Aggarwal et al., 2006).

The COX-2 enzyme is a cytoplasmic protein that catalyzes the synthesis of prostaglandins from arachidonic acid (AA). In the initial enzymatic reaction, AA is converted to prostaglandin G2 by COX-2 or Cyclooxigenase-1 (COX-1), which is subsequently converted to prostaglandin H2, also by COX-2. Prostaglandin H2 is then converted to several structurally related prostaglandins by prostaglandin synthases. These include the eicosanoids: prostaglandin E2 (PGE2), prostaglandin D2, prostaglandin F2α, prostaglandin I2, and thromboxane A2. These eicosanoids then act through specific G-protein-coupled-receptors to maintain normal physiological processes, which include immune function, maintenance of gastrointestinal mucosa, blood clotting, and renal homeostasis. However, prostaglandins also promote inflammation, swelling, pain, and fever (Greenhough et al., 2009). Baseline levels of prostaglandins are maintained by COX-1 and have been shown not to be up regulated in colonic tumors, whereas the COX-2 enzyme is regulated in colorectal cancer (Sano et al., 1995).

Transcription of the COX-2 gene is activated by pro-inflammatory cytokines, which include IL-1 and TNF-α. Its

expression is increased at inflammatory sites, and over expressed in colorectal cancer in 80 percent of carcinomas and 40 percent of adenomas. COX-2 is believed to contribute to colorectal cancer promotion by inducing resistance to apoptosis, and stimulating angiogenesis and invasiveness (McConnell and Yang, 2009).

Cyclooxygenase enzymes were first implicated in colorectal cancer when patients with Gardner's syndrome were treated with Non-steroidal Anti-inflammatory Drugs (NSAIDs) and displayed reduced numbers of adenomas after treatment. This was also the first clinical study that suggested NSAIDs could be used to treat colorectal cancer (Waddell et al., 1989; Greenhough et al., 2009).

4. NSAIDs as a Colon Cancer Treatment

Data from epidemiological studies and laboratory experiments demonstrate NSAIDS may be protective against colorectal cancer. From 1982 to 1988, more than 662,000 adults participated in a prospective cohort study investigating the relationship between aspirin use and mortality. Colon cancer death rates decreased in both men and women who frequently consumed aspirin. The relative risk of death resulting from colon cancer with aspirin consumption at least 16 times/month was 0.60 (95% CI: 0.40-

0.89) and 0.58 (95%CI: 0.37-0.90) for men and women, respectively. The investigators concluded that regular aspirin use might reduce colon cancer via the inhibition of prostaglandins (Thun et al., 1991). In a second prospective mortality study of more than 635,000 people by the same research group, duration of aspirin use was also shown to reduce the relative risk of developing colon cancer. Men and women, adjusted for age, race, and sex, who consumed aspirin at least 16 times/month for 1-9 years had a relative risk of 0.71, whereas those who consumed it for 10 years or more with the same frequency had a significantly lower relative risk of 0.36 compared to all nonusers of aspirin (Thun et al., 1993).

In animal experiments, aspirin has been shown to reduce colonic tumor incidence, burden and expression of PGE2 in rats (Reddy et al., 1993). For example, the NSAIDs, sulindac and piroxicam, protect against colon tumor development in mice and rats (Moorghen et al., 1988; Skinner et al., 1991; Pollard and Luckert, 1989; Reddy et al., 1987).

5.Dietary Factors Associated with Colon Cancer

The western diet, a diet with high intakes of red meats, sweets, processed foods and refined grains, has long

been suggested to influence development of colon cancer (Fung et al., 2003). Epidemiological studies have associated an increased risk for developing colorectal cancer in people who consume western style diets. Using dietary information collected over 20 years from more than 76,000 women, the western diet was shown to have a relative risk for colon cancer of 1.46 (95% CI, 0.97-2.19) when the highest and lowest quintiles of the western style diet were compared. However, diets with higher intakes of fruits, vegetables, legumes, fish, poultry, and whole grains were only observed to have the possibility of being inversely associated with colon cancer risk (Fung et al., 2003).

In a case control study conducted over three years in California, Utah, and Minnesota, researchers demonstrated an increased risk of colon cancer in men and women consuming a western diet in 1,993 cases matched with 2,410 controls. In colon cancer cases diagnosed before age 67, males had an odds ratio of 1.96 (95% CI: 1.22-3.15) and women had an odds ratio of 2.02 (95% CI, 1.21-3.36)(Slattery et al., 1998).

However, in a Swedish study involving more than 61,000 women over approximately 10 years, a western style diet was not identified as a risk factor for developing colon cancer (Terry et al., 2001).

Fung et al. (2003) defined western style diets as being higher in sweets and refined grains. Epidemiological data support the association of carbohydrate consumption and risk of colon cancer development. Franceschi et al. (2001) demonstrated colon cancer risk was associated with diets rich in high glycemic index foods with an odds ratio of 1.7 (95% CI: 1.4-2.0) when the highest and lowest quintiles were compared and data were adjusted for age, sex, and other confounders.

Results from case control studies have also suggested sugar consumption may be a risk factor for developing colon cancer. Bristol et al. (1985) demonstrated the relative risk of developing colon cancer increased by a factor of 3.6 (95% CI: 1.2-10.9) in persons consuming diets with sugar consumption greater than 99 g/day. Additionally, results of the Iowa Women's Health Study cohort also indicated that women who consumed diets rich in sucrose had an increased risk of colon cancer (Bostick et al., 1994).

In a study conducted in India, heavy sugar consumption also was shown to be a colon cancer risk factor. Based on dietary data collected using a food frequency questionnaire, consumption of sugar was determined by a subject's interest or non-interest in sweet foods. A positive colon cancer association was observed for those

who consumed sweets daily when compared to those who did not like sweets (Nayak et al., 2009).

Finally, in a case control study investigating the association between simple carbohydrates and risk of colon cancer, high sucrose intake was associated with increased colorectal cancer risk in men less than 67 years old. The odds ratio when the highest and lowest quintiles were compared was 1.59 (95% CI: 1.07-2.37). Men and women consuming diets with a high glycemic index were also observed to have a greater risk for proximal colon tumors with odds ratios of 1.58 (95% CI: 1.06-2.36) for men and 1.72 (95% CI: 1.11-2.67) for women when the highest and lowest quintiles for glycemic index from dietary sugars were compared (Slattery et al., 1997).

However, other case-control studies have shown a less significant relationship between sugar consumption and colon cancer (La Vecchia et al., 1988; Michaud et al., 2005).

Experiments using animals have been useful in confirming the results elucidated from epidemiological studies. For example, results from animal studies have shown an association between sucrose intake and colon cancer development. Researchers investigated the effect of 10 g/kg of body weight of sucrose, glucose, or fructose

consumption on colonic epithelial cell proliferation and aberrant crypt foci number in CF1 mice injected with azoxymethane (AOM). Increased aberrant crypt foci formation was observed in mice given gavages of sucrose (18.4 ± 1.5) and fructose (13.1 ± 1.8) compared to water (8.6 ± 1.1) (Stamp et al., 1993).

Microadenoma (MA) development was also shown to increase in AOM injected mice consuming cooked sucrose compared to the control-uncooked diet (48 ± 16 vs. 28 ± 13 MA) (Corpet et al., 1990). Additionally, colonic crypt proliferation and aberrant crypt foci were significantly greater in rats consuming sucrose versus starch (Caderni et al., 1993). In studies by Caderni, one showed that rats consuming sucrose-rich diets had greater mucosal cell proliferation than rats that consumed starch-rich diets after 105 days. Treatment with the carcinogen 1,2-dimethylhydrazine (DMH), which was used to increase colonic cell proliferation, did not influence outcome in diets containing either 46 percent sucrose or starch (Caderni et al., 1991). In a subsequent study wherein rats were fed diets containing 46 percent sucrose or starch for three months, the number of colonic ACF were the same between the groups, but ACF were larger in size in the sucrose group (Caderni et al., 1997). Additionally, a mixed diet

consisting of sucrose and starch has also been shown to enhance ACF development in rats (Poulsen et al, 2001; Kristiansen et al., 1995; Kristiansen et al., 1996). Although these diets contained mixed carbohydrates, the study demonstrated that diets based on refined carbohydrates stimulate cell proliferation.

In a study investigating colon tumor incidence and burden in *Apc*$^{Min/+}$ mice, mice fed sucrose for 10 weeks did not show significantly different colonic adenoma development from mice fed starch. However, mice consuming sucrose were observed to have greater cell proliferation in the colon as determined by Ki-67 antigen labeling (Wang, 2005). However, in a 16-week follow-up study, sucrose, when compared to starch, influenced proximal small intestinal adenoma number and size, and colonic adenoma incidence (Wang et al., 2009; Wang 2005). In another study using *Apc*$^{Min/+}$ mice, colon tumor number, average size, burden and incidence were not influenced by carbohydrates when compared to mice fed diets containing primary carbohydrates of starch, fructose, glucose, sucrose, or an equal glucose and fructose mixture (Powell, 2011). It is important to note that studies conducted by Powell (2011) and Wang (2005) did not utilize a carcinogen, such as DMH or AOM, to induce cell proliferation. Additionally, as noted by Powell

(2011), the *Apc*$^{Min/+}$ mouse is prone to developing a significant number of small intestinal tumors, which lead to morbidity and can limit the effectiveness of the model developing colonic tumors with short diet protocols.

6. Dietary Factors and Positive Health Outcomes

While diets rich in carbohydrates have been associated with increased risk of colorectal cancer, diets rich in fruits and vegetables reduce this risk. For example, the diets of more than 492,00 men and women were analyzed to study associations between dietary patterns and colorectal cancer in the National Institutes of Health American Association of Retired Persons (AARP) Diet and Health Study. Fruits and vegetables were associated with a significantly lower risk of colorectal cancer in an age-adjusted multivariate-adjusted model for men with high scores for fruit and vegetable consumption, but there was no association observed in women (Flood et al., 2008). However, association between fruit and vegetable consumption was less consistent, and hence another group of researchers conducted a pooled analysis of 14 cohort studies. A total of 5,838 cases out of 756,217 men and women were diagnosed with colon cancer during a period of 6-20 years of follow-up. The results showed a multivariable

relative risk of colon cancer of 0.91 (95% CI: 0.82-1.01) when the highest and lowest quintiles of fruit and vegetable intake were compared (Koushik et al., 2007).

In a meta-analysis, 28 case-control studies and 12 cohort studies were analyzed to determine whether fruits and vegetables were protective against colon cancer. Of the studies identified, 13 were excluded for insufficient vegetable consumption data, and 21 were excluded for insufficient fruit consumption data. The authors concluded a moderate decreased risk of colorectal cancer in persons consuming diets containing greater than 100 g/day of fruits or vegetables. However, in their analysis, data derived from the case-control studies demonstrated a significant protective effect due to consumption of fruits and vegetables, but a non-significant protective effect for the cohort studies (Riboli and Norat, 2003).

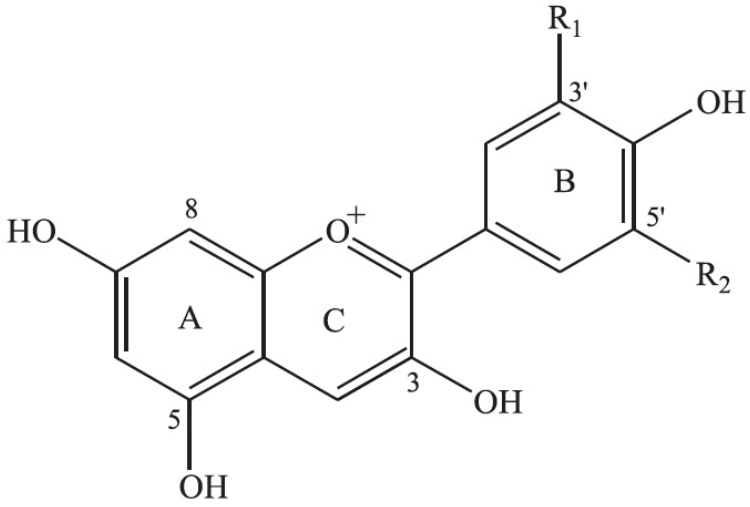
7. Polyphenols

Anthocyanins are non-nutritive phytochemicals belonging to the class of compounds called phenolics and occur naturally in plants, flowers, fruits and vegetables (Prior, 2003). Water-soluble and known for their bright red, purple and blue colors, it has been hypothesized that the purpose of anthocyanins in plants is to attract

pollinating insects and birds. Additionally, they are indicators of ripeness in fruits, serve as plant defense compounds, and reduce photo-oxidative stress by savaging free radicals (Beckwith et al., 2004; Quina et al., 2009).

Anthocyanins are the most abundant dietary flavonoid (Jing et al., 2008) of the 8000 or more phenolics that have been identified (Pietta, 2000). Derived from the amino acids phenylalanine and tyrosine, they consist of two benzene rings joined by a three-carbon chain (Pietta, 2000). The basic structure is a flavillium cation with rings labeled A, B and C (Figure 1). Anthocyanins occur naturally as glycosides. In reference to anthocyanins, the aglycones are classified as anthocyanidins, whereas the glycosides are classified as anthocyanins. The most common aglycone structures are cyanidin, delphinidin, pelargonidin, malvidin, and petunidin (Figure 1) (Harborne, 1958; Wang and Stoner, 2008). Hundreds of anthocyanins have been identified, with sugars conjugated to the C_3 hydroxyl group on the C ring. The most commonly conjugated sugars are: glucose, galactose, rhamnose, xylose and arabinose (Harborne, 1958; Wang and Stoner, 2008).

Figure 1. Chemical Structures of Common Anthocyanidins



Anthocyanidin	**R_1**	**R_2**
Cyanidin	OH	H
Delphinidin	OH	OH
Pelargonidin	H	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

8. Bioavailability of Anthocyanins

Bioavailability is defined as the fraction of an orally administered dose that is digested, absorbed and metabolized through normal metabolic pathways (McGhie and Walton, 2007).

Animal studies have shown anthocyanins are absorbed as intact glycosides. Their absorption can be detected as early as 15 minutes after ingestion in the plasma of rats (Miyazawa et al., 1999; Ichiyanagi et al., 2006). In rats given doses of cyanidin-3-glucoside at 160 mg/kg or 320 mg/kg of body weight by stomach tube, plasma cyanidin-3-glucoside was detected by high-performance liquid chromatography at concentrations of 406 μg/L and 669 μg/L, respectively. Cyanidin-3,5-diglucoside was also detected in plasma, and plasma concentration was determined to be dose dependent for both glycosides tested (Miyazawa et al., 1999). The bioavailability in rats orally administered or intravenously injected with individual bilberry anthocyanins was determined to range between 0.61 to 1.82 percent, and 0.93 percent for an anthocyanin mixture (Ichiyanagi et al., 2006).

In human studies, Murkovic et al. (2001) reported very low anthocyanin absorption based on low urinary excretion of the intact glycoside. However, Ohnishi et al. (2006) demonstrated that six of the 12 cranberry anthocyanins they identified were absorbed and excreted in urine, and absorption was estimated to be 5 percent of the amount consumed. In a study with tart cherries, twelve healthy human volunteers ingested a relative dose of 45 or 90 whole

cherries in powder form to determine anthocyanin bioavailability. Unmodified anthocyanins, methylated anthocyanins and glucuronic acid-conjugated derivatives were detected in plasma and urine samples. The unmodified anthocyanins detected in plasma were cyanidin-3-glucosylrutinoside and cyanidin-3-rutinoside (Kirakosyan et al., 2010). Additionally, cyanidin-3-glucoside and cyanidin-3,5-diglucoside were detected in the plasma of 12 human subjects who orally ingested anthocyanins from elderberries and black currents. However, the agylcone, cyanidin, was not detected (Miyazawa et al., 1999).

The absorption mechanisms for anthocyanins and anthocyanidins have not been clearly delineated to this point. However, unmodified blueberry and grape anthocyanins are rapidly absorbed in rat stomachs (Talavera et al., 2003; Passamonti et al., 2003). The acidic environment of the stomach and anthocyanins' interaction with bilitranslocase has been hypothesized as a mechanism for anthocyanin transportation and detection of intact anthocyanin in rat plasma (Passamonti et al. 2002). In studies investigating specific regions of absorption in the gastrointestinal tract, the jejunum, but not the duodenum, ileum or colon, has also been shown to be the site of absorption of boysenberry derived cyanidin-3-glucoside in

Swiss mice (Matuschek et al., 2006). The authors concluded the specific location of anthocyanin transport may indicate a site of active transport. Additionally, the glucose transporter (GLUT2), which is found on both the apical and basolateral membrane of the small intestine, has been demonstrated to transport anthocyanins. In Caco-2 cells, treatment with anthocyanins was also shown to increase GLUT2 expression (Faria et al., 2009).

9. Anthocyanins as Anti-inflammatory Compounds

Anthocyanins have been shown to be inhibitors of cyclooxygenase enzymes, which are involved in the synthesis of prostaglandins. Isomers of the enzyme, COX-1 and COX-2, have different physiological functions. Cyclooxygenase-1 is considered to act as a constitutive enzyme in many tissues and is important for producing prostaglandins required for maintaining the gastric mucosa, renal blood flow and platelet function (Crofford, 1997; Greenhough et al., 2009). Conversely, COX-2 is an inducible enzyme present in most cells and is responsible for inflammatory responses (Hawkey, 1999).

Several studies investigating the anti-inflammatory properties of tart cherries have demonstrated that anthocyanins inhibit COX enzyme activity. Balaton and

Montmorency tart cherry extracts were shown to inhibit COX-1 activity by 84 and 91 percent, respectively, and COX-2 activity by 76 and 87 percent when tested at 250 μg/ml in a COX enzyme activity assay (Mulabagal et al., 2009). Anthocyanin mixtures from cherries and berries were also shown to inhibit COX enzyme activity at 125 μg/ml, but to varying degrees. Additionally, purified cyanidin was shown to inhibit COX-1 and COX-2 activity by 38.7 and 46.8 percent, respectively, when tested at a concentration of 5 μmol/L. However, purified cyanidin glycosides, when tested at 10 μmol/L, had limited COX inhibitory activity (Seeram et al., 2001). Purified cyanidin also has been shown to inhibit COX-1 and COX-2 activity at 90 and 60 mmol/L, respectively, but was also effective at 15 mmol/L (Wang et al., 1999). In all experiments, COX enzyme inhibition by cyanidin was comparable to the non-steroidal anti-inflammatory drugs (NSAIDs) used as controls.

10. Anthocyanins as Antioxidant Compounds

The antioxidant activity of anthocyanins has been shown to be comparable to commercially available antioxidants such as butylated hydroxytoluene and *tert*-butylhydroquinone (Wang et al., 1999). Additionally, antioxidant capacity of anthocyanins was inversely related

to the number of sugar residues attached at the C_3 position. Therefore, the greater the number of sugars residues attached resulted in less antioxidant capacity being observed. Additionally, cyanidin, when compared to tart cherry glycosides, demonstrated the greatest antioxidant activity. This was attributed to the aglycone having a more stable aryloxyl radical (Wang et al., 1999).

The antioxidant activity of anthocyanins and anthocyanidins has been attributed to radical-scavenging capacity of the catechol structure of the flavillium cation's B ring (Figure 1) (Pietta, 2000).

11. *In Vitro* Studies Using Human Cancer Cell Lines

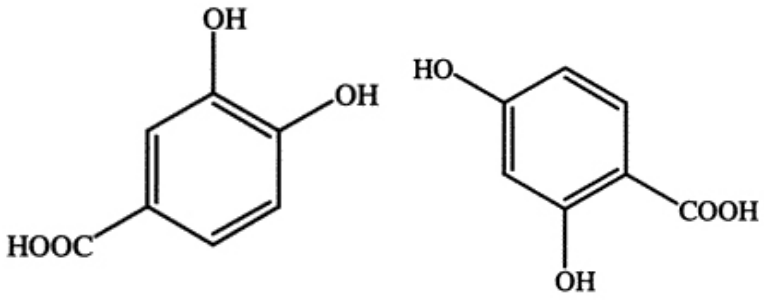
Treatment of HCT 116 and HT 29 human colon cancer cells with anthocyanins or cyanidin causes a dose-dependent reduction in cell growth without cytotoxicity. The IC_{50} values in these experiments for a mixture of anthocyanins were 260 and 585 μmol/L for HCT 116 and HT 29 cells. However, the IC_{50} values for cyanidin demonstrated it was more effective in inhibiting cell growth. The cyanidin IC_{50} values for HCT 116 and HT 29 cells were 85 and 63 μmol/L, respectively (Kang et al., 2003). Similarly, Yi et al. (2005) demonstrated cancer cell proliferation was inhibited

by blueberry extracts in HT 29 and Caco-2 cells. The anthocyanin fraction resulted in the greatest growth inhibition in HT-29 cells, with an IC_{50} of approximately 25-50 μg/ml depending on the blueberry variety. With Caco-2 cells, blueberry anthocyanins inhibited cell growth with concentrations as low as 15 μg/ml. Although anthocyanins and anthocyanidins have been shown to inhibit cell proliferation, their stability at physiological pH has been debated. At a pH < 3, the primary tart cherry anthocyanidin is the red flavylium cation (Seeram et al., 2001). As pH increases, the blue colored quinonoidal structure forms due to deprotonization of oxygen. Hydration of the flavylium cation at higher pH leads to the formation of chalcones with cis and trans structures. At physiological pH, the primary bioactive compounds have been hypothesized to be quinonoidal bases, hemiketals and chalcones (McGhie and Walton, 2007).

The bioactive components of Balaton cherries are cyanidin 3-glucosylrutinoside, cyanidin 3-rutinoside, and cyanidin 3-glucoside. Since anthocyanins are unstable at higher pH and temperature, Seeram et al. (2001) investigated their stability in cell culture media at physiological conditions. The degradation products of the glycosides and the aglycone, cyanidin, identified in cell

culture media were benzoic acid derivatives: protocatechuic acid, 2,4-dihydroxybenzoic acid, and 2,4,6-trihydroxybenzoic acid (Figure 2).

Figure 2. Chemical Structures of Anthocyanin Metabolites



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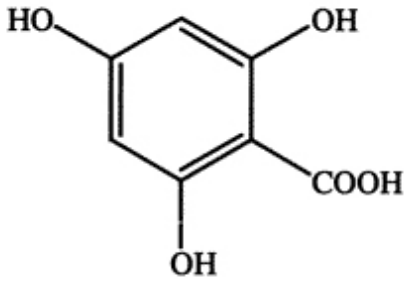


3

1	Protocatechuic acid
2	2,4-dihydroxybenzoic acid
3	2,4,6-trihydroxybenzoic acid

12. Plant Phenolics in Animal Studies

Animal studies investigating the effects of flavonoids on tumor promotion have shown promising results. Issa et al. (2007) demonstrated that green tea inhibited new colonic adenomas in *Apc*$^{Min/+}$ mice injected with AOM; however, it was not effective against larger tumors. Previously, Kang et al. (2003) demonstrated that *Apc*$^{Min/+}$ mice that consumed diets containing anthocyanins, cyanidin, or tart cherries had fewer cecal adenomas than mice that consumed the control diet or sulindac. However, colonic adenomas were not found to be significantly different among treatment groups. Additionally, Bobe et al. (2006) demonstrated that anthocyanins, when fed with sulindac to *Apc*$^{Min/+}$ mice, were more effective in reducing small intestinal tumors than sulindac alone. Similarly, Hagiwara et al. (2001) demonstrated that purple corn anthocyanins inhibited colorectal carcinogenesis in rats treated with DMH and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP).

In a study investigating the impact of anthocyanins on obesity, inflammation, and hyperlipidemia, which are components of metabolic syndrome, researchers demonstrated a significant link between cherry consumption and reduced

inflammatory markers. For 90 days, Zucker fatty rats were fed a high fat diet with or without tart cherry powder. Rats fed diets with cherry powder had reduced fat mass, retroperitoneal TNF-α mRNA, plasma TNF-α, and decreased NF-kB activity. The researchers also noted there was an overall reduction of systemic and local inflammation (Seymour et al., 2009).

An anthocyanin mixture from bilberries, and isolated cyanidin-3-glucoside (C3G), were fed to *Apc*$^{Min/+}$ mice at 0.03, 0.01 or 0.3% in a standard AIN-93G diet for twelve weeks. The researchers observed that the anthocyanin mixture and the isolated C3G reduced colon tumors in a dose-dependent manner when compared to mice consuming the control diet. At the highest dose, tumor burden was decreased 30 percent by the anthocyanin mixture, and 45 percent by C3G (Cooke et al., 2006).

In a murine experiment investigating the potential of tart cherry anthocyanins to suppress inflammation-induced behavior in rats, researchers demonstrated that anthocyanins, in a dose-dependent manner, reduced pain induced by heat, mechanical stimulation and edema (Tall et al., 2004).

Finally, in a study using Wistar rats, cyanidin 3-*O*-β-D-glycoside was shown to be rapidly absorbed and detected

in plasma. Its metabolites, protocatechuic acid and methyl cyanidin 3-*O*-β-D-glycoside, were also detected in plasma and tissue. The authors concluded these components may have the potential to act as antioxidants in plasma and tissue (Tsuda et al., 1999).

13. *Apc*$^{Min/+}$ Mouse Model and Azoxymethane

The *Apc*$^{Min/+}$ (multiple intestinal neoplasia) mouse model has been extensively employed by this laboratory and others for colorectal cancer research. These mice are highly susceptible to intestinal adenoma formation, due to the Min germline mutation, which is analogous to the human mutation in FAP (Su et al., 1992). This similarity makes *Apc*$^{Min/+}$ mice a suitable model to study dietary interactions and colorectal cancer, and to relate observations from animal studies to human colorectal cancer. However, *Apc*$^{Min/+}$ mice also commonly develop more than 30 adenomas throughout the gastro-intestinal tract and have a life span of approximately 120 days (Jackson Laboratory, 2013).

Azoxymethane is a potent colon-selective carcinogen and stimulates colonic adenoma multiplicity (Issa et al., 2007). The use of AOM increases the sensitivity of the

Apc$^{Min/+}$ mouse model for detecting effects on colonic adenoma development (Mollersen et al., 2004).

RATIONALE AND SPECIFIC AIMS

Colorectal cancer is a major health problem in the United States and the world. In the U.S., colorectal cancer is the second most common cause of cancer mortality; however, as much as 60 percent of colorectal cancers are preventable. Diet can play a significant role in reducing cancer risk. Previous research indicates eating a diet rich in fruits and vegetables reduces the risk of developing colorectal cancer. On the other hand, some dietary factors increase colon cancer risk. For example, the relationship between a high sucrose diet and the development of colonic tumors or small-intestinal tumors has been demonstrated epidemiologically and in previous research in this laboratory and others.

Plant phenolics, such as anthocyanins, have been demonstrated to have many health-promoting benefits. Anthocyanins are natural red, purple, and blue flavonoid pigments that impart color to fruits, vegetables, flowers and plants. They have a wide variety of health-promoting benefits attributed to their antioxidant and anti-inflammatory properties. Research investigating the role of anthocyanins for cancer prevention has shown promise. However, much more information is needed to elucidate the

mechanisms whereby anthocyanins influence cancer risk. This research aims to further understand the impacts of anthocyanin consumption on intestinal tumor development in animal models to relate to human colon cancer. This research specifically investigated the efficacy of anthocyanins to reduce colorectal tumorigenesis associated with diets rich in sucrose and other dietary carbohydrates, which have been shown to stimulate intestinal tumor development.

The overall goal of this research was to determine the effects of anthocyanin-rich tart cherry extracts on intestinal adenoma development associated with feeding carbohydrate-rich diets based on glucose, sucrose and starch to *Apc*$^{Min/+}$ mice, a model of human colon cancer development.

The specific aims of this research were to:

1) Determine the efficacy of anthocyanin-rich extracts to reduce intestinal adenoma development in *Apc*$^{Min/+}$ mice consuming high-carbohydrate diets (based on starch, sucrose and glucose), and
2) Relate changes in intestinal adenomas to alterations in body weight and body composition.

The working hypothesis of specific aim one was that anthocyanins will reduce intestinal adenoma development in

Apc$^{Min/+}$ mice compared to mice consuming control diets based on dietary starch and diets not containing anthocyanins. We further hypothesized that dietary sucrose and glucose will increase intestinal adenoma development compared to dietary starch, and that feeding anthocyanins will attenuate the increase in tumorigenesis caused by these carbohydrate sources. To test this hypothesis, the numbers and sizes of intestinal adenomas was quantified at the end of a feeding study wherein these carbohydrates were administered as the sole carbohydrate sources in the diets fed to *Apc*$^{Min/+}$ mice for nine weeks.

The working hypothesis of specific aim two was that diet-induced differences in intestinal adenoma development will be related to changes in body weight and body composition. To test this hypothesis, body weights of mice were measured weekly and total body fat and lean percentages were measured at study termination. It was anticipated that mice consuming diets rich in anthocyanins will have increased body weight, lean body mass, and body fat compared to mice not consuming anthocyanins.

At the conclusion of these studies, we expected to have demonstrated that dietary anthocyanins reverse the stimulatory effects of high-carbohydrate diets on intestinal

tumor development in *Apc*$^{Min/+}$ mice. These observations will demonstrate the potential health benefits of anthocyanins in human diets.

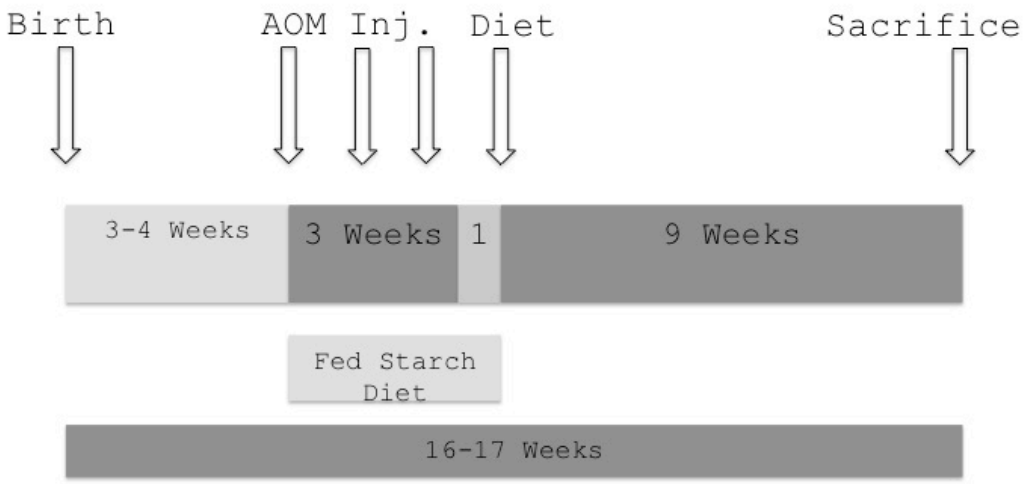
MATERIALS AND METHODS

1. Animals

An *Apc*$^{Min/+}$ mice breeding colony, originally purchased from the Jackson Laboratory (Bar Harbor, ME), was maintained in the University Research Containment Facility at Michigan State University. This study was approved by the Michigan State University All-University Committee on Animal Use and Care. Mice were housed in a room with constant humidity (40-50%), temperature (71-73°F), and a 12-hour day:night cycle. Mice used in this study were the offspring produced when male C57BL/6J *Apc*$^{Min/+}$ mice were mated with normal female C57BL/6J mice. Male *Apc*$^{Min/+}$ mice, when not paired with normal female mice, where given drinking water containing sulindac (200 mg/L) to reduce morbidity caused by intestinal adenoma development. The resulting offspring were randomly assigned to one of six treatment groups at weaning, but were fed the control diet from weaning until conclusion of the azoxymethane (AOM) injection protocol (Figure 3). Mice were weaned between 21 and 28 days after birth and housed in plastic cages (1-4 mice/cage). After weaning, they received a series of intraperitoneal AOM injections (8 mg/kg) once a week for

three weeks. After the three AOM injections, they were given one week to recover before beginning their respective experimental diets, which they were fed *ad libitum* for nine weeks or until weight loss of 10 percent or greater was detected. Approximately 13 males and 13 females were assigned to each diet treatment. Genotype was determined based on presence of small-intestinal adenomas observed at the end of the study. At total of 159 mice were used in this study. The numbers of female and male $Apc^{Min/+}$ mice were 36 and 23, respectively. The numbers of female and male wild type C57BL/6J mice were 49 and 51, respectively. Mice were weighed weekly to track growth and monitor health status. Six $Apc^{Min/+}$ mice and one wild type C57BL/6J mouse were sacrificed early due to weight loss.

Figure 3. Experimental Design

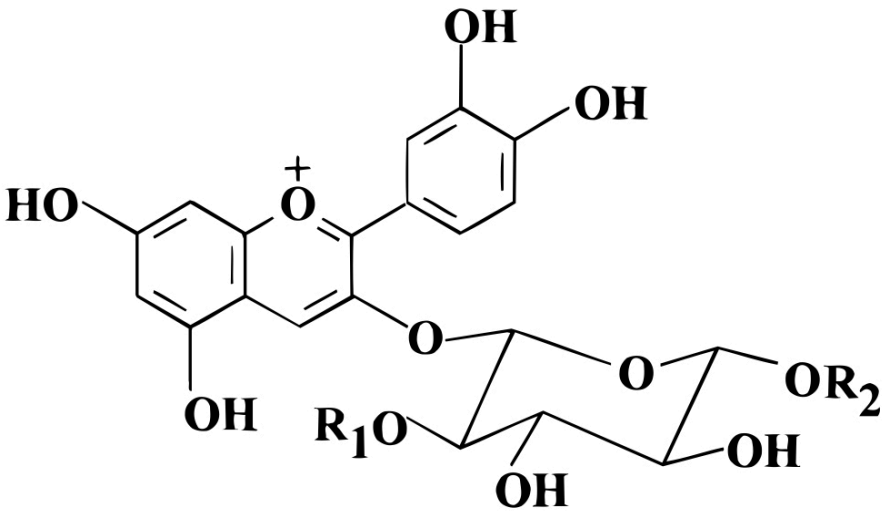


2. Diets

Isocaloric experimental diets were based on the AIN-93G diet (Reeves et al., 1993) and contained sucrose, glucose, or starch as the sole carbohydrate source (Table 1). Fat content was increased from 7 to 15 percent to reflect a typical human diet (approximately 31% of total energy from fat). Micronutrients were increased to account for the anticipated decreased food consumption caused by the increased energy density of the diets. These diet formulations were the three primary diets, of which each contained 0 or 1500 parts per million (mg/kg) (PPM) of 50 percent pure anthocyanins from tart cherries. The anthocyanins were supplied from the laboratory of Dr. Muralee Nair, Michigan State University, and previously used in experiments in this laboratory. The anthocyanins used this project (Figure 4) were isolated from Montmorency cherries and identified (Wang et al., 1997) as: 1) 3-cyanidin 2”–O-β-D-glucopyranosyl-6”–O-α-L-rhamnopyranosyl-β-D-glucopyranoside; 2) 3-cyanidin 6”–O-α-L-rhamnopyranosyl-β-D-glucopyranoside; and 3) 3-cyanidin O-β-D-glucopyranoside (Figure 4). Fresh and pitted cherries contained between 12.5-25.0 mg of anthocyanins per 100 g (Wang et al., 1999). The composition of anthocyanins used in this experiment was

65 percent 3-cyanidin 2”–O-β-D-glucopyranosyl-6”–O-α-L-rhamnopyranosyl-β-D-glucopyranoside and 35 percent 3-cyanidin 6”–O-α-L-rhamnopyranosyl-β-D-glucopyranoside (Kang et al., 2003).

Figure 4. Chemical Structures of Anthocyanins from Tart Cherries



Anthocyanin	**R_1**	**R_2**
3-cyanidin 2”–O-β-D-glucopyranosyl-6”–O-α-L-rhamnopyranosyl-β-D-glucopyranoside	Glucose	Rhamnose
3-cyanidin 6”–O-α-L-rhamnopyranosyl-β-D-glucopyranoside	H	Rhamnose
3-cyanidin O-β-D-glucopyranoside	H	H

Table 1. Composition of Diets

	Starch (g/kg) (control)	Sucrose (g/kg)	Glucose (g/kg)
Casein (85% protein)	221	221	221
Corn Starch	523	0	0
Sucrose	0	523	0
Glucose	0	0	523
Soybean Oil	150	150	150
Cellulose	50	50	50
AIN-93G-MX	39	39	39
AIN-93-VX	11	11	11
L-Cysteine	3	3	3
Choline Bitartrate	3	3	3
Tert-Butylhydroquinone	0.03	0.03	0.03
+/- Anthocyanins	1.5	1.5	1.5

3. Weight and Body Composition

Mice were weighed weekly starting from the day prior to the first AOM injection until the day prior to sacrifice. Body composition analysis was also performed the day prior to sacrifice using the EchoMRI-100 body composition analysis system for live animals (Echo Medical Systems LLC, Houston, TX).

4. Sample Collection

Mice were euthanized after nine weeks of dietary treatment by carbon dioxide asphyxiation. The terminal age of mice was between 16-17 weeks. Blood samples were immediately collected via cardiac puncture, transferred to

K_2EDTA blood collection tubes, and centrifuged for 10 minutes at 1,300 (g). The plasma was transferred into fresh microtubules and stored at -40°C for future analysis. Liver and abdominal fat sections also were collected and stored at -40°C for future analysis. The entire gastro-intestinal tract from the small intestine to rectum was removed, opened longitudinally and rinsed with cool water and phosphate buffered saline (PBS). Individual sections (small intestine, cecum, and colon) were pinned on cardboard and fixed with 10 percent neutral buffered formalin overnight. After fixation, a one-centimeter medial colonic section, void of visible adenomas, was removed and preserved in paraffin. The remaining fixed tissue segments were stained with 0.3 percent methylene blue for three minutes, rinsed with PBS, and stored in 1 percent neutral buffered formalin.

5. Quantification of Intestinal Adenomas

Quantification of adenomas in the intestinal sections was determined visually by using a Nikon SMZ stereomicroscope by an observer blind to treatment. The small intestine for each mouse was divided into three equal sections (proximal, medial, and distal) at the time of

sacrifice. The colon was divided into two equal sections: proximal and distal. Small intestinal adenomas were quantified by total count and by two-dimensional size using the formula: area = $(\pi * d_1 * d_2)/4$. Colonic and cecal adenomas were quantified by number and three-dimensional size using the following formula: volume = $(\pi * d_1 * d_2 *$ height$)/6$. The dimensions of the adenomas were measured to the nearest 0.25 mm with a transparent grid placed under the samples. Small intestinal adenomas are reported by sections as: adenoma number, average size, and adenoma burden (area). Colon tumors are reported as: adenoma number, average size, adenoma burden (volume), adenomas per tumor-bearing mouse, and adenoma incidence.

6. Statistical Analyses

Statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC, Version 9.2). Body weights, lean body mass percent, fat mass percent; and tumor number, average adenoma size, and adenoma burden of each intestinal segment were analyzed using analysis of variance (ANOVA) with a 2 x 3 factorial arrangement, which represented two anthocyanin concentrations x three dietary carbohydrates. Parameters used in the statistical analysis included sex,

Apc status, anthocyanin, carbohydrate, and the interactions between these parameters. When significant main effects were detected, appropriate means were compared by the least significant difference procedure. Statistical differences were detected using a critical value of $P<0.05$ with the F statistic. Results in tables are presented as least-square means ± standard error of the mean (SEM). Colonic adenoma incidence was analyzed with Fisher's exact test using the SAS frequency procedure.

RESULTS

1. Body Weights and Body Composition

Weekly average body weights of wild type C57BL/6J mice and *Apc*$^{Min/+}$ mice, averaged across all dietary treatments, are presented in Figure 5. *Apc*$^{Min/+}$ mice weighed less than wild type C57BL/6J mice throughout the feeding period, with significant differences ($P<0.05$) observed in every week except weeks three and five. Final body weights of pooled wild type C57BL/6J mice and *Apc*$^{Min/+}$ mice by sex are presented in Figure 6. Final bodyweights of mice were influenced by sex (Figure 6), and *Apc* gene status (Figures 7 and 8) ($P<0.05$).

Sex did not significantly influence mouse weight at the start of the experiment. However, beginning with the second week of dietary treatment, male mice weighed significantly more than female mice. This trend continued for the duration of the experiment. At termination of this study, male *Apc*$^{Min/+}$ mice weighed more than their female counterparts (Figure 7), with terminal weights of 24.30 ± 0.86 and 20.64 ± 0.76 g, respectively.

Weekly average body weights of pooled wild type C57BL/6J mice and *Apc*$^{Min/+}$ mice by carbohydrate source are presented in Figure 9. Carbohydrate source did not have a significant effect on body weights of pooled mice at the conclusion of the study; however significant ($P<0.05$) differences were detected in weeks seven, eight and ten. During these weeks, mice consuming sucrose weighed more than mice consuming starch or glucose.

Weekly average body weights of female and male *Apc*$^{Min/+}$ mice consuming different diets are presented in Figures 10 and 11. No significant sex x carbohydrate x anthocyanin interactions were detected for body weight during the duration of the experiment.

Terminal body fat and lean body mass percentages are presented in Figures 12 and 13. *Apc* status was the only factor that significantly ($P<0.05$) influenced these parameters. Wild type C57BL/6J mice had significantly more body fat and less lean mass on a percentage basis than *Apc*$^{Min/+}$ mice.

2. Small Intestinal Adenomas

Adenoma number, average size, and burden were assessed in the proximal, medial, and distal segments of the small

intestine. Values for each segment were summed to obtain a composite value for the whole small intestine and these data are presented in Table 2. Total small intestinal adenoma number was significantly influenced by sex ($P<0.05$). Female mice (51.68 ± 4.02) had a significantly greater number of small intestinal adenomas than males (38.67 ± 5.03). However, mean adenoma size and total adenoma burden were not significantly influenced by sex. A sex x carbohydrate x anthocyanin interaction was detected for total small intestinal adenoma number and burden ($P<0.05$), but not average adenoma size. Females consuming glucose had the greatest number of adenomas and burden compared to all groups. Female mice that consumed anthocyanin-glucose had significantly fewer adenomas (56.50 ± 11.42) and significantly less adenoma burden (84.12 ± 25.13 mm^2) than females consuming glucose (105.60 ± 10.21) and (196.51 ± 22.47 mm^2), respectively. Males consuming glucose had the fewest adenomas and lowest burden, but this was not significantly different from males that consumed the control or anthocyanin-glucose diets. No other differences within carbohydrate sources were detected.

Proximal small intestinal data are presented in Table 3. Adenoma number in females (11.92 ± 0.82) was

significantly ($P<0.05$) greater in the proximal small intestine than in males (7.97 ± 1.06), but a significant difference for average size or burden was not detected based on sex. Proximal small intestinal burden was significantly ($P<0.05$) influenced by a sex x carbohydrate x anthocyanin interaction, but adenoma number and average size were not. Female mice consuming glucose had the greatest adenoma burden (50.96 ± 7.54 mm^2), which was significantly ($P<0.05$) different from females consuming anthocyanin-glucose (27.49 ± 8.43 mm^2) and sucrose (25.92 ± 5.96). Males consuming glucose had the lowest proximal small intestinal adenoma burden (6.49 ± 6.89 mm^2) of all mice, which was significantly ($P<0.05$) different than males consuming anthocyanin-glucose, sucrose, and anthocyanin-sucrose. Average adenoma burden in males consuming glucose was also significantly different ($P<0.05$) from all female diet groups except those that consumed anthocyanin-glucose.

Medial small intestinal adenoma data are presented in Table 4. Medial small intestinal adenoma number and burden tended ($P<0.10$) to be influenced by a sex x carbohydrate x anthocyanin interaction, but average size was not. No significant differences were detected for average adenoma

number, average adenoma size, or burden for any interaction analyzed.

Distal small intestinal adenoma data are presented in Table 5. No significant differences were associated with sex. However, distal small intestinal adenoma number tended ($P<0.10$) to be influenced by a sex x carbohydrate x anthocyanin interaction. Additionally, a sex x carbohydrate x anthocyanin interaction was detected for mean burden ($P<0.05$). Female mice consuming glucose showed the greatest adenoma burden (80.27 ± 10.87 mm^2) compared to all treatment groups for both sexes ($P<0.05$). Female mice consuming sucrose had the lowest adenoma burden (6.33 ± 8.59 mm^2), but this difference was only observed to be different when compared to females consuming glucose and to males consuming cornstarch or anthocyanin-sucrose.

3. Colonic and Cecal Adenomas

Colonic adenoma number, incidence, average size, and burden are presented in Tables 6, 7, and 8. No cecal adenomas were observed in any mouse in this study. Colonic adenomas were only observed in *Apc*$^{Min/+}$ mice.

Adenoma number and incidence are presented in Table 6.

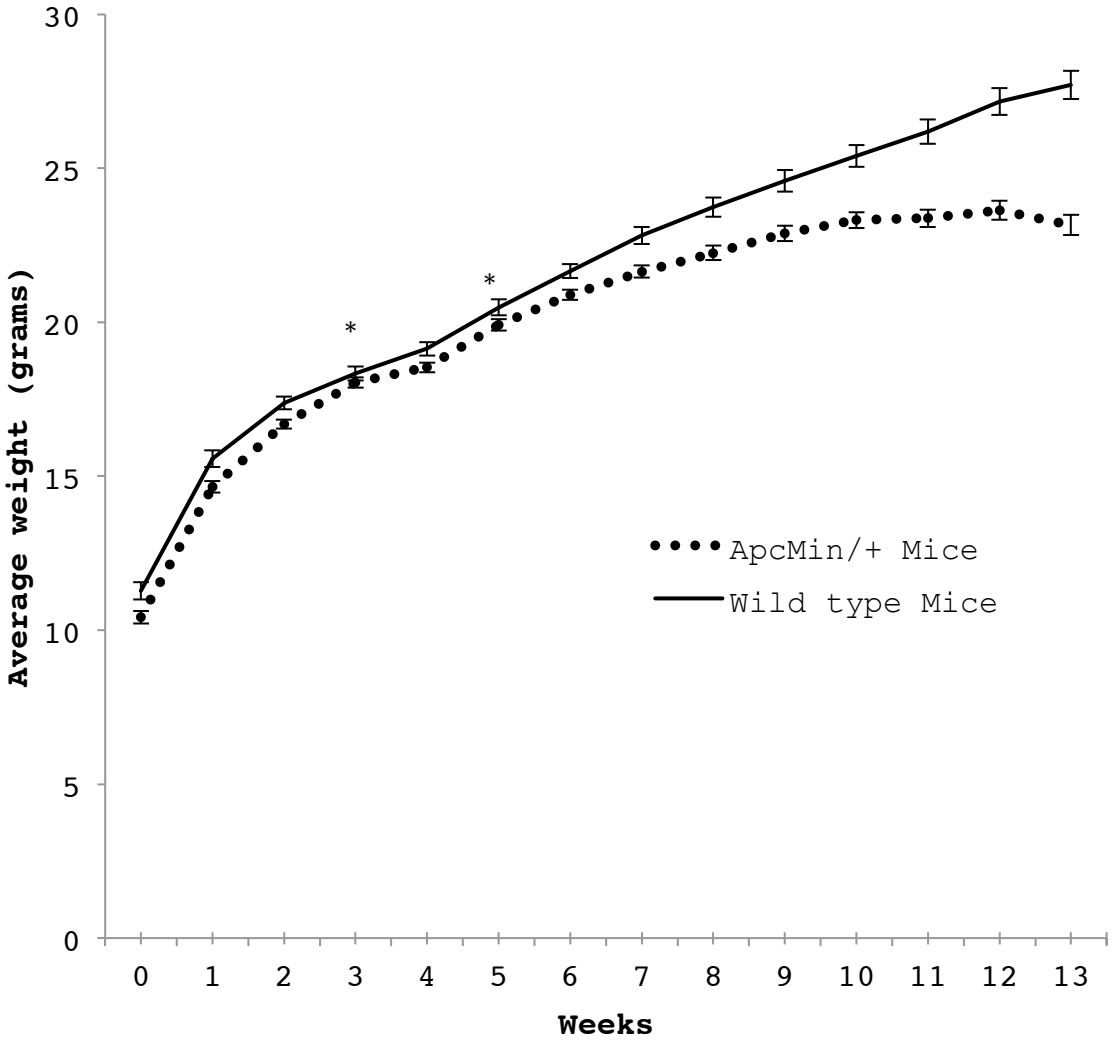
Adenoma number and incidence were not significantly influenced by sex or dietary treatment.

Average adenoma size and burden for all *Apc*$^{Min/+}$ mice are presented in Table 7. Sex did not influence average size or burden; however, an anthocyanin x carbohydrate interaction significantly ($P<0.05$) influenced mean colonic adenoma size in all *Apc*$^{Min/+}$ mice. Mice that consumed sucrose had significantly larger adenomas (16.41 ± 3.06 mm^3) than all mice except those that consumed anthocyanin-cornstarch (8.67 ± 3.06 mm^3).

Average adenoma size and burden for adenoma-bearing *Apc*$^{Min/+}$ mice are presented in Table 8. Sex did not influence average size or burden; however, an anthocyanin x carbohydrate interaction significantly ($P<0.05$) influenced average colonic adenoma size in adenoma bearing *Apc*$^{Min/+}$ mice. Mice that consumed sucrose had significantly ($P<0.05$) larger adenomas (24.94 ± 3.61 mm^3) than all mice. Mice that consumed anthocyanin-sucrose had the smallest average adenoma size (6.05 ± 3.74 mm^3), but this was only significantly different from mice that consumed sucrose ($P<0.05$). Mice that consumed anthocyanin-sucrose had

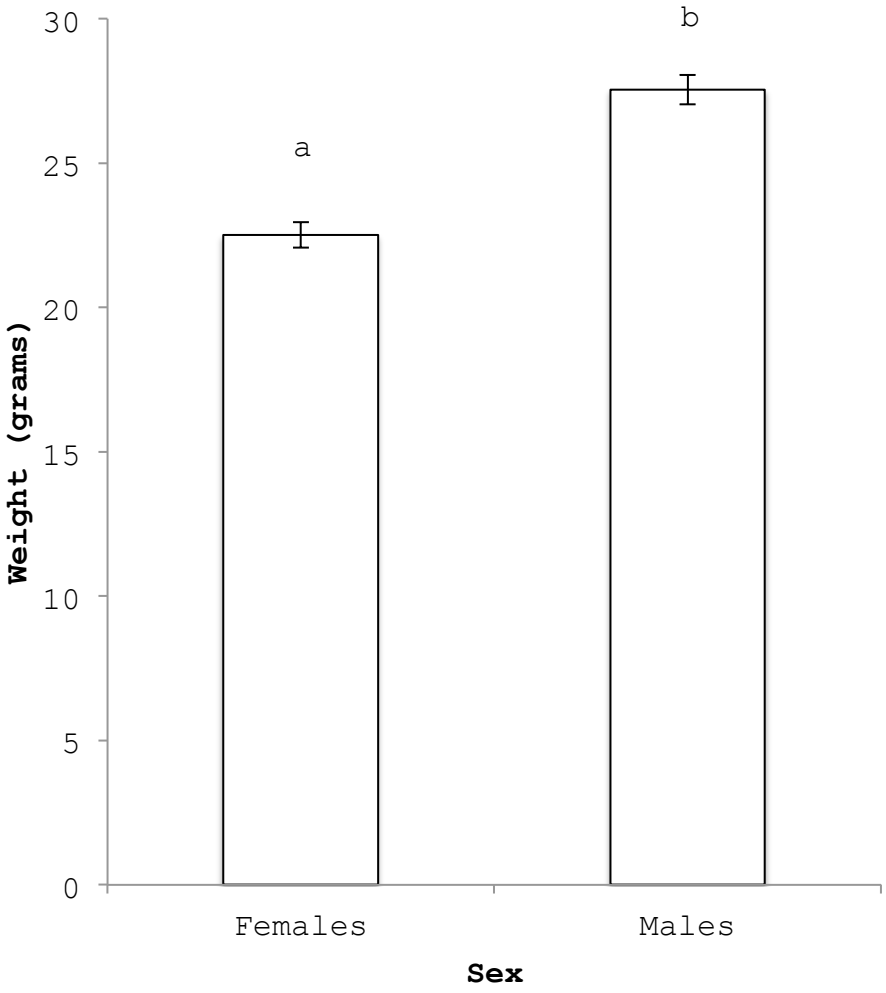
adenomas that were 75.8 percent smaller compared to mice only consuming sucrose. Adenoma burden among adenoma-bearing mice was not influenced by dietary treatment.

Figure 5. Weekly average body weights of wild type C57BL/6J and *Apc*$^{Min/+}$ mice.



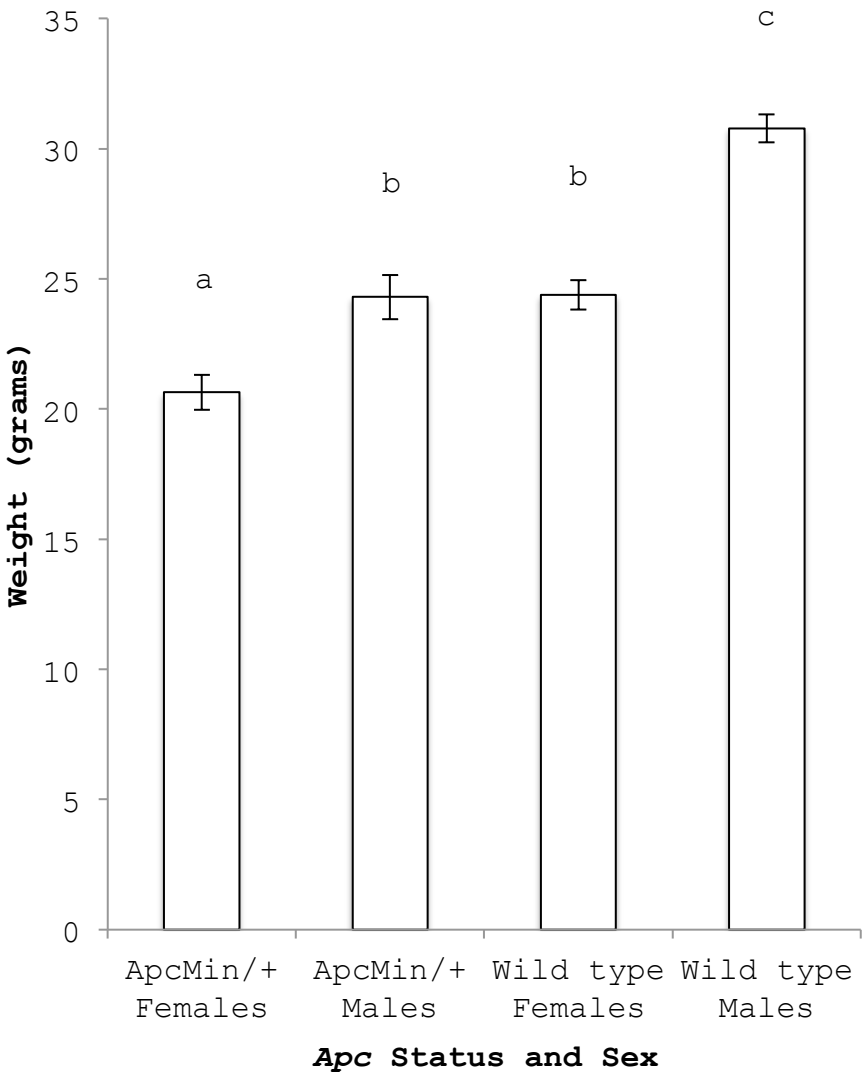
* Denotes weeks without significant difference (P>0.05).

Figure 6. Final body weights of pooled male and female wild type C57BL/6J and *Apc*$^{Min/+}$ mice.



[ab] Means not sharing a common superscript are different (P<0.05).

Figure 7. Final body weights of male and females by wild type C57BL/6J and *Apc*$^{Min/+}$ status.



[abc] Means not sharing a common superscript are different (P<0.05).

Figure 8. Weekly average body weights of male and female wild type C57BL/6J and *Apc*$^{Min/+}$ mice.

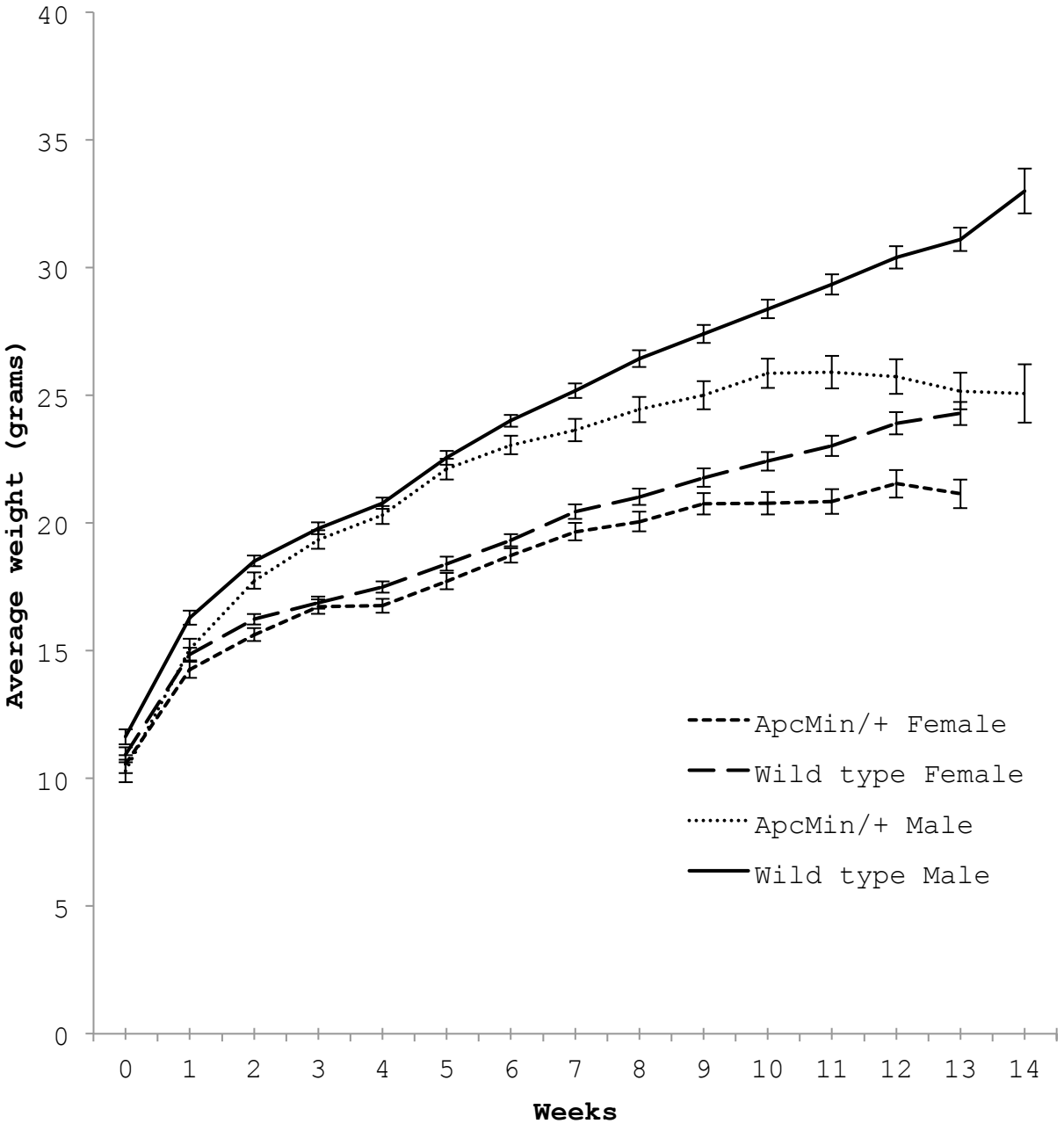
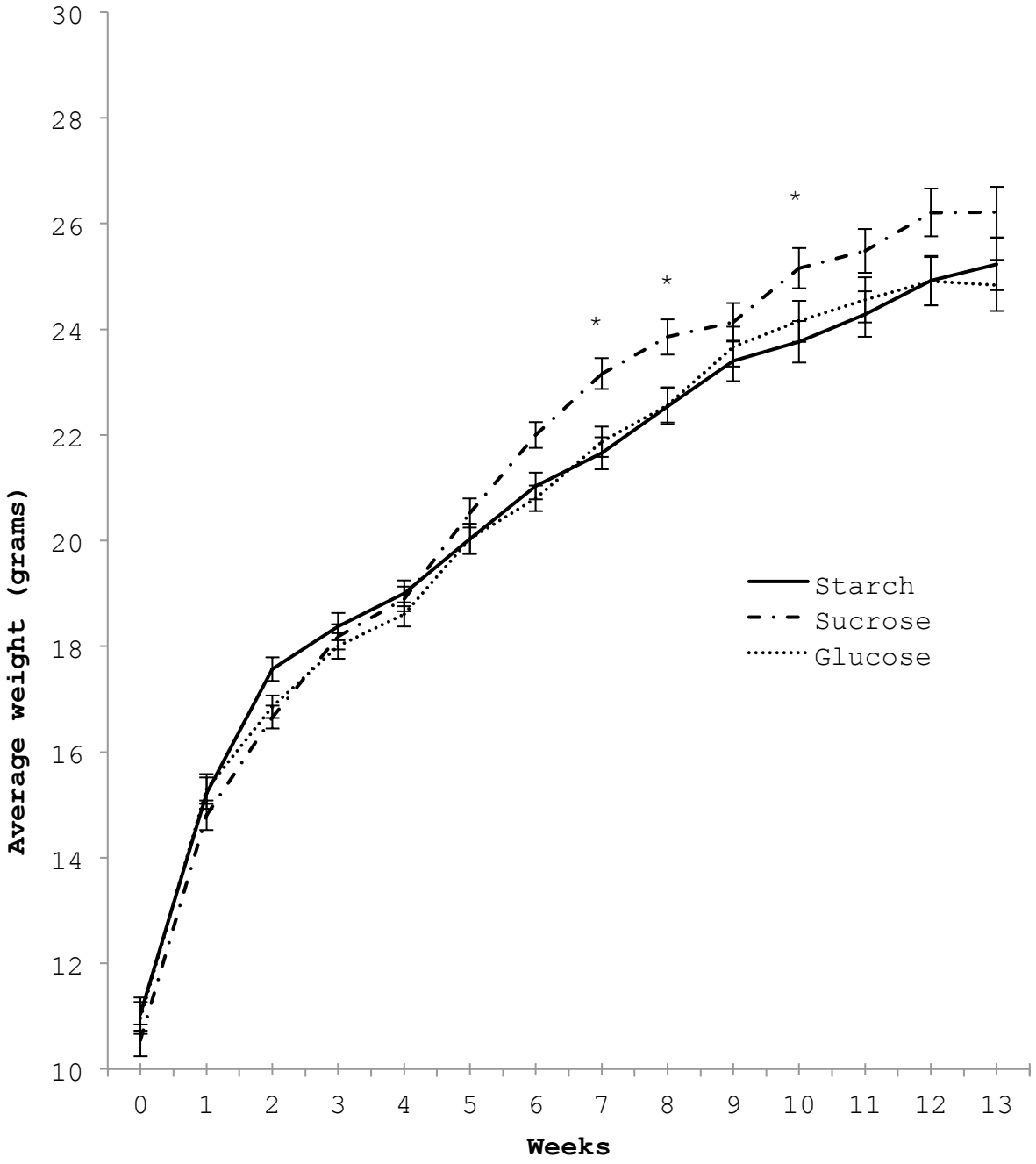


Figure 9. Carbohydrate effect on pooled C57BL/6J mice and *Apc*$^{Min/+}$ mice weekly mean body weights.



* Denotes significant difference between sucrose fed mice and mice fed starch or glucose ($P<0.05$).

Figure 10. Weekly average female *Apc*$^{Min/+}$ mice body weights when fed diets without (-) or with (+) 1500 ppm anthocyanins: (-) cornstarch, (-) sucrose, (-) glucose, (+) cornstarch, (+) sucrose, or (+) glucose.

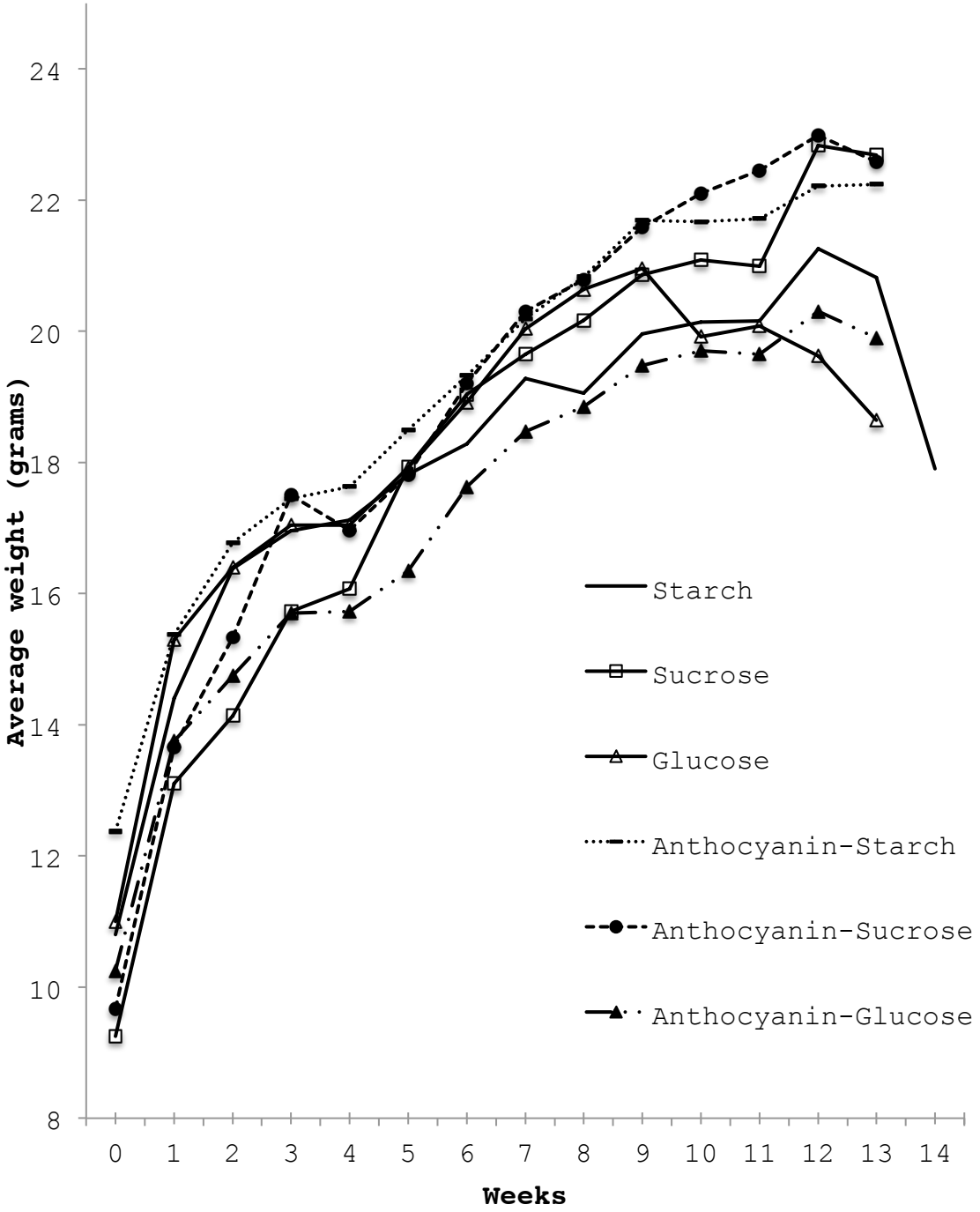


Figure 11. Weekly average male *Apc*$^{Min/+}$ mice body weights when fed diets without (-) or with (+) 1500 ppm anthocyanins: (-) cornstarch, (-) sucrose, (-) glucose, (+) cornstarch, (+) sucrose, or (+) glucose.

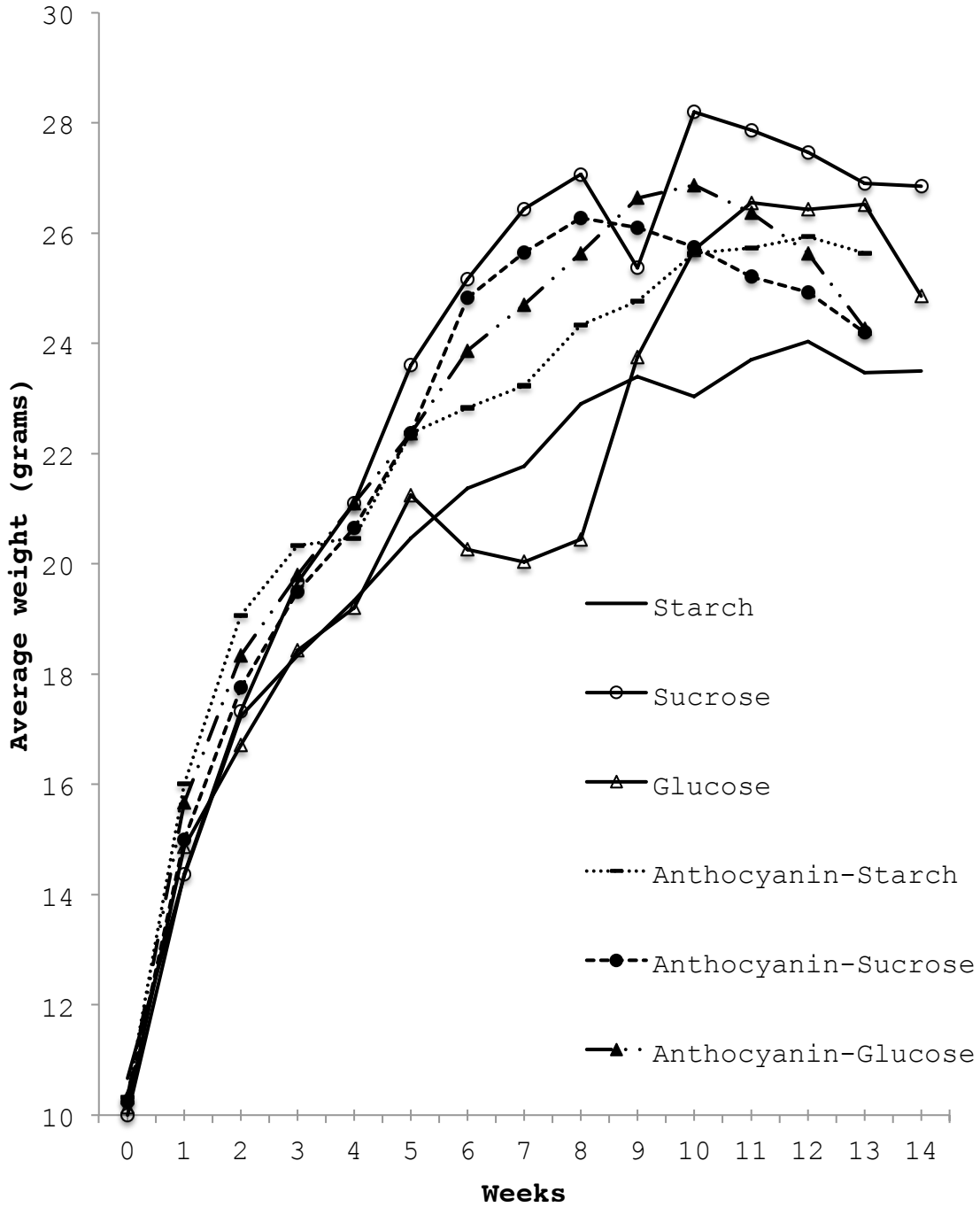
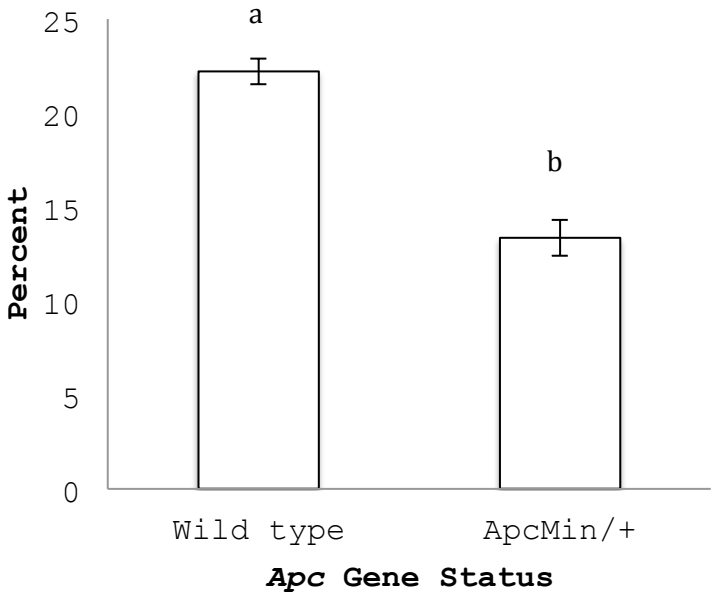
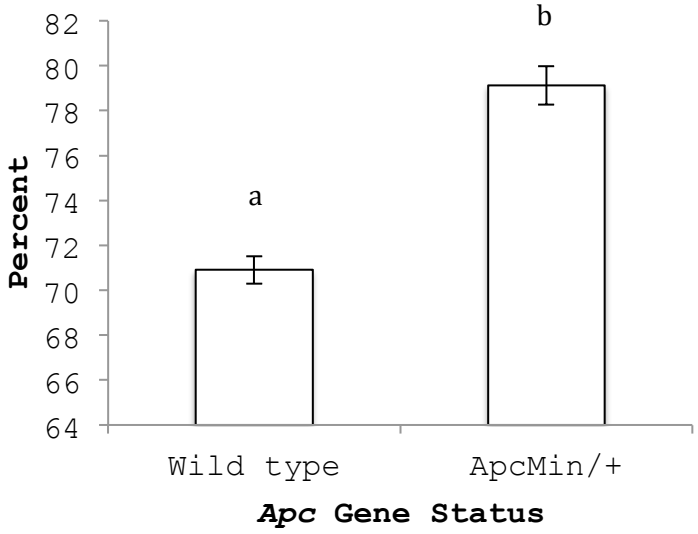


Figure 12. Mean lean body mass percentage of wild type C57BL/6J and *Apc*$^{Min/+}$ mice.



[ab] Means not sharing a common superscript are different (P<0.05).

Figure 13. Mean lean body mass percentage of wild type C57BL/6J and *Apc*$^{Min/+}$ mice.



[ab] Means not sharing a common superscript are different (P<0.05).

Table 2. Total small intestine adenoma number, average size, and burden in *Apc*$^{Min/+}$ mice fed diets without (-) or with (+) 1500 ppm anthocyanins: (-) cornstarch, (-) sucrose, (-) glucose, (+) cornstarch, (+) sucrose, or (+) glucose.[#]

TOTAL SMALL INTESTINE	**Adenoma Number**	**Average Size (mm^2)**	**Total Burden*** **(mm^2)**
Sex[+]			
Female	51.68 ± 4.02^{a}	1.96 ± 0.13	96.36 ± 8.85
Male	38.67 ± 5.03^{b}	1.91 ± 0.16	79.81 ± 11.08
Treatment (Female)			
(-) Cornstarch	35.00 ± 11.42^{bcd}	2.26 ± 0.37	78.60 ± 25.13^{bc}
(-) Sucrose	23.88 ± 8.08^{cd}	2.06 ± 0.26	46.71 ± 17.77^{bc}
(-) Glucose	105.60 ± 10.21^{a}	1.90 ± 0.31	196.51 ± 22.47^{a}
(+) Cornstarch	40.25 ± 8.08^{bcd}	2.38 ± 0.26	90.84 ± 17.77^{b}
(+) Sucrose	48.83 ± 9.32^{bc}	1.73 ± 0.30	81.36 ± 20.52^{bc}
(+) Glucose	56.50 ± 11.42^{b}	1.46 ± 0.37	84.12 ± 25.13^{bc}
Treatment (Male)			
(-) Cornstarch	41.67 ± 13.19^{bcd}	2.02 ± 0.42	94.47 ± 29.01^{bc}
(-) Sucrose	34.67 ± 13.19^{bcd}	2.51 ± 0.42	86.98 ± 29.01^{bc}
(-) Glucose	23.00 ± 9.32^{c}	1.15 ± 0.30	30.44 ± 20.52^{c}
(+) Cornstarch	39.33 ± 13.19^{bcd}	1.80 ± 0.42	75.31 ± 29.01^{bc}
(+) Sucrose	53.00 ± 11.42^{b}	1.91 ± 0.37	97.86 ± 25.13^{b}
(+) Glucose	40.33 ± 13.19^{bcd}	2.09 ± 0.42	93.82 ± 29.01^{bc}

[#] Data presented as least square mean ± SEM.

* Total burden equal to the sum of all adenomas within the intestinal segment.

[+] Sex is considered a different category from sex x treatment.

[abcd] Means not sharing a common superscript within a column and category are different ($P<0.05$).

Table 3. Proximal small intestine adenoma number, average size, and burden in *Apc*$^{Min/+}$ mice fed diets without (-) or with (+) 1500 ppm anthocyanins: (-) cornstarch, (-) sucrose, (-) glucose, (+) cornstarch, (+) sucrose, or (+) glucose.[#]

PROXIMAL SMALL INTESTINE	**Adenoma Number**	**Average Size (mm^2)**	**Total Burden[*] (mm^2)**
Sex[+]			
Female	11.92 ± 0.82[a]	3.28 ± 0.31	34.25 ± 2.90
Male	7.97 ± 1.06[b]	3.22 ± 0.40	26.28 ± 3.72
Treatment (Female)			
(-) Cornstarch	8.40 ± 2.14	4.20 ± 0.81	31.38 ± 7.54[ab]
(-) Sucrose	7.13 ± 1.69	3.70 ± 0.64	25.92 ± 5.96[b]
(-) Glucose	20.80 ± 2.14	2.78 ± 0.81	50.96 ± 7.54[a]
(+) Cornstarch	8.75 ± 1.69	4.43 ± 0.64	36.51 ± 5.96[ab]
(+) Sucrose	12.67 ± 1.96	2.69 ± 0.74	33.26 ± 6.89[ab]
(+) Glucose	13.75 ± 2.40	1.93 ± 0.91	27.49 ± 8.43[bc]
Treatment (Male)			
(-) Cornstarch	5.67 ± 2.77	4.89 ± 1.05	30.30 ± 9.74[abc]
(-) Sucrose	10.00 ± 2.77	3.58 ± 1.05	34.23 ± 9.74[ab]
(-) Glucose	4.83 ± 1.97	1.60 ± 0.74	6.49 ± 6.89[c]
(+) Cornstarch	6.33 ± 2.77	3.52 ± 1.05	22.01 ± 9.74[bc]
(+) Sucrose	12.75 ± 2.40	2.56 ± 0.91	29.13 ± 8.43[ab]
(+) Glucose	8.33 ± 2.77	3.16 ± 1.05	35.51 ± 9.74[ab]

[#] Data presented as least square mean ± SEM.

[*] Total burden equal to the sum of all adenomas within the intestinal segment.

[+] Sex is considered a different category from sex x treatment.

[abc] Means not sharing a common superscript within a column and category are different ($P<0.05$).

Table 4. Medial small intestine adenoma number, average size, and burden in *Apc*$^{Min/+}$ mice fed diets without (-) or with (+) 1500 ppm anthocyanins: (-) cornstarch, (-) sucrose, (-) glucose, (+) cornstarch, (+) sucrose, or (+) glucose.[#]

MEDIAL SMALL INTESTINE	**Adenoma Number**	**Average Size (mm^2)**	**Total Burden**[*] **(mm^2)**
Sex			
Female	18.58 ± 1.76	2.04 ± 0.18	35.51 ± 3.51
Male	13.88 ± 2.25	1.75 ± 0.23	26.02 ± 4.50
Treatment (Female)			
(-) Cornstarch	13.00 ± 4.56	2.28 ± 0.47	30.25 ± 9.12
(-) Sucrose	8.25 ± 3.60	1.74 ± 0.37	14.46 ± 7.21
(-) Glucose	36.20 ± 4.56	1.85 ± 0.47	65.27 ± 9.12
(+) Cornstarch	15.25 ± 3.62	2.60 ± 0.37	35.56 ± 7.21
(+) Sucrose	18.50 ± 4.17	1.96 ± 0.43	30.21 ± 8.33
(+) Glucose	20.25 ± 5.10	1.79 ± 0.52	37.31 ± 10.20
Treatment (Male)			
(-) Cornstarch	16.33 ± 5.89	1.28 ± 0.61	24.34 ± 11.77
(-) Sucrose	10.33 ± 5.89	2.46 ± 0.61	26.77 ± 11.77
(-) Glucose	10.00 ± 4.17	1.27 ± 0.43	15.41 ± 8.33
(+) Cornstarch	11.67 ± 5.89	1.63 ± 0.61	23.30 ± 11.77
(+) Sucrose	17.25 ± 5.10	1.76 ± 0.52	29.89 ± 10.20
(+) Glucose	17.67 ± 5.89	2.07 ± 0.61	36.39 ± 11.77

[#] Data presented as least square mean ± SEM.

[*] Total burden equal to the sum of all adenomas within the intestinal segment.

Means in this table are not different (P>0.05).

Table 5. Distal small intestine adenoma number, average size, and burden in *Apc*$^{Min/+}$ mice fed diets without (-) or with (+) 1500 ppm anthocyanins: (-) cornstarch, (-) sucrose, (-) glucose, (+) cornstarch, (+) sucrose, or (+) glucose.[#]

DISTAL SMALL INTESTINE	**Adenoma Number**	**Average Size (mm^2)**	**Total Burden**[*] **(mm^2)**
Sex[+]			
Female	21.50 ± 1.97	1.20 ± 0.15	27.36 ± 4.28
Male	16.81 ± 2.47	1.35 ± 0.18	27.52 ± 5.36
Treatment (Female)			
(-) Cornstarch	15.50 ± 5.60	1.33 ± 0.41	21.56 ± 12.15^{bc}
(-) Sucrose	8.50 ± 3.96	1.20 ± 0.30	6.33 ± 8.59^{c}
(-) Glucose	48.60 ± 5.01	1.58 ± 0.38	80.27 ± 10.87^{a}
(+) Cornstarch	16.25 ± 3.96	1.40 ± 0.30	18.77 ± 8.59^{bc}
(+) Sucrose	17.67 ± 4.57	0.87 ± 0.34	17.89 ± 9.92^{bc}
(+) Glucose	22.50 ± 5.60	0.80 ± 0.42	19.31 ± 12.15^{bc}
Treatment (Male)			
(-) Cornstarch	19.67 ± 6.47	1.53 ± 0.48	39.83 ± 14.03^{b}
(-) Sucrose	14.33 ± 6.47	1.82 ± 0.48	25.98 ± 14.03^{bc}
(-) Glucose	8.17 ± 4.57	0.84 ± 0.34	8.54 ± 9.92^{bc}
(+) Cornstarch	21.33 ± 6.47	1.15 ± 0.48	29.99 ± 14.03^{bc}
(+) Sucrose	23.00 ± 5.60	1.31 ± 0.42	38.83 ± 12.15^{b}
(+) Glucose	14.33 ± 6.47	1.44 ± 0.48	21.93 ± 14.03^{bc}

[#] Data presented as least square mean ± SEM.

[*] Total burden equal to the sum of all adenomas within the intestinal segment.

[+] Sex is considered a different category from sex x treatment.

[ab] Means not sharing a common superscript within a column and category are different ($P<0.05$).

Table 6. Colon adenoma number per mouse, adenoma number per adenoma bearing mouse, and percent incidence in *Apc*$^{Min/+}$ mice fed diets without (-) or with (+) 1500 ppm anthocyanins: (-) cornstarch, (-) sucrose, (-) glucose, (+) cornstarch, (+) sucrose, or (+) glucose.[#]

COLON	Adenoma Number/Mouse	Adenoma Number/Tumor Bearing Mouse	Incidence (%)
Sex			
Female	1.67 ± 0.41	2.63 ± 0.55	63.8 ± 8.12
Male	0.89 ± 0.53	1.42 ± 0.80	57.1 ± 9.52
Treatment			
(-) Cornstarch	0.47 ± 0.87	1.00 ± 1.42	50.0 ± 18.9
(-) Sucrose	2.52 ± 0.81	4.00 ± 1.03	63.6 ± 15.2
(-) Glucose	1.05 ± 0.72	1.75 ± 1.07	54.5 ± 15.7
(+) Cornstarch	1.06 ± 0.81	1.39 ± 0.99	81.8 ± 12.2
(+) Sucrose	1.66 ± 0.77	2.50 ± 1.07	60.0 ± 16.3
(+) Glucose	0.92 ± 0.91	1.50 ± 1.42	42.9 ± 20.2

[#]Data presented as least square mean ± SEM.
Means in this table are not different (P>0.05).

Table 7. Average colon adenoma size and adenoma burden in all *Apc*$^{Min/+}$ mice fed diets without (-) or with (+) 1500 ppm anthocyanins: (-) cornstarch, (-) sucrose, (-) glucose, (+) cornstarch, (+) sucrose, or (+) glucose. [#]

COLON	Average Adenoma Size (mm^3)	Adenoma Burden* (mm^3)
Sex		
Female	5.82 ± 1.55	13.26 ± 3.52
Male	7.78 ± 1.99	9.71 ± 4.51
Treatment		
(-) Cornstarch	4.68 ± 3.29[b]	4.68 ± 7.47
(-) Sucrose	16.41 ± 3.06[a]	29.79 ± 6.92
(-) Glucose	3.15 ± 2.73[b]	4.96 ± 6.19
(+) Cornstarch	8.67 ± 3.06[ab]	10.06 ± 6.92
(+) Sucrose	3.80 ± 2.91[b]	9.76 ± 6.60
(+) Glucose	4.11 ± 3.45[b]	9.64 ± 7.81

[#] Data presented as least square mean ± SEM.

* Total burden equal to the sum of all adenomas within the intestinal segment.

[ab] Means not sharing a common superscript within a column and category are different ($P<0.05$).

Table 8. Average colon adenoma size and adenoma burden in adenoma bearing *Apc*$^{Min/+}$ mice fed diets without (-) or with (+) 1500 ppm anthocyanins: (-) cornstarch, (-) sucrose, (-) glucose, (+) cornstarch, (+) sucrose, or (+) glucose.[#]

COLON	**Average Adenoma Size (mm^3)**	**Adenoma Burden*** **(mm^3)**
Sex		
Female	8.80 ± 1.91	20.65 ± 4.79
Male	13.07 ± 2.79	15.55 ± 7.00
Treatment		
(-) Cornstarch	8.85 ± 4.98[b]	8.85 ± 12.52
(-) Sucrose	24.94 ± 3.61[a]	46.35 ± 9.07
(-) Glucose	7.18 ± 3.74[b]	10.63 ± 9.39
(+) Cornstarch	11.31 ± 3.46[b]	13.12 ± 8.69
(+) Sucrose	6.05 ± 3.74[b]	15.05 ± 9.39
(+) Glucose	7.22 4.98[b]	14.60 ±12.52

[#]Data presented as least square mean ± SEM.

* Total burden equal to the sum of all adenomas within the intestinal segment.

[ab] Means not sharing a common superscript within a column and category are different ($P<0.05$).

DISCUSSION

Apc$^{Min/+}$ mice weighed less than wild type C57BL/6J mice throughout the feeding period, with significant differences observed in every week except weeks three and five (Figure 5); however, body weights of both groups were significantly different ($P<0.05$) at conclusion of this study. Body weight differences also were observed to be influenced by a significant sex x *Apc* status interaction, with *Apc*$^{Min/+}$ males weighing less than wild type C57BL/6J males, and *Apc*$^{Min/+}$ females weighing less than wild type C57BL/6J females at the conclusion of this study. At termination, *Apc*$^{Min/+}$ males weighed approximately four grams more than *Apc*$^{Min/+}$ females (Figures 7 and 8). This observation is consistent with previous findings in our laboratory using several dietary interventions. The weight loss observed in *Apc*$^{Min/+}$ mice is highly correlated to morbidity associated with adenoma development in *Apc*$^{Min/+}$ mice. Weight loss increases as the average size of small intestinal tumors increases. When adenomas are approximately 1.5 mm in diameter or greater, adenomas have the tendency to hemorrhage and increase the risk of morbidity (Kang, 2005).

These observations also are consistent with a study conducted by The Jackson Laboratory (JAX) where mean weights of male and female wild type C57BL/6J mice were 29.71 and 21.99 g at 16 weeks of age (Jackson Laboratory, 2013). The mice in this study were also 16-17 weeks of age at termination, and body weights of wild type C57BL/6J male and female mice were 30.79 and 24.38 g respectively, suggesting AOM treatment did not influence body weight in wild type C57BL/6J mice

Body weights of *Apc*$^{Min/+}$ mice were less than wild type C57BL/6J mice in this study; however, it cannot be determined if this can be specifically attributed to tumorigenesis associated to the *Apc* knockout or AOM treatment, or a combination of both factors. However, these observations do suggest that tumorigenesis begins as early as three to four weeks of life and influences body weight early in development.

Dietary treatment did not have a significant influence on body weights of *Apc*$^{Min/+}$ mice at the conclusion of the study. Previous studies in this laboratory demonstrated mice fed sucrose weighed significantly more than mice fed starch (Wang, 2005; Powell, 2011). Although similar trends

were observed in this study, they were not statistically significant.

Mouse body fat and lean mass were only influenced by *Apc* status in this study, with $Apc^{Min/+}$ mice having less body fat and higher lean mass compared to their wild type counterparts on a percentage basis. This was a similar trend observed by Powell (2011); however, Powell (2011) also observed significant differences associated with similar dietary treatments based on starch, fructose, glucose, and sucrose.

In this study, *Apc* status and/or sex were the only factors that significantly influenced weight and body composition. These results contrast with previous findings from this laboratory, which can be attributed to the use of an aggressive treatment, AOM treatment in an $Apc^{Min/+}$ mouse model, and a longer dietary treatment period compared to Powell (2011). Although mice tolerated AOM injections well, AOM injections may have contributed to weight loss, and mice that lost weight tended to lose weight during the final three weeks and this may have contributed to results with non-significant differences.

The number of total small intestinal adenomas was significantly greater in females than males. This

difference was also observed in the proximal section of the small intestine, but not the medial or distal sections. Average adenoma size and burden were not different between sexes. The net effect of this observation is sex influenced tumor multiplicity. This observation is consistent with research results from this laboratory using the same mouse model (Powell, 2011; Kang, 2002; Cao, 2012).

In this study, a sex x carbohydrate x anthocyanin interaction was detected for total small intestinal adenoma number and adenoma burden. Female mice consuming sucrose had the second fewest total small intestinal adenomas among all treatment groups. However, females that consumed anthocyanin-sucrose did not show a significantly different number of adenomas, average size or burden from those that consumed only sucrose. Distal small intestinal adenoma burden was the lowest in females consuming sucrose, which can likely be the reason females had second lowest total small intestinal burden of all treatment groups. Distal small intestinal adenoma burden was lower than what was observed in the proximal and medial small intestine of females. This may be attributed to sucrose's components, D-glucose and fructose, primarily being absorbed in the proximal and medial sections (Wright, 1993; Douard and Ferraris, 2008). Additionally, sucrose has been shown to

promote intestinal proliferation in murine models (Wang, 2005; Caderni et al., 1991; Caderni et al., 1997). This may explain why female mice consuming sucrose had a greater burden in the upper two-thirds of the small intestine compared to the distal third, and why total small intestinal burden was lower. However, this observation was only significantly different for females that consumed glucose.

Female mice that consumed glucose showed the greatest total small intestinal adenoma number among all groups. Many types of cancer cells have increased glucose uptake (Taubes, 2012). This observation may be explained by the Warburg effect, as described by Otto Warburg in the 1920s. He observed that cancer cells utilize glucose in a process called aerobic glycolysis. In this process, cancer cells convert glucose -> pyruvate -> lactate, which is used as a primary energy source in cells' cytoplasm (Taubes, 2012). Researchers have shown the enzyme M2-pyruvate kinase (M2PK), which converts phosphoenolpyrutave to pyruvate, can be used as a biomarker for colorectal cancer because it is highly expressed in cancer cells (Kumar et al, 2007; Hardt et al, 2004). Reducing the activity of this enzyme can reverse the Warburg effect and thus implicates (M2PK) as a key component of glucose metabolism in cancer cells

(Christofk et al., 2008). The Warburg effect also may explain why females consuming glucose had the greatest burden in the distal small intestine (Table 5). Since cancer cells have been shown to prefer glucose as an energy source, it is possible adenomas in the distal small intestine had increased glucose utilization in this study, which may lead to an increased capacity for proliferation.

Dietary polyphenols have been shown *in vitro* to decrease glucose uptake in human cancer cell lines and in animal cells (Kobayashi et al., 2000; Johnston et al., 2005). In Caco-2 cancer cells, activity of sodium-dependent glucose transporters was inhibited by flavonoid glycosides and naturally occurring dietary aglycones. However, aglycones (those that are produced by hydrolysis during digestion) and phenolic acids did not inhibit glucose transportation. In sodium-free conditions, aglycones inhibited glucose transportation, whereas the glycosides and phenolic acids did not. These results suggest aglycones may inhibit glucose transporters like those in the GLUT family, while the glycosides may inhibit sodium-dependent active transporters (Johnston, 2005). In rat tissue, green tea (-)-epicatechin gallate and (-)-epigallocatechin gallate has been shown to inhibit glucose transport by acting as a sodium-dependent glucose transporter 1 (SLGT1)

antagonist *in vitro* (Kobayashi et al., 2000). Additionally, the glucose transporter (GLUT2), which is found on both the apical and basolateral membrane of the small intestines (Wright, 1993), has been demonstrated to transport anthocyanins *in vitro* (Kobayashi et al., 2000). In Caco-2 cells, treatment with anthocyanins decreased glucose transport while increasing GLUT2 expression (Faria et al., 2009).

In this study, anthocyanins consumption was associated with a decrease in tumorigenesis in females consuming glucose, with a site-specific influence in the proximal and distal small intestine. No other relationships between diet and adenoma development for female mice can be established based on the results of this study.

The results for male mice are less defined. There were no significant differences in adenoma number when comparing mice consuming cornstarch, sucrose, or glucose to their corresponding anthocyanin containing diets in the whole small intestine or individual intestinal sections. Adenoma burden in male mice was not influenced by diet in the whole small intestine; however, adenoma burden in the proximal small intestine was significantly greater for males consuming anthocyanin-glucose compared to glucose. This observation is the opposite of what was observed in female

mice. There may be two reasons for this difference. Firstly, three male mice consuming glucose were sacrificed five weeks early due to a weight loss of 10 percent. Secondly, only four male mice completed the dietary treatment for this group. These factors contributed to an incomplete data set for this treatment group.

The differences between sexes for mice consuming glucose with or without anthocyanins are too inconsistent to formulate a hypothesis for the difference observed between sexes in this study.

Additionally, it has been demonstrated anthocyanins and anthocyanidins act as antioxidants and anti-inflammatory agents (Seeram et al., 2001; Seeram et al., 2003, Mulabagal et al., 2009), which may explain how anthocyanins in this study reduced tumor number and burden in female mice consuming glucose.

Anthocyanins have been shown to be inhibitors of COX activity and inflammation. Since inflammation has not been strongly linked to sporadic colorectal cancer, it is unlikely to be a significant initiator of colorectal cancer (Terzic et al., 2010). However, chronic inflammation and up-regulation of COX-2 and increased levels of its prostaglandin products have been linked to tumor promotion (Taketo, 1998; Yang et al., 1998). Furthermore, NSAIDs such

as sulindac and Licofelone ([2,2-dimethyl-6-(4-chloropheny-7-phenyl-2,3-dihydro-1H-pyrrazoline-5-yl] acetic acid) have been shown experimentally to reduce small intestinal adenoma number in the same mouse model used in this study (Kang, 2005; Mohammed et al, 2011). The combination of anthocyanins and sulindac also has been shown to reduce tumor number and total adenoma burden, but not average size (Bobe et al., 2006). This combination may be more effective at reducing COX activity than anthocyanins alone, which was also observed by Wang et al. (1999).

In this study, it is hypothesized that anthocyanins are absorbed in the small intestine by GLUT2 and inhibit SGLT1 and GLUT2 glucose absorption (Faria et al., 2009; Kobayashi et al., 2000; Johnston et al., 2005). Then, anthocyanins, or anthocyanidins after removal of sugar residues, may act as anti-inflammatory and antioxidant agents (Tsuda et al., 1999; Seeram et al., 2003; Wang et al., 1999); thereby reducing small intestinal adenoma burden associated with glucose consumption.

In this study, carbohydrate source did not significantly influence colon adenoma number, burden or incidence. These results are consistent with those observed by Wang (2005) in his first study and by Powell (2011). Additionally, no cecal adenomas were observed in any mouse,

which is in contrast to the results observed by Kang (2002). However, other researchers have also observed a low number of cecal tumors in studies using *Apc*$^{Min/+}$ mice (Erdman et al., 2005; and Yang et al., 2003).

Previous research in this laboratory has yielded consistent results with colonic tumor multiplicity in diets with varying carbohydrate sources. Mice that consumed sucrose had the same number of adenomas compared to mice consuming starch (Wang et al., 2009; Powell, 2011; and Wang, 2005). The numbers of colonic adenomas in mice consuming varying carbohydrates in this study was generally greater than the number observed by Powell (2011) and Wang (2005), which indicates the AOM injections may have influenced tumor multiplicity as anticipated. This increase in tumor multiplicity associated with AOM treatment is consistent with findings by Suzui et al. (2002) and Issa et al. (2007).

A significant carbohydrate x anthocyanin interaction was observed for average colonic adenoma size (Tables 7 & 8). Mice consuming sucrose had significantly larger adenomas compared to all other diets. Wang et al. (2009) also observed that sucrose significantly influenced adenoma incidence and size when compared to mice consuming starch. However, Wang's observation was not significant in adenoma-

bearing mice (Wang et al., 2009). It has been hypothesized that sucrose consumption, in this model and other murine models, promotes intestinal proliferation. Increased cell proliferation by sucrose has been demonstrated with Ki67 labeling (Wang, 2005). Enhanced aberrant crypt formation has also been demonstrated in rodents fed sucrose (Caderni et al., 1991; Caderni et al., 1997). Short-term high-sucrose consumption increases oxidative stress in rats (Busserolles et al., 2002). Therefore, it is reasonable to suggest that long-term consumption of high-sucrose diets may have contributed to consistent oxidative stress in this study thereby increasing the likelihood for additional genetic mutations and adenoma promotion.

The link between sucrose consumption and elevated inflammatory markers also was observed in a dietary intervention study involving 41 overweight patients. The study demonstrated that consumption of high sucrose diets increased serum levels of haptoglobin and transferrin (Sorensen et al., 2005). Similarly, epidemiological data from the Nurses' Health Study positively correlated sugar and refined grain consumption with inflammatory markers such as IL-6 and TNF-α (Schulze et al., 2005).

In this study, mice consuming the anthocyanin-sucrose diet demonstrated a reduced colonic adenoma burden when compared to the mice consuming only sucrose.

Anthocyanins can be absorbed as intact glycosides (Miyazawa et al., 1999). Cyanidin 3-*O*-β-D-glycoside (C3G) and one of its metabolites, protocatechuic acid, has also been detected in the plasma of rats given C3G orally by gavage (Tsuda et al., 1999). Additionally, methylated C3G has been detected in liver and kidney tissue (Tsuda et al., 1999). In a human study, anthocyanins were detected in plasma and urine samples after test subjects consumed cherry powder, and antioxidant capacity of plasma increased after cherry ingestion (Kirakosyan et al., 2010). Finally, Zucker rats fed cherry powder had decreased plasma inflammatory markers (Seymour et al., 2009). These results support the potential of anthocyanins to be absorbed and act as antioxidants or anti-inflammatory agents *in vivo*.

The hypothesized mechanism by which anthocyanins reduce colonic adenoma promotion is by inhibiting COX-I and COX-II enzymatic activity and thereby reducing inflammation, a hallmark of cancer. Animal studies investigating this hypothesis have shown that consumption of tart cherries reduces plasma TNF-α and IL-6, and mRNA

TNF-α and IL-6, which are key regulators of COX enzyme expression (Seymour et al., 2009).

Additionally, cell culture studies investigating the effect of various anthocyanins and anthocyanidins on COX enzyme expression support this hypothesis (Seeram et al., 2001; Seeram et al, 2003; Wang et al., 1999).

SUMMARY AND CONCLUSIONS

This study demonstrated that intestinal tumor development in *Apc*$^{Min/+}$ mice was influenced by feeding diets with varying carbohydrate sources and by anthocyanins. Consumption of glucose led to the greatest small intestinal adenoma number and burden in female mice, and the stimulatory effect of glucose was inhibited by the addition of dietary anthocyanins. The hypothesized mechanism of action of anthocyanins is by inhibiting glucose absorption and by acting as anti-inflammatory and anti-oxidative agents.

Consumption of sucrose led to the greatest colonic tumor size in both male and female mice. The stimulatory effect of sucrose in this model is consistent with research published previously by this laboratory. Additionally, anthocyanins reduced average adenoma size in mice fed sucrose. The hypothesized mechanism of action of anthocyanins in the colon is their ability to act as anti-inflammatory and antioxidative agents, and to reduce inflammation-induced cell proliferation associated with sucrose consumption.

In this study, 1.5 grams of approximately 50 percent pure anthocyanins were added to each kilogram of diet. This

in effect resulted in an actual anthocyanin dose of 750 PPM Assuming mice consumed 15 g per 100 g of bodyweight and had a mean body weight of 21 grams from the start of dietary treatment to termination, *Apc*$^{Min/+}$ mice would have consumed an average of 2.4 mg/day of anthocyanins per day. This would be the equivalent of 6.0 g/day of the cherry variety used to extract the anthocyanins in this study, based on an anthocyanin content of 0.4 mg/g (Kang et al., 2003). However, it is important to note that a dose-dependence was not investigated in this study, and doses lower than 750 ppm may be equally effective in this model.

In conclusion, these results support the health promoting properties of anthocyanins; however, additional research is needed to determine the mechanisms hypothesized by these research findings and to investigate the potential of anthocyanins to influence human cancer biomarkers.

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