Dietary Supplementation With Tart Cherries for Prevention of Inflammation-Associated Colorectal Cancer in Mice

Ashlie Hunter
Utah State University

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DIETARY SUPPLEMENTATION WITH TART CHERRIES FOR PREVENTION OF INFLAMMATION-ASSOCIATED COLORECTAL CANCER IN MICE

by

Ashlie Hunter

Thesis submitted in partial fulfillment of the requirements for the degree of

UNIVERSITY HONORS

in

Biology (Cellular/Molecular Emphasis) in the Department of Biology

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Departmental Honors Advisor
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Honors Program Director
Dr. Kristine Miller

UTAH STATE UNIVERSITY
Logan, UT

Spring 2017
ABSTRACT

Dietary Supplementation with Tart Cherries for Prevention of Inflammation-Associated Colorectal Cancer in Mice

by

Ashli Hunter, Bachelor of Science
Utah State University, 2017

Advisor: Abby D. Benninghoff
Departments: Degree, Department of Biology; Research activity, Department of Animal, Dairy and Veterinary Sciences

The cherry fruit is a nutrient-dense food with comparatively low caloric content and significant amounts of key nutrients and bioactive food chemicals. Much of the health benefit of cherries is attributed to their high amounts of anthocyanins, which have anti-oxidant and anti-cancer properties that contribute to changes in cell signaling pathways involved in inflammation, carcinogenesis and angiogenesis. In this project, we aimed to determine whether dietary supplementation with tart cherries prevents colon tumor development in mice consuming a Western diet compared to a prudent diet. Previously, our research team developed the Total Western Diet (TWD) for mice to emulate typical U.S. nutrition with respect to macro- (sugars, fats, and protein) and micronutrient (vitamin and mineral) contents. Incorporating the TWD in pre-clinical studies allows for the analysis of the impact dietary bioactives or functional foods on tumor development in a physiological environment that reflects nutritional patterns of the average American. We hypothesized that dietary supplementation with freeze-dried whole tart cherries would suppress development of colon tumors in a model of inflammation-associated colorectal cancer. A 2x2 factorial design was employed, whereby mice were fed either AIN93G
(optimized for rodent health) or the TWD, each with and without Montmorency tart cherry powder added to the diet for a total anthocyanin content of 188 mg/kg diet. Mice were initiated with 10 mg/kg azoxymethane and provided 1% dextran sodium sulfate (DSS) in their drinking water for 4 weeks to promote colonic inflammation and tumorigenesis. Necropsy and tumor assessment was performed after 15 weeks of treatment. TWD consumption markedly enhanced colitis activity (40-fold increase) compared to mice fed AIN93G. Moreover, TWD-fed mice had significantly higher histopathology scores for inflammation and mucosal injury during the period of colitis and, importantly, during the recovery phase of this disease model. Colonic inflammation in TWD-fed mice persisted to the end of the study (day 105), whereas mucosal injury (save for sites of neoplasia) had resolved. Also, as expected based on prior studies, mice fed TWD had higher tumor multiplicity (near 6-fold increase) compared consumption of the AIN93G diet. The most important observation in this study was that supplementation with tart cherry powder caused a significant 40% reduction ($p<0.05$) in tumor incidence in mice fed AIN93G. However, tart cherries had no effect on tumor incidence in mice fed TWD. Also, tart cherry powder supplementation did not significantly affect histopathology scores for inflammation or mucosal injury nor tumor multiplicity or size as compared to AIN93G- or TWD-fed counterparts. Moreover, addition of the tart cherry supplement did not significantly affect colitis disease activity. These data contrast with a prior observation by our group that green tea supplementation was effective at reducing development of aberrant crypts, but only in mice fed TWD. These observations point to important interactions between basal diets and dietary bioactive supplements and underscore the need for careful consideration of the role of basal diet in dietary chemoprevention studies in rodents.
DEDICATION

This work is dedicated to my parents Greg and Christie Hunter with great appreciation for their many years of true support and love. I also dedicate this work to my siblings Justin, Brenna, Erika and John who helped me keep a positive perspective through this long journey. Finally, I want to dedicate this work to Mr. Rawlins, a favorite teacher in high school who sparked my life-long interest in science.
ACKNOWLEDGEMENTS

I would like to express my heartfelt thanks to my undergraduate mentor, Dr. Abby Benninghoff. She created the opportunity for a young, inexperienced freshman to get involved in research. Her support and encouragement fostered my curiosity and shaped me into the young scientist I am today. This thesis and all the work it culminates were only possible because her keen eyes saw the potential in me. Thank you for taking notice, creating an experience, and allowing me to flourish in your laboratory.

I would also like to extend my gratitude to Dr. Korry Hintze who has also allowed me to grow and develop in his laboratory. Thank you for your encouragement and guidance. My experiences in the laboratory have also been greatly shaped by the wonderful people with whom I have been blessed to work. I would like to thank the members of the Benninghoff Laboratory past and present with whom I have been fortunate to work. I would like to especially thank Deanna Larson, Sumira Phatak, Tess Armbrust, and Veronica Martel who assisted in the completion of this project.

Finally, I would like to thank the people who have been there since day one, my wonderful parents, Greg and Christie Hunter. Thank you for teaching me to reach for the stars and helping me as I stumble along the journey. I have only gotten this far because of you.
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<th>Description</th>
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<tbody>
<tr>
<td>AIN</td>
<td>American Institute of Nutrition</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AOM</td>
<td>azoxymethane</td>
</tr>
<tr>
<td>CAC</td>
<td>colitis associated colorectal cancer</td>
</tr>
<tr>
<td>CRC</td>
<td>colorectal cancer</td>
</tr>
<tr>
<td>DAI</td>
<td>disease activity index</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sodium sulfate</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin stain</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error measure</td>
</tr>
<tr>
<td>TC</td>
<td>tart cherry</td>
</tr>
<tr>
<td>TWD</td>
<td>total western diet for rodents</td>
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**Symbols**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>mg</td>
<td>milligrams</td>
</tr>
<tr>
<td>kcal</td>
<td>kilocalories</td>
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</table>
INTRODUCTION

Approximately 25% of all deaths in countries with a Westernized lifestyle are attributed to cancer (Boyle and Langman, 2000). Colorectal cancer (CRC) is the second leading cause of cancer-related death in men and the third leading cause in women in the United States, with 135,430 estimated new cases projected for 2017 (American Cancer Society, 2017). In 2010 the estimated cost of medical care for CRC was $14 billion (Kim and Milner, 2007). Risk for developing CRC is determined by many factors including genetic susceptibility and lifestyle choices. Incidence of CRC is highest in whites and African Americans, and the disease primarily affects those over 50. Lifestyle factors, such as lack of exercise and poor nutrition, also promote the development of CRC.

Diet modification represents a safe and cost-effective strategy to decrease the incidence of cancer and/or delay the onset of the disease. Several foods, micronutrients, and bioactive food components have been identified that may help reduce cancer risk, including chemicals present in certain fruits, vegetables and whole grains. However, many individuals consume a diet that is deficient in these food items and the beneficial micronutrients and bioactive chemicals they provide. The Western diet is characterized by a high consumption of simple carbohydrates and fat, with a low intake of many essential micronutrients (Cordain et al., 2005). Consumption of a Westernized-diet is associated with an increased risk of developing chronic degenerative diseases, including cancer (Carrera-Bastos et al., 2011). Accurately modeling dietary intakes is a major research challenge for scientists studying the role of diet in modifying disease state or risk to improve translation from animal models to clinical studies (Lai et al., 2014). Functional foods that prevent development of CRC using an animal model that represents typical U.S. nutrition, which is energy-dense and nutrient-poor, need to be identified.
In most studies investigating the contribution of functional foods, bioactive food components or micronutrients for cancer prevention, researchers routinely employ standard diets that are balanced with respect to macro- and micronutrient levels to optimize rodent health, such as the AIN diets formulated by the American Institute of Nutrition. Although numerous studies have examined the effects of bioactive chemicals on colorectal cancer development, few of these studies have incorporated the typical American diet as part of the experiment design.

The typical diet consumed by Americans lacks essential micronutrients and contains an abundance of simple carbohydrates, fats, and sodium. Mouse studies using dextran sodium sulfate (DSS) to induce colitis have shown that high fat and high sugar diets exacerbate the disease state. However, these studies have focused on dietary macronutrients only (Agus et al., 2016; Risio et al., 1996). The total Western diet (TWD) for mice was developed to emulate typical U.S. nutrition with respect to macro- (sugars, fats, and protein) and micronutrient (vitamin and mineral) contents (Hintze et al., 2012). Incorporating the TWD in pre-clinical studies allows for the analysis of the impact of dietary bioactives or functional foods on tumor development in a physiological environment that reflects nutritional patterns of the average American. Previous studies by our group demonstrated a promoting effect of TWD on development of pre-neoplastic lesions and colon tumors in A/J or C57BL/6J mice (Perez Monsanto, 2013; Ward et al., 2017). Addition of green tea, a beverage with anti-cancer and anti-inflammatory properties, significantly reduced tumor multiplicity and volume in mice fed TWD (Ward et al., 2017). However, green tea supplementation had no significant effect on tumor development in mice fed the control diet. This observation reinforced the idea that researchers should consider basal nutrition when examining putative bioactives or functional foods in pre-clinical studies.
Anthocyanins have anti-oxidant and anti-cancer properties that contribute to changes in cell signaling pathways involved in inflammation, carcinogenesis and angiogenesis (Domitrovic, 2011; Wang and Stoner, 2008) (Fig. 1). The purported health benefits of anthocyanins extend from prevention of obesity and type-II diabetes to promotion of cardiovascular and neural health (Tsuda, 2012). Much of the health benefit of cherries is attributed to their high amounts of

![Base structure of anthocyanins](base_structure.png)

**Figure 1. Example structures of select anthocyanins.** Anthocyanins are commonly found in brightly colored fruits, such as berries and tart cherries. Anthocyanidins are the sugar-free components of the parent bioactive compound.
anthocyanins, which are water-soluble, bioavailable chemicals responsible for the fruit’s bright red color (McCune et al., 2011). Cyanidin 3-glucosylrutinoside, cyanidin 3-rutinoside, and cyanidin 3-sophoroside provide most of the anthocyanin content found in Montmorency tart cherries (Kirakosyan et al., 2015). Cherry anthocyanins potentially modulate several cellular processes including cell cycle arrest, apoptosis, and cellular differentiation promotion (McCune et al., 2011; Tsuda, 2012). In vitro experiments illustrate protection of cells from oxidative stress and inflammation by anthocyanins (Lim et al., 2011; Song et al., 2013). Reductions in COX-2 and COX-2 promoters along with attenuation of several proteins including NF-kB by stopping activation of the Fyn signaling pathway have been shown with administration of cyanidin 3-glucoside in vitro (Lim et al., 2011). Reduction in tissue NF-kB and other inflammatory cytokines has also been shown in vivo (Seymour et al., 2015). Cyanidin 3-glucoside has also been shown to have antioxidant properties, reducing ROS production and increasing recovery of glutathione in human breast cancer cells (Song et al., 2013). Malvidin, another major anthocyanin found in many berries and other anthocyanin-rich foods, also has been shown to have anti-proliferative and anti-inflammation properties (Bontempo et al., 2013; Bunea et al., 2013; Huang et al., 2014; López de Las Hazas et al., 2016; Oliveira et al., 2016).

The role of tart cherry consumption in prevention of CRC remains relatively unexplored. Bobe et al. examined the effect of dietary supplementation with tart cherries using the APC\textsuperscript{Min/+} mouse, a genetic model of intestinal cancer (Bobe et al., 2006). In this study, mice were fed tart cherries in combination with the anti-inflammatory drug sulindac. This combination treatment resulted in a modest reduction of multiplicity and tumor burden in the small intestine, but not in the colon. In a separate study, APC\textsuperscript{min/+} mice consuming cherries had reduced cecal tumor burden and multiplicity; however, no significant effect of cherry consumption on colon
tumorigenesis (Kang et al., 2003). However, neither study incorporated gut inflammation, which often accompanies the Western style diet. Thus, the goal of this experiment was to determine whether dietary supplementation with tart cherries, a functional food rich in anthocyanins, suppressed colon tumor development in mice consuming a Western diet compared to a prudent diet. We hypothesized that consumption of tart cherries would reduce tumor incidence, burden, and multiplicity in a murine model of CAC incorporating TWD.
MATERIALS AND METHODS

Chemicals

All chemicals were used according to standard operating protocols approved by the Utah State Environmental Health and Safety Office. Azoxymethane (AOM) at a concentration of 13.4 M and purity of ≥98% was obtained from Sigma-Aldrich (St. Louis, MO; CAS No. 25843-45-2). Dextran Sodium Sulfate salt (DSS) with a molecular weight ca. 40,000 was obtained from VWR (Radnor, PA; CAS No. 9011-18-1). Other reagent grade chemicals were purchased from general laboratory suppliers.

Animals

Procedures and handling of the mice were approved by the Utah State University Institutional Animal Care and Use Committee (protocol #2404). Male C57BL/J mice were obtained from Jackson at three weeks of age. The mice were quarantined for one week with free access to food and water before being assigned to one of the four experimental diet groups. The mice were group housed, four to a cage, in ventilated cages with Bed-o’cobs® bedding at Utah State University’s Utah Science Technology and Research (USTAR) BioInnovation facility vivarium. A 12:12 hour dark:light cycle was employed and the room was maintained at a constant temperature and humidity.

Diet Composition

AIN93G is a purified diet formulated for to support growth, lactation, and pregnancy of rodents (Reeves et al., 1993). This standard diet is frequently used in cancer pre-clinical studies as a basal diet and has an energy density of 3.8 kcal/g. The total Western diet for rodents (TWD) was formulated as previously described by Hintze et al. by employing the principle of nutrient density (Hintze et al., 2012). The energy density of the TWD is 4.4 kcal/g.
A powder made from freeze-dried, homogenized Montemorrency tart cherries was purchased from Shoreline Fruit (Traverse City, MI) under the brand name CherryPure®. Polyphenol content of the tart cherry powder was determined by HPLC and UV spectroscopy by Atlas Bioscience Labs (Tuscon AZ) and is reported in Table 1. The tart cherry powder (TC) was incorporated into the two basal diets (TWD and AIN93G) to achieve an estimated total anthocyanin content of 188 mg/kg. All four diets (AIN93G, TWD, AIN93G + TC, and TWD + TC) were formulated by Envigo® (Huntington, UK). The diets were stored at 4°C in the dark until use.

**Table 1. Bioactives in CherryPure® tart cherry powder from Shoreline Fruit**

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Amount</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total flavonoids (as rutin)</td>
<td>77.01 mg/g</td>
<td>HPLC</td>
</tr>
<tr>
<td>Melatonin</td>
<td>38.20 µg/g</td>
<td>HPLC</td>
</tr>
<tr>
<td>Polyphenolics</td>
<td>85.10 mg/g</td>
<td>UV-Vis</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malvidin</td>
<td>52.56 mg/g</td>
<td>HPLC</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>10.42 mg/g</td>
<td>HPLC</td>
</tr>
<tr>
<td>Pelargonidin</td>
<td>1.267 mg/g</td>
<td>HPLC</td>
</tr>
<tr>
<td>Peonidin</td>
<td>0.761 mg/g</td>
<td>HPLC</td>
</tr>
<tr>
<td>Delphinidin</td>
<td>3.033 mg/g</td>
<td>HPLC</td>
</tr>
<tr>
<td>Petunidin</td>
<td>0.624 mg/g</td>
<td>HPLC</td>
</tr>
<tr>
<td>Total anthocyanins</td>
<td>68.67 mg/g</td>
<td>HPLC</td>
</tr>
</tbody>
</table>

**Note:** Identification and quantitation of bioactives was performed by Atlas Bioscience Labs. HPLC, high performance liquid chromatography; UV-Vis, UV visual spectroscopy.

**Experimental Design**

A 2x2 factorial study design was employed with mice fed AIN93G or TWD basal diets with or without TC (Fig. 3). The mice were group-housed with four animals per cage. Body weight was recorded weekly, and freshly voided feces were collected from individual mice weekly. Fecal samples were frozen at -80°C for later analysis. Starting week 1, mice in groups 1-
Figure 3. **Experiment design.** The x-axis shows time in days and each horizontal bar represents a group of mice necropsied together. The white bars (G1 and G2) represent the AIN93G diet groups with and without tart cherry supplementation respectively, while the gray bars (G3 and G4) represent the TWD groups with and without tart cherry supplementation. Along the x-axis there are markers indicating endpoint measurements, treatments, and disease progression.

2 were fed AIN93G, whereas mice in groups 3-4 were fed TWD. Food was available to mice *ad libitum* and fresh food was provided twice weekly; food intake was estimated by differential weight. Mice were provided standard drinking water *ad libitum*, and fresh water was provided weekly. Following a 1-week diet run in period, mice in groups 2 and 4 were provided AIN93G or TWD diets, respectively, supplemented with TC. On study day 21, mice were initiated with a 10 mg/kg dose of azoxymethane (prepared in phosphate-buffered saline) by subcutaneous injection and 1% (w/v) dextran sodium sulfate (DSS) in their drinking water for 10 days. Colitis
disease activity was recorded on day 31 using a modified index scoring system which incorporated body weight loss, stool consistency, blood in the stool, and rectal bleeding on the last day of DSS treatment for all four treatment groups (De Fazio et al., 2014).

The study design included time points for subsampling mice from each group for assessment of histopathology. On study days 7, 31, and 45, two cages were randomly selected from each diet group for necropsy. All remaining mice were necropsied on day 105. Prior to necropsy, body composition measurements were recorded by EchoMRI-700 (Houston, TX) and freshly voided fecal samples were obtained. At necropsy, body, liver, kidney, spleen, and cecum weights were recorded. Blood was obtained by cardiac puncture, and serum was collected after centrifugation (10,000 × g) for 5 minutes using z-gel tubes (Sarstedt, Nümbrecht, Germany). Liver tissue, blood plasma and cecal contents were collected and stored at -80°C. Colons were excised from cecum to anus, and the lengths recorded prior to being split longitudinally. The flattened colon tissues were stored in 70% ethanol solution at 4°C until assessment of tumors by light microscopy. Randomly selected colon tissue samples were assessed within 24 h of collection, then transferred to 10% phosphate-buffered formalin for histopathology (details below).

**Colonic Tumor Assessment**

A dissecting microscope was used to visualize colon tissues, which were stained in 0.2% methylene blue and rinsed with phosphate buffered saline (PBS) to aid tumor visualization. Prior to assessment, tissues were blinded to the researcher to avoid detection bias. Tumors were measured in three dimensions, length (L), width (W) and depth (D), using digital calipers. The distance from the distal end of the colon was also noted. Tumor incidence was calculated as the percent of mice with tumors. Tumor multiplicity was calculated as the number of tumors per mm
of colon. Tumor volume was calculated by the equation \( V = \frac{\pi}{6} (L \times W \times D) \). Tumor burden was calculated as the total volume of tumors per tumor bearing animal.

**Histopathology**

Tissues selected for histopathology assessment were stored using the Swiss-roll method for 24 hours in 10% formalin and then transferred to 70% ethanol prior to embedding in paraffin. Microscopy slides were prepared from 3 \( \mu \)m sections of the paraffin block and stained with hematoxylin and eosin per standard protocol. These samples were submitted to the Utah Veterinary Diagnostic Laboratory for histopathological analysis by a board-certified pathologist. Tissues were assessed for inflammation and mucosal injury using a grading scheme previously described (Boivin et al., 2003; Washington et al., 2013). Sections were scored on a 3- to 4-point scale for three parameters: inflammation/cellular infiltration, epithelial regeneration and crypt damage. Inflammation parameters were summed together and then multiplied by a factor reflecting the percentage of the colon involved, 0 to 25% (1), 26 to 50% (2), 51 to 75% (3), and 76 to 100% (4), to obtain the overall score (up to 24). For inflammation, severity and distribution were separately assessed and combined into one score; assessment of the epithelium was evaluated by averaging the severity of crypt loss or ulceration over 15 high power fields (400×). For mucosal injury, crypt damage and regeneration were also summed and multiplied by a factor reflecting the percentage of the colon involved to obtain an overall score (up to 32).

**Statistical Analysis**

Statistical analysis for tumor incidence was performed using the Fisher’s exact test (GraphPad Prism), followed by the Bonferroni adjustment to correct for multiple post-hoc testing (SAS). For all other parameters, data were analyzed by mixed model ANOVA with Tukey HSD
post-hoc tests (JMP v.12, SAS Institute) with cage as a random factor nested within diet and supplement in the statistical model. Food and energy intakes were assessed on a per cage basis.
RESULTS

Estimated food and energy intakes

Food consumption was reported as total grams of food consumed for the entire 15-week study per mouse per cage (Fig. 4A). No effect of diet or supplementation on food consumption was observed. Total energy intake in kilocalories per mouse per cage was calculated and is reported in (Fig 4B). The total energy intake differed between the AIN93G and TWD groups (main effect of diet, p=0.0056). The TWD group consumed 15% more total kilocalories than the AIN93G group. Addition of TC to the TWD resulted in a 24% increase in total energy intake when compared to the AIN93G group. No significant difference was seen between the TWD and TWD+TC groups. The AIN93G diet has an energy density of 3.8 kcal/g, while the energy density for the TWD is 4.4 kcal/g. No difference in total energy intake due to tart cherry supplementation or interaction between TC and basal diet was observed.

Figure 4. Estimated food and energy consumption in mice fed AIN93G or TWD basal diets, with and without tart cherry supplementation. Values shown are the mean total food consumed (A) or mean total energy consumed (B) over the course of the 15-week study on a per cage basis + SEM (n=5 to 7 cages per group). Different letters indicate significant difference as determined by one-way ANOVA with Tukey post-hoc test for multiple comparisons. ANOVA main effects for diet, tart cherry and the interaction are shown.
**Body weight and composition**

Body weight gain over the 15-week study was not significantly affected by either AIN93G or TWD basal diets, nor supplementation with tart cherry powder (Fig 5A-C). The depression in growth rate and slight depression in body weight seen around 5 weeks corresponded with onset of colitis during treatment with DSS. Moreover, the final lean mass was not affected by either basal diet nor tart cherry (Fig. 5D), whereas a small, but significant ($p=0.0347$) increase of about 0.8 to 1.2 g fat mass, or an increase of 17% to 19% fat mass as percentage of body weight, was evident in mice fed TWD compared to those fed AIN93G (Fig.

![Figure 5](image)

Figure 5. Body weight and body composition in mice fed AIN93G or TWD basal diets, with and without tart cherry supplementation. Values shown are the weekly body weight (A), weekly body weight gain (B), final body weight (C), final lean mass (D) and final fat mass (E) + SEM ($n=20$ to 24 mice per group). Different letters indicate significant difference as determined by one-way ANOVA with Tukey post-hoc test for multiple comparisons. ANOVA main effects for diet, tart cherry and the interaction are shown.
Of note, fat mass gain was significantly greater in mice fed TWD+TC compared to both AIN93G and AIN93G+TC diet groups (Fig. 5E).

**Organ and cecum weights**

Liver weight, as mass or relative to body weight, was not significantly affected by either basal diet nor supplementation with tart cherry powder (Table 2). However, as has been observed previously in this diet model of inflammation-associated colorectal cancer, mice fed the TWD had a slightly higher kidney weight and markedly higher spleen weight compared to their AIN93G-fed counterparts (Table 2), likely a consequence of the high tumor burden in these mice. Interestingly, the mouse cecum weight (measured full at time of necropsy), varied depending upon both basal diet and tart cherry supplement, with a significant interaction between these two factors of $p=0.0006$ (Fig. 6).

![Figure 6. Effect of basal diet and tart cherry supplement on final cecum weight. Values shown are the mean weight of the full cecum at necropsy + SEM (n=20 to 24). Different letters indicate significant difference as determined by one-way ANOVA with Tukey post-hoc test for multiple comparisons. ANOVA main effects for diet, tart cherry and the interaction are shown.](image)
Table 2. Organ weights for mice fed AIN93G or TWD with and without tart cherry supplementation

<table>
<thead>
<tr>
<th>Diet</th>
<th>Liver weight (g)</th>
<th>Relative liver weight (%)</th>
<th>Kidney weight (g)</th>
<th>Relative kidney weight (%)</th>
<th>Spleen weight (mg)</th>
<th>Relative spleen weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIN93G</td>
<td>0.99±0.015&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.82±0.067&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.30±0.005&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.14±0.019&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>70±2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.27±0.011&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TWD</td>
<td>0.99±0.036&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.81±0.072&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.31±0.007&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>1.22±0.029&lt;sup&gt;b&lt;/sup&gt;</td>
<td>127±15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.49±0.056&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>AIN93G+TC</td>
<td>0.96±0.023&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.64±0.054&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.29±0.004&lt;sup&gt;cab&lt;/sup&gt;</td>
<td>1.11±0.020&lt;sup&gt;c&lt;/sup&gt;</td>
<td>72±6.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.28±0.025&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TWD+TC</td>
<td>1.02±0.019&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.74±0.066&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.33±0.006&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.21±0.019&lt;sup&gt;b&lt;/sup&gt;</td>
<td>139±18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.53±0.080&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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*Note: Values are the mean weight in g or the relative weight (mass as percentage of body weight) for the liver, kidney and spleen at necropsy ± SEM (n=20 to 24 mice per group). Different letters indicate significant difference as determined by one-way ANOVA with Tukey post-hoc multiple test.*
Colitis, inflammation, and mucosal injury

Colitis was assessed by visual inspection of stool samples on day 31, immediately after cessation of DSS treatment. A disease activity index (DAI) score was calculated based on stool quality and observed weight loss. A remarkable near 40-fold increase (p < 0.0001) in DAI was observed for mice in the TWD group compared to the AIN93G group (Fig 7). Tart Cherry supplementation had no apparent effect on the disease activity index score in mice fed either basal diet. To further investigate the interaction of diet and disease stage in inflammation, H&E-stained colon tissues were prepared from colon tissue samples obtained from mice necropsied at pre-initiation, colitis, recovery, and terminal time points (see Fig. 3). Tissues from mice collected at the pre-initiation state showed normal morphology, with distinct, well-organized crypt structure, with no apparent morphological changes due to basal diet (Fig. 8A,E). At colitis, this

Figure 7. Colitis disease activity index for mice fed AIN93G and TWD with and without TC supplementation. Colitis was assessed immediately following cessation of treatment with DSS on day 31; values shown are the calculated disease activity index + SEM (n=40 mice per group). Different letters indicate significant difference as determined by one-way ANOVA with Tukey post-hoc test for multiple comparisons. ANOVA main effects for diet, tart cherry and the interaction are shown.
Figure 8. Histopathology of colon tissues from mice fed AIN93G or TWD basal diets, with and without tart cherry supplementation, at pre-initiation, colitis, recovery and terminal disease stages. Representative microscopy images of H&E-stained colon tissues are shown for mice fed AIN93G or TWD basal diets, with and without tart cherry supplementation. Tissues were obtained at day 7 (pre-initiation), colitis (day 31), recovery (day 45) and terminal (day 105) disease stages. Labels are: L, lamina propria; C, crypt, E, epithelium; M, tunica muscularis.

Normal structure was disrupted, with mild inflammation evident in AIN93G-fed mice (Fig. 8B,I). However, in mice fed TWD, this inflammation was moderate to severe and a loss of epithelium
was apparent (Fig. 8G,M). Two weeks after cessation of DSS treatment, recovery of colon tissue epithelial structure was evident in AIN93G-fed mice (Fig. 8C,J), whereas evidence of moderate inflammation and mucosa damage was still evident in tissues from TWD-fed mice. At the study end, colon tissues from AIN93G mice had recovered their normal epithelial structure (Fig.

![Graph showing inflammation and mucosal injury scores](image.png)

**Figure 9. Scoring of inflammation and mucosal injury of colonic tissues.** Scores assessing inflammation (A) and mucosal injury (B) were calculated based on histopathological assessment of colon tissues from AIN93G- and TWD-fed mice, with and without TC supplementation. Values are the mean score ± SEM (n=6 to 8 mice per group). Tissues from each of the four disease stages were assessed, but no samples for TC-supplemented mice were available for pre-initiation per the experiment design. Within each disease stage, different letters indicate significant differences among diet/supplement groups according to two-way ANOVA with Tukey post-hoc tests for multiple comparisons. For comparisons of effect of diet/supplement groups across disease stage, ***, p<0.001; **** p<0.0001 as compared to the pre-initiation stage as determined by two-way ANOVA with Tukey post-hoc test for multiple comparisons.
8D, K), save for the few sites where adenomas had developed. Alternatively, tissues from TWD-fed mice maintained a high level of inflammation and adenocarcinomas were abundant (Fig. 8H, N). Of note, the veterinary pathologist did not discern any impact of TC supplementation on histological features of colon tissues at any of the disease stages assessed.

As outlined in the methods, the pathologist scored each blinded sample for markers of inflammation and mucosal injury. No significant change in inflammation score was observed in mice fed AIN93G across all four stages (Fig. 9A). Mice in the TWD group had a comparable inflammation score to the AIN93G diet group during the pre-initiation stage. However, a marked 6-fold increase in inflammation was evident in TWD-fed mice during the colitis stage, and this high level of inflammation persisted into recovery. At the terminal time point inflammation had decreased in severity with respect to early stages, but remained 2.5-fold above pre-initiation levels. Tart cherry supplementation had no effect on the inflammation score in mice fed either basal diets.

These same tissue sections were blindly scored by a pathologist based on regeneration of the epithelium, crypt damage, and the percent of colon involved as measure of mucosal injury (Fig. 9B). At the study beginning, no mucosa injury was evident for mice fed AIN93G, whereas a slight, but not significant increase was apparent for mice fed TWD. At the colitis, recovery and terminal stages of disease development, mucosa injury was elevated in AIN93G-fed mice, but these increases were not significantly different than the pre-initiation stage nor each other. Alternatively, mucosal injury was markedly higher (near 40-fold) in mice fed TWD during colitis with a substantial 55% improvement during the recovery phases (Fig. 9B). By the study end, mucosal injury had returned to levels similar pre-initiation and to their AIN93G counterparts.
Figure 10. Colon tumorigenesis in mice fed AIN93G or TWD diets, with and without tart cherry supplementation. Values shown are tumor incidence (A), multiplicity (number of tumors per colon length) (B), volume (C) and burden (total tumor volume) (D) ± SEM (n=20-24 mice per group). For A, different letters indicate a significant difference determined by Fisher’s exact test followed by Bonferroni adjustment to correct for multiple testing. For C-D, different letters indicate significant difference as determined by one-way ANOVA with Tukey post-hoc test for multiple comparisons. ANOVA main effects for diet, tart cherry and the interaction are shown.

Colon tumorigenesis

The primary objective of this study was to investigate the impact of tart cherry supplementation on colorectal carcinogenesis on mice fed two basal diets, AIN93G and TWD. Typically, this model of inflammation-associated colorectal cancer yields tumor incidence values near 100%. In this study, colon tumor incidence in mice fed AIN93G and TWD diets was 75% and 90%, respectively (Fig. 10A). Addition of tart cherry powder to the AIN93G diet significantly reduced tumor incidence 42% (Fisher’s exact $p$ value = 0.0084; Bonferroni-adjusted $p$ value = 0.0336), whereas tart cherry did not alter tumor incidence in TWD-fed mice (Fisher’s exact $p$ value = 1.000).
Colon tumor multiplicity was calculated as the number of tumors per mm colon, which accounts for the common observation of reduction in colon length in DSS-treated or high tumor-bearing mice. Basal diet had a substantial effect on multiplicity, with mice fed TWD bearing a substantial greater number of tumors (5.6-fold increase) compared to their AIN93G-fed counterparts ($p<0.001$) (Fig. 10B). Contrary to observations for tumor incidence, no effect of tart cherry supplementation was evident for mice fed either basal diet. Similar trends were apparent for tumor volume, for which mice fed TWD harbored larger tumors (5.8-fold increase in volume) compared to AIN93G-fed mice (Fig. 10C). This trend persisted when assessing tumor burden, which accounts for the total tumor load in an animal. A striking 40-fold increase in tumor burden was evident for TWD-fed mice compared to their AIN93G counterparts (Fig. 10D). Again, no effect of tart cherry was evident for tumor volume or burden.
DISCUSSION

We report for the first time a reduction in the incidence of CAC in mice fed the standard AIN93G basal diet supplemented with tart cherry powder to achieve a total anthocyanin content of 188 ppm. Interestingly, this same effect was not apparent in mice fed TWD supplemented with TC, suggesting that dietary chemoprevention with tart cherries may not be effective for individuals consuming a typical Western type diet. On the other hand, consumption of TWD led to a marked, 40-fold increase in colonic inflammation and a substantial increased tumor burden compared to mice fed AIN93G. This observation supports the notion that consumption of a Western type diet promotes intestinal inflammation and exacerbates colon tumorigenesis.

Contrary to our hypothesis that consumption of TC would ameliorate diet-induced inflammation and suppress colon tumorigenesis, no effect of TC was evident for measurements of gut inflammation, mucosal injury, tumor multiplicity, or tumor burden. Previously, we showed a reduction of aberrant crypt foci in mice fed TWD and provided green tea, but not in mice fed AIN93G and provided green tea (Ward et al., 2017). These observations point to important interactions between basal diets and dietary bioactive supplements and underscore the need for careful consideration of the role of basal diet in dietary chemoprevention studies in rodents.

Others have investigated tart cherry anthocyanins as cancer preventive bioactives against intestinal cancer. For example, Thomasset et al. showed that adding tart cherries to the diet decreased both cecal and small intestine tumors in mice (Thomasset et al., 2017). Addition of tart cherry extract to sub-optimal levels of sulindac, an anti-inflammatory drug, was shown to decrease small intestine tumorigenesis in APC\(^{min/+}\) mice (Bobe et al., 2006). Interestingly, a stronger treatment effect was observed in the proximal and medial sections of the small intestine as compared to the distal section of mice fed tart cherry extract with sub-optimal levels of
sulindac compared to sulindac alone. Kang, et al. (Kang et al., 2003) showed a decrease in cecal tumor incidence and burden in APC^{min/+} mice supplemented with tart cherries, cyanidin, or anthocyanins; however, no significant effect was seen for tumors in the colon. Interestingly, an increase in small intestinal tumor size in mice fed tart cherries was observed in comparison to the control and anthocyanin groups.

Western-style diets have been associated with an increased risk of colitis-associated colorectal cancer, irritable bowel disorder, and other inflammatory diseases (Agus et al., 2016; Bultman, 2017; Chung et al., 2017; Dolan and Chang, 2017) Components of the Western-style diet have been investigated to elucidate their role in CRC. Red and processed meats, commonly consumed in America and other Westernized countries, have been linked to higher colorectal cancer incidence (Chao et al., 2017; Chiang and Quek, 2015; Giovannucci et al., 1994). In 2015, the International Agency of Research on Cancer (IARC) reclassified red and processed meats to group 1 (carcinogenic to humans). Studies investigating the role of fat, specifically the Western-diet fat profile, on carcinogenesis in the colon have produced contradictory results (Enos et al., 2016; Giovannucci et al., 1994; O'Neill et al., 2016; Reddy, 2002). Evidence suggests consumption of saturated fats and n-6 polyunsaturated fatty acids increases risk of carcinogenesis, while consumption of n-3 polyunsaturated fatty acids interact with several cellular processes to inhibit carcinogenesis (Reddy, 2002). Conversely, another study suggested that increased consumption of saturated fat had a protective effect against colorectal cancer in the azoxymethane/dextran sodium sulfate model of carcinogenesis (Enos et al., 2016).

Micronutrients also play a role in disease development (La Vecchia et al., 1997; Levi et al., 2000; Perez Monsanto, 2013). Work by Newmark and colleagues suggested that key micronutrients calcium and vitamin D along with various B vitamins and contributors to one-
carbon metabolism are also linked to colon carcinogenesis (Newmark et al., 2009). Indeed, previous work by our group suggested that the micronutrient fraction of the TWD was largely responsible for the elevated tumor burden in mice fed this model diet of Western nutrition (Perez Monsanto, 2013). Results of this study concur with our prior work in that mice fed TWD experienced a marked increase in tumor multiplicity, size and burden. Importantly, new findings from this study suggest that the promotion of colon tumorigenesis in C57BL/6J mice is linked to prolonged elevated inflammation and mucosal damage after cessation of DSS treatment to induce acute, transient colitis. The elevation in symptoms of colitis (e.g., stool consistency, blood in stool and body weight loss) corresponded with histological findings of severe inflammation and disorganization and/or loss of the gut epithelium. Our findings are consistent with other reports that consumption of a Western-type diet exacerbates inflammatory disease (Agus et al., 2016; Giugliano et al., 2006; Manzel et al., 2014).

Based on observations of colitis symptoms and tissue histopathology, the observed decrease in tumor incidence by consumption of TC in mice fed AIN93G appeared to be via a different mechanism than suppression of colonic inflammation. It is possible that the bioactive chemicals in tart cherries may act by a mechanism other than inflammation to reduce colon tumor incidence in mice fed AIN93G. Anthocyanins have been shown to downregulate several cytochrome P450 enzymes which are involved in the metabolism of pro-carcinogenic compounds, such as azoxymethane (Hoek-van den Hil et al., 2015; Srovnalova et al., 2014). Altered hepatic metabolism of the pro-carcinogen AOM by CYP2E1 to its carcinogenic metabolite, methylazoxymethanol, may explain the decreased incidence in mice fed AIN+TC, although this experiment was not designed to test that hypothesis. Another consideration that may explain different effects of TC in mice fed different basal diets is the contribution of the gut
microbiome to metabolism of bioactives from functional foods. Others have shown that microbiota population structure and diversity shifts with changes in diet (Moschen et al., 2012; Turnbaugh et al., 2009). The different basal diets used in this study may or may not support gut microbiome populations that differentially metabolize anthocyanins or other bioactives present in the tart cherry powder. Such differences in metabolism could result in different bioactive profiles and differential bioavailability of these bioactives to colon tissues (Fernandes et al., 2015; Kay et al., 2017). Analysis of the gut microbiome over the course of disease development was part of the study, but is not within the scope of this thesis.

Many brightly colored fruits and some vegetables contain anthocyanins, although the profile of specific anthocyanins present and their relative concentrations vary (Redondo et al., 2017). Previously Kirakosyan reported that cyanidin was the major anthocyanin component of tart cherries (Kirakosyan et al., 2015), yet HPLC analysis of our tart cherry powder obtained from Shoreline Fruit revealed an anthocyanin profile with malvidin as the major component, present at five-fold the concentration of the next most abundant form, cyanidin. It is important in dietary chemoprevention studies to recognize that different varieties, cultivars, and hybrids of cherries likely display differences in anthocyanin profile and content. Different anthocyanins are likely to have different modes of action, including capacity to scavenge reactive oxygen species and modulate inflammation pathways, which may explain why some cyanidin-rich berries are effective at suppressing colitis (Montrose et al., 2011; Shin et al., 2014), whereas this malvidin-rich tart cherry powder was apparently ineffective in this animal model.

In conclusion, results from this study indicate a pronounced increase of colonic inflammation, mucosal injury and promotion of inflammation-associated colorectal cancer with consumption of a diet emulating typical American macro and micronutrient intakes (TWD).
Whereas consumption of tart cherry powder reduced incidence of colon tumors in mice fed a standard diet designed to support animal health, TC was without effect in mice fed the Western-type diet. These distinct results between basal diet groups suggest an interaction between basal diet and bioactive supplements and support careful consideration and employment of model diets in rodent studies. Though the original hypothesis that TC would suppress TWD-promoted tumorigenesis via suppression of inflammation proved incorrect, the finding that TC reduced tumor incidence is significant and warrants further study. Further investigation into the mechanism of cancer prevention by tart cherry anthocyanins is necessary to better understand potential benefits of supplementation and to identify target populations that would benefit from this dietary intervention.
AUTHOR’S REFLECTIONS

Undergraduate research has played a pivotal role in my education at Utah State University. Although the idea of working with mice did not initially appeal to me, in the fall of 2013, I began volunteering in Dr. Benninghoff’s laboratory by assisting with mouse housekeeping and laboratory maintenance. This decision to step outside my comfort zone was one of the best decisions I have made during my undergraduate career. While participating in research, I have learned laboratory techniques, developed presentation skills, learned about laboratory management, and developed relationships with professors and other young scientists.

My capstone project grew out of a contract I began with Dr. Benninghoff. This project involved a mouse study looking at the effects of tart cherry supplementation on markers of colorectal cancer development. Involved from day one, I set-up and ran this study with the assistance of the laboratory technician, Deanna Larson. We ran into some possible errors in experimental design which were recognized after the initiation of the study. We realized mice fed the TWD were consuming more water while on DSS than mice fed AIN93G. Differences in consumption could alter inflammation severity and disrupt proper comparisons between the two groups. Another cohort of mice with adjusted DSS was added to the study. Attending laboratory meetings gave me the opportunity to learn more about the process of experimental design and the process of formulating a solution. I was regularly involved in gathering data on food consumption, water intake, and body weight was monitored throughout the course of the study. Weekly fecal samples were also collected from each mouse. At the conclusion, tissue samples were collected and analyzed. I performed tumor counts and measurements and data entry.

Working on this study and on other studies in Dr. Benninghoff and Dr. Hintze’s laboratories has provided me with marketable skills: mouse handling, necropsy, subcutaneous injection, oral gavage, PCR, DNA extraction, data analysis and interpretation, and presentation
skills. After graduation, I will be continuing my research training at the National Institutes of Health in a post-baccalaureate position. This wonderful opportunity was available to me because of the research experiences I have had at Utah State University. My honors capstone project has been a springboard into my future.

Along with these laboratory skills, I have learned several important life skills through my own experiences and through the wonderful guidance of my lab peers and mentors. Through her additional summer lecture series and her feedback, Dr. Benninghoff has taught me how to put together and present an aesthetically pleasing poster or presentation. Her guidance has also been pivotal in the composition of this Honor’s thesis. I encourage all future honors students to develop a relationship with their mentor. You will gain valuable insight into areas requiring improvement along with areas you may not have realized are your strengths. You will also have their support throughout your undergraduate journey.

My interactions with my lab colleagues have also taught me several lessons, including the importance of organization, communication, and preparation. Running any study is a lot of work, and any prior preparation performed will increase the productivity of the task you need to perform. Every person in our lab has been involved in preparation, whether it was by labeling tubes, helping set up the room, taking an inventory of materials, or organizing a schedule. Busy hands make light work; however, the hands must be told what to do. I have seen the laboratory process run smoothly or be very trying, based upon the quality of communication.

I believe these three processes of preparation, organization, and communication are also relevant in the honors thesis/capstone project process. Preparation comes from getting engaged and finding what you are passionate about. The Honors program challenges its students to dare to know. So be curious. Get involved in a different field. My research was conducted in a
different department than my degree major. The contract experiences and honors courses assist you in finding your interest. Take advantage of these wonderful opportunities to meet faculty and other students from across the disciplines. Once you are involved in something, you will need to plan and organize a project. Do not be afraid to approach a faculty mentor with an idea or to try something that may be a little outside of your comfort zone. Then continue to communicate with him or her on your progress, whether you have discovered something exciting or hit a very large roadblock.

Finally, do not procrastinate your writing or presentation. I have had the opportunity to present posters and talks at several conferences including the Biology Department’s Research Symposium, Utah State University’s Student Research Symposium, the Utah Conference on Undergraduate Research, the National Conference of Undergraduate Research, the Mountain West Society of Toxicology, and Experimental Biology. There are so many opportunities for young researchers to share their experiences. Take advantage of the opportunities available. At these conferences, individuals learn about other areas of research, have opportunities to network, and improve professional presentation skills. Writing for many is the hardest part of their honors thesis/capstone project. Future students should get started as soon as they can on organizing their data and starting a rough draft of their thesis. Write the methods section as you are performing the work and begin writing summaries of results as you analyze data. Then when the semester of actual thesis writing arrives, you have already written portions from which you can pull information.

This process is a lot of work. Make sure you have set aside the time and resources to make it happen, but usually the projects arise from something you are already involved with or are interested in. So be curious, explore new fields, and dare to know.
LITERATURE CITED


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AUTHOR BIOGRAPHY

Ashli Arné Hunter was born in Boise, Idaho and attended Melba High School in Melba, Idaho. After graduating high school in 2013, Ashli attended Utah State University from the fall of 2013 to the spring of 2017 where she graduated with a Bachelor of Science in Cellular and Molecular Biology and with a minor in Chemistry. While attending USU, Ashli was involved in undergraduate research and presented her work at several conferences including the regional Mountain West Society of Toxicology meeting and the international 2016 Experimental Biology conference, at which she was recognized as an Emerging Leader in nutrition science by the American Society of Nutrition. Ashli was nominated by USU for the Goldwater Scholars Award in 2016 and received the John R. Simmons scholarship from the Department of Biology in 2015. As a recipient of an NIH Postbaccalaureate Intramural Research Training Award, Ashli will continue her training at the National Institute of Diabetes and Digestive and Kidney Diseases studying the connection of the gut microbiome and risk of hepatitis. She plans to continue her research career by pursing a PhD in the health science arena.