THE EFFECT OF DIGESTIVE MODIFICATION ON THE ANTICANCER ACTIVITY OF TEA CATECHINS IN THE HT-29 HUMAN COLON CANCER CELL LINE

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

Marti A. Cenky, B.S.

****

The Ohio State University
2008

Master’s Examination Committee:

Professor Joshua Bomser, Advisor

Professor Earl Harrison

Professor Steven Schwartz

Approved by

______________________________

Advisor
College of Education and Human Ecology
ABSTRACT

Epidemiological evidence suggests that increased consumption of catechin-rich tea is associated with reduced risk of gastrointestinal cancers. However, the effects of digestive conditions on the anticancer activity of tea and catechins have been largely ignored. The present study compared the anticancer activities of undigested (raw) and digested tea extracts/catechins in colon cancer cell line HT-29. Cell viability was measured by the MTT assay and the concentration of extracts required to reduce cell viability by 50% (IC50) was determined. Raw material green tea and (-)epigallocatechin-3-gallate (EGCG) extracts showed significantly lower IC50 than the same extracts after undergoing digestive modification. There was no difference in IC50 between raw and digested black tea. Intracellular oxidation was measured by the 2',7'-dichlorofluorescein (DCF) assay. No difference in intracellular oxidation was observed between raw and digested extracts. These data suggest that digestive conditions impact the anticancer activity of tea catechins; however the mechanism impacted by digestion is not yet known.
Dedicated to my husband, Ryan. I love you!

And to the rest of my family for all of their support. Thank you!
I wish to thank my advisor, Joshua Bomser, for all of his help and guidance throughout this whole process. I appreciate his patience with a part-timer and could not have done this without him.

I wish to thank my lab mates, Fabiola Gutierrez Orozco and Elizabeth Clubbs. I really could not have survived the last four years without either of you. I cannot thank you girls enough.
VITA

September 4, 1981…………………………Born – Columbus, Ohio, USA

2003…………………………………………B.S. Biological Sciences, Ohio University

FIELDS OF STUDY

Major Field: Education and Human Ecology

Specialization: Human Nutrition
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td></td>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td></td>
<td>Acknowledgments</td>
<td>iv</td>
</tr>
<tr>
<td></td>
<td>Vita</td>
<td>v</td>
</tr>
<tr>
<td></td>
<td>List of Figures</td>
<td>viii</td>
</tr>
<tr>
<td></td>
<td>List of Tables</td>
<td>ix</td>
</tr>
<tr>
<td></td>
<td>List of Abbreviations</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>Chapters</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Literature Review</td>
<td>6</td>
</tr>
<tr>
<td>2.1</td>
<td>Colon Cancer</td>
<td>6</td>
</tr>
<tr>
<td>2.1.1</td>
<td>Introduction to the colon</td>
<td>6</td>
</tr>
<tr>
<td>2.1.2</td>
<td>Colon cancer epidemiology</td>
<td>7</td>
</tr>
<tr>
<td>2.1.3</td>
<td>Colon cancer risk factors</td>
<td>7</td>
</tr>
<tr>
<td>2.1.4</td>
<td>Colon cancer progression</td>
<td>8</td>
</tr>
<tr>
<td>2.1.5</td>
<td>Colon cancer treatment</td>
<td>10</td>
</tr>
<tr>
<td>2.1.6</td>
<td>Genetic alterations implicated in colon cancer</td>
<td>12</td>
</tr>
<tr>
<td>2.1.7.1</td>
<td>Dietary agents promoting colon Cancer</td>
<td>13</td>
</tr>
<tr>
<td>2.1.7.2</td>
<td>Dietary agents inhibiting colon Cancer</td>
<td>14</td>
</tr>
<tr>
<td>2.2</td>
<td>Tea</td>
<td>15</td>
</tr>
<tr>
<td>2.2.1</td>
<td>Tea production</td>
<td>15</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.</td>
<td>Progression of colon cancer</td>
<td>10</td>
</tr>
<tr>
<td>2.2.</td>
<td>Major components of green and black teas</td>
<td>16</td>
</tr>
<tr>
<td>2.3.</td>
<td>Absorption of EGCG by HT-29 cells</td>
<td>22</td>
</tr>
<tr>
<td>3.1.</td>
<td>HPLC chromatograms of extract digestions</td>
<td>33</td>
</tr>
<tr>
<td>3.2.</td>
<td>IC_{50} – Green Tea, Black Tea and EGCG</td>
<td>36</td>
</tr>
<tr>
<td>3.3.</td>
<td>Percent viability – MTT Assay for Green Tea, Black Tea and EGCG</td>
<td>37</td>
</tr>
<tr>
<td>3.4.</td>
<td>Intracellular oxidation – DCF Assay for Green Tea, Black Tea and EGCG</td>
<td>41</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1. Concentration of catechins in tea extracts</td>
<td>ix</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

AAPH………………………………………2,2’-axobis-(2-amidinopropane)dihydrochloride
AMPK………………………………………………AMP-activated protein kinase
APC………………………………………………………adenomatosis polyposis coli
COX-2……………………………………………………cyclooxygenase-2
DCC………………………………………………..deleted in colorectal carcinoma
DCF……………………………………………………2’,7’-dichlorofluorescein
DCFDA………………………………………………2’,7’-dichlorofluorescein-diacetate
DCFH…………………………………………………2’,7’-dichlorohyrdofluorescein
DNA…………………………………………………………deoxyribonucleic acid
EC…………………………………………………………(-)epicatechin
ECG…………………………………………………(-)epicatechin-3-gallate
EGC…………………………………………………………(-)epigallocatechin
EGCG………………………………………………………(-)epigallocatechin-3-gallate
ERK-1…………………………………………extracellular signal-related kinase-1
ERK-2…………………………………………extracellular signal-related kinase-2
FAP…………………………………………………………familial adenomatous polyposis
GAE…………………………………………………………gallic acid equivalent
GI……………………………………………………………gastrointestinal
HCl……………………………………………………………………….hydrochloric acid
HPLC…………………………………………...high performance liquid chromatography
MAPK……………………………………………………mitogen-activated protein kinase
mRNA…………………………………………………………messenger ribonucleic acid
MTS………………………………………………………………………………………...

\[3-(4,5\text{-dimethylthiazol-2-yl})-5-(3\text{-carboxymethoxyphenyl})-2-(4\text{-sulfophenyl})\text{-2H-tetrazolium}\]

MTT…………..(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
p53………………………………………………………………………………tumor protein 53
PPP2R1B………protein phosphatase 2 (formerly 2A), regulatory subunit A, beta isoform
ROS………………………………………………………………..reactive oxygen species
THSN A……………………………………………………………………..theasinensin A
THSN D……………………………………………………………………..theasinensin D
VEGF…………………………………………………..vascular endothelial growth factor
Colorectal cancer is the 3\textsuperscript{rd} most common cancer for men and women worldwide. There will be approximately 108,000 new cases in the year 2008, and it is expected that approximately 49,000 will die from colon cancer in 2008, accounting for 9\% of all cancer deaths (American Cancer Society, 2008). This type of cancer affects men and women equally in which 51\% of new cases annually are occurring in men and 49\% are occurring in women (Boyle et al., 2000; Hong et al., 2002). Colon cancer is preventable and is curable if detected at an early stage. The development of colon cancer is a slowly evolving process, and typically starts with one or more genetically altered cells (Lipkin et al., 1999).

Colon cancer starts in the epithelial cell lining of the intestinal wall. It is slow growing and usually shows no symptoms until the tumor is large in size. A colon polyp is a fleshy growth that occurs on the lining of the colon. They can be detected by a colonoscopy and removed. Colon polyps that go untreated almost always progress into colon cancer, but if treated, polyps can be removed and never become colon cancer.

Many studies have been performed to show the relationship between diet and colon cancer. They have looked at different dietary components including fat, fiber and
tea consumption. Studies have shown that diets rich in polyunsaturated fats, such as omega-3 fatty acids found in fish, may decrease the risk of colon cancer. There have been many fewer studies done on these types of fats as compared to animal fat studies; however, the studies that have been completed have shown an inverse relationship between the consumption of fish and colon cancer incidence (Caygill et al., 1995). Other studies have also shown that fish and fish oil consumption have the same inverse relationship with colon cancer even in populations with overall high fat intake, suggesting that the effect of polyunsaturated fats on colon cancer overcome the effects of saturated fats (Caygill et al., 1996).

There have also been studies that have looked at the effect of dietary fiber on colon cancer. These studies have shown that the risk of colon cancer in populations with high consumption of total fat can be decreased by also having high consumption of total fiber, fibrous foods and specific whole grain foods (Lipkin et al., 1999). In the Lipkin et al. review, they stated that out of 19 case-control studies, three studies showed dietary fiber having no affect and two studies showing an adverse affect. The other thirteen studies showed that dietary fiber reduced colon cancer risk.

Research has suggested that tea consumption positively affects cancer risk and outcomes in the gastrointestinal (GI) tract. Tea catechins, such as (-)epigallocatechin-3-gallate (EGCG), have been shown to exhibit chemopreventative activities. Previous studies have used a proliferation assay with MTS to determine the antiproliferation activity that EGCG has on colon cancer cell line HT-29. The data from this study suggested that EGCG inhibited cell proliferation of the HT-29 cells in a dose dependent
manner (Chen et al., 2003). Tea catechins have been shown to possess chemopreventative ability in vitro as a pure extracts. Whether or not tea catechins possess this same ability once the digestive process has occurred has not been determined.

Tea is one of the most commonly consumed beverages in the world. There are many types of tea, all classified based on the way that they are produced. Green tea and black tea are two of the major commercial types of tea. Green tea makes up approximately 10% of the world’s tea. Green tea is produced by steaming fresh tea leaves and then drying them. The steaming process heats inactivated oxidative enzymes. Green tea has a greenish-yellowish color. Black tea is the most common type of tea worldwide. Black tea is produced by fermentation. This process involves crushing fresh tea leaves and allowing enzyme-mediated oxidation to occur. The process allows the caffeine amount to triple. Black tea has a reddish-brown color. Oolong tea is the most expensive type of tea. It is produced similar to black tea, but is allowed to oxidize only half way through the fermentation process. This gives the tea leaves a reddish color. White tea is the rarest of all tea types. It is produced the same as green tea, but uses the buds of the tea plant instead of the leaves. The buds are white in color and therefore result in a colorless liquid.

Due to the difference in preparation, there is a difference in catechin levels within the two tea types. Common catechins found in tea are (-)epigallocatechin-3-gallate (EGCG), (-)epigallocatechin (EGC), (-)epicatechin-3-gallate (ECG), and (-)epicatechin (EC). Green tea contains 30-40% catechins while the black tea contains 3-10% catechins.
Fermentation of black tea converts catechins to oligomeric theaflavins and polymeric thearubigins (Lambert et al., 2003).

The hypothesis of this study is that digestive modification will affect the anticancer properties of tea and tea catechins. The first part of the study will show the effect of digestive modification on tumor cell viability. The second part of the study will look at mechanisms which could be altered by digestive modification.

For this study, the Mario Ferruzzi and Rodney Green lab group of Purdue University, Food Science Department, West Lafayette, Indiana, prepared the raw and digested extracts. For this study, digestive modification process included simulation of the gastric and intestinal phases of digestion. In the gastric phase, the raw extracts were exposed to porcine pepsin and pH level of 2. The extracts were incubated for one hour. The intestinal phase of digestive simulation involved the adjustment of pH levels to 5. The extracts were treated with pancreatin, lipase, bile and the pH was adjusted to 7.2. They were incubated for 2 hours and the catechin levels were determined by using high performance liquid chromatography (HPLC).

The first aim is to determine the effect digestive modification has on cancer cell viability. Chen et al. determined the inhibitory effect of EGCG on colon cancer cell line HT-29 (Chen et al., 2003). They treated the HT-29 cells with varying concentrations of EGCG. They determined that EGCG does inhibit the growth of the HT-29 cells in a dose dependent manner. They also determined that while the HT-29 cells were growth inhibited by EGCG, EGCG did not exhibit a significant amount of cytotoxicity. This suggests that EGCG inhibited the colon cancer cell growth by a mechanism other than
cytotoxicity. It is not known whether the digestion of tea catechins removes the ability to reduce cancer cell viability that the native compound has been shown to possess. This study used both raw material and digested tea extracts to determine if there is a difference in cell viability caused by the digestive process.

The second aim is to determine the effect digestive modification has on intracellular oxidative stress. Tea catechins are widely known to be very potent antioxidants. They are able to scavenge reactive oxygen species (ROS) before ROS can cause damage to the cell. Some of the harmful effects ROS can cause include DNA damage, oxidation of fatty acids in lipids and oxidation of amino acids in proteins. These changes can lead to cancer. The antioxidant ability of tea catechins have been show to help protect many cells from the ROS damage inhibiting progression to cancer. However, some studies suggest that tea catechins can impose oxidative stress in many types of tumor cell lines, leading to cytotoxicity and apoptosis through the induction of ROS (Yamamoto et al., 2004). Yamamota et al found that high concentrations of EGCG damaged tumor cells, but not normal cells. Another study showed that EGCG significantly generated ROS in HT-29 cells (Hwang et al., 2007). The study suggests that the generation of ROS activates AMPK, inhibiting the tumor promotion enzyme COX-2 (Hwang et al., 2007). It has not been shown if the amount of intracellular oxidative stress differs between pre- and post digestion. This study will use both raw material and digested extracts to determine if digestive modification of the compounds alters the generation of ROS in HT-29 cells.
CHAPTER 2
LITERATURE REVIEW

2.1. Colon Cancer

2.1.1. Introduction to the colon

The digestive system is where nutrients such as vitamins, minerals, carbohydrates, fats, proteins and water from consumed foods are processed, and the waste material is passed out of the body. The digestive system is composed of the esophagus, stomach and the small and large intestines. The first six feet of the large intestine is called the colon. The colon is a storage tube for solid wastes. The main function is to regulate water and solid extraction from feces. The luminal surface of the colon is made up of crypts, which are deep cavities embedded in connective tissue, forming a very large surface area needed for absorption (Lipkin et al., 1999). The cells that make up the crypts are undergoing constant regeneration. At the base of the crypt, the newly generated cells are formed, and during maturation, the cells move to the upper crypt regions. Once there, they die and are sloughed off into the lumen. In a human, the colon epithelium is regenerated every four to eight days.
2.1.2. Colon cancer epidemiology

Colorectal cancer is the 3rd most common cancer for men and woman worldwide. There will be approximately 108,000 new cases in the year 2008, and it is expected that approximately 49,000 will die from colon cancer in 2008, accounting for 9% of all cancer deaths (American Cancer Society, 2008). This type of cancer affects men and women equally where 51% of new cases annually are occurring in men and 49% are occurring in women (Boyle et al., 2000; Hong et al., 2002). Colon cancer is preventable and is curable if detected at an early stage. The development of colon cancer is a slowly evolving process, and typically starts with one or more genetically altered cells (Lipkin et al., 1999).

Colon cancer is widely believed to be an environmental disease. This is due to the differences seen in ethnic and racial groups and in migrants (Boyle et al., 2000). Studies have shown that the risk of colon cancer for Japanese offspring who have migrated to the United States is three to four times higher than that among the Japanese living in Japan (Boyle et al., 2000). This is considered to be due to the difference in diet and lifestyle between the United States and Japan (Boyle et al., 2000).

2.1.3. Colon cancer risk factors

Lifestyle is an important factor in the development of colon cancer. Studies have shown that the western diet, high in red meat and processed meats, along with low consumption of fresh fruit, vegetables, poultry and fish can increase the risk of developing colon cancer (Caygeill et al., 1996; Graham et al., 1988). Some studies have
suggested that high intake of alcohol also increases colon cancer risk (Marchand et al., 1997). Smoking and living in industrialized countries are also risk factors for colon cancer (Marchand et al., 1997). Lifestyles that incorporate healthy eating, such as fresh fruits and vegetables, increased consumption of fish, and exercise have been shown to reduce the risk of colon cancer.

Family history is a risk factor in the development of colon cancer. Individuals with relatives having colon cancer before the age of 60 or multiple relatives having colon cancer are considered high risk (American Cancer Society, 2008). Familial adenomatous polyposis (FAP) carries a 100% risk of developing colon cancer by age 40 if it remains untreated. FAP is a genetically inherited mutation in the adenomatosis coli (APC) gene. Along with FAP, having the hereditary non-polyposis colorectal cancer genetic mutation also increases the risk of colon cancer development (Nishisho et al., 1991).

Individuals who have had recurring colon polyps have an increased risk of developing colon cancer (American Cancer Society, 2008). Individuals who have had other cancers also have an increased risk.

Age is another factor that influences the risk of colon cancer. The most common age range for the development of colon cancer is 60-70 years old (American Cancer Society, 2008). The presence of colon cancer before the age of 50 is uncommon unless there is a family history of early onset colon cancer.
2.1.4. Colon cancer progression

Colon cancer starts in the epithelial cell lining of the intestinal wall. It is slow growing and usually shows no symptoms until the tumor is large in size. A colon polyp is a fleshy growth that occurs on the lining of the colon. They can be detected by a colonoscopy and surgically removed. Colon cancer almost always starts as an untreated colon polyp, but not all polyps will progress into cancer.

Cancer within the epithelial lining is called carcinoma in situ. It is only found within these cells and has not progressed outside of the innermost lining. This is considered stage 0. As the cancer migrates to the second and third layers of the inner wall of the colon, it is now considered to be stage I, or Dukes’ A colon cancer. At this stage, the cancer has not spread to the outer wall or outside of the colon. Stage II occurs when the cancer has spread to the outer walls and to the surrounding tissue. However, the cancer has not yet spread to any lymph nodes. This is also called Dukes’ B colon cancer. The cancer will then spread to the lymph nodes, but will not yet have migrated to other parts of the body. This is stage III or Dukes’ C colon cancer. The final stage of colon cancer progression is stage IV, or Dukes’ D colon cancer. This is when the cancer spreads to other parts of the body such as the liver or lungs (Figure 2.1. National Cancer Institute, 2008).

The early stages of colon cancer are highly treatable. If the tumor is still localized within the colon walls, the five year survival rate is about 93% (American Cancer Society, 2008). However, later stages of colon cancer, successful treatment becomes
much more difficult, and the five year survival rate can range from 64% to 8% (American Cancer Society, 2008). Therefore, early screening for high risk populations is highly recommended.

Figure 2.1. The stages of progressing colon cancer. Figure obtained from The National Cancer Institute website
http://www.nic.nih.gov/cancertopics/pdq/treatment/colon/Patient/page2

Figure 2.1. Progression of colon cancer
2.1.5. Colon cancer treatment

Polyps can be treated during a colonoscopy. A wire loop is used to cut the stalk of the polyp and cauterize it to prevent bleeding. More invasive surgery to remove polyps may be necessary, but is typically not performed unless the polyp has developed into localized colon cancer.

Colon cancer is treated based on the stage of progression. Three types of treatment are generally used. They include surgery to physically remove the cancer cells, chemotherapy in which drugs are used to kill the cells and radiation therapy in which x-rays or other types of radiation are used to kill the cells.

For stage 0 colon cancer, when the cells are contained within the lining of the colon, a local excision is used to remove the tumor and a small section of tissue surrounding it. Complete removal of the tumor is considered to be curative.

For patients with stage I, when the tumor has spread beyond the inner lining but has not spread to the outer colon wall or outside of the colon, surgery is used to remove the tumor and additional treatment is not usually needed. 93% of patients survive beyond 5 years (American Cancer Society, 2008).

Stage II, which involves much larger tumors that extend beyond the colon wall but have not invaded the lymph nodes, usually rely on surgery as the recommended treatment and the 5 year survival rate is about 78% (American Cancer Society, 2008).

Stage III colon cancers, which have spread outside the colon and into the lymph nodes, require that patients undergo surgery to remove the tumor within the colon and invaded tissues. These patients will then receive chemotherapy and may have radiation
therapy depending on the size of the tumor and amount of invasion of surrounding tissue. Around 64% of patients survive beyond 5 years (American Cancer Society, 2008).

Stage IV, which is the final and most aggressive stage of colon cancer, meaning that these cancers have metastasized to other parts of the body such as the liver or lungs, and may have invaded the lymph nodes, requires surgery to remove the tumor in the colon and also in the parts of the body where the tumor has invaded. Chemotherapy is utilized along with other drugs recently used for treating stage IV colon cancer such as Erbitux or Avastin. These drugs work to inhibit angiogenesis through blocking vascular endothelial growth factor (VEGF), which is a potent angiogenic factor important for colon cancer neovascularization (Jung et al., 1999). These patients may also be involved in clinical trials and will also receive radiation therapy to relieve symptoms. Only about 8% of patients survive beyond 5 years with stage IV colon cancer (American Cancer Society, 2008).

Individuals who are at high risk for colon cancer are urged to be screened on a regular basis in order to diagnose at an early stage for a better chance of survival. They are also encouraged to develop healthy lifestyle habits, including consuming diets that have been shown to reduce the risk of colon cancer.

2.1.6. Genetic alterations implicated in colon cancer development

Multiple genetic alterations have been implicated in the development of colon cancer (Geyat et al., 2001). Many of these alterations are seen in a majority of colon cancer tumors and some are very rare. They include inactivation of tumor suppressor
genes, activation of oncogenes, mismatch repair defects and epigenetic silencing by methylation of gene promoters (Geyat et al., 2001). The entire realm of genetic alterations remains to be found.

Previous research has suggested that colon cells develop into small polyps after the loss of the tumor suppressor gene, adenomatosis polyposis coli (APC) (Kinzler et al., 1996). The researchers went on to say that k-Ras is activated and the polyp becomes a small, benign, adenoma. The small adenoma can be removed before growing into a malignant tumor. The research done by Kinzler et al suggests that a malignant adenocarcinoma of the colon results from a loss of the deleted in colorectal carcinoma (DCC) and tumor protein 53 (p53) tumor suppressor genes.

Other studies have also shown genetic alterations leading to the onset of colon cancer. One study suggested that the protein phosphatase 2 (formerly 2A), regulatory subunit A, beta isoform (PPP2R1B) tumor suppressor may lead to colon cancer with one deletion mutation (Wang et al., 1998). This mutated gene generates a truncated form of the serine/threonine protein phosphatase 2A-Aβ, which then is unable to bind to its catalytic subunit. This can inhibit cell cycle regulation and cellular growth control. The study showed that 15% of primary colon tumors possessed the deletion mutation on the PPP2R1B gene (Wang et al., 1998).
2.1.7. Diet and colon cancer

2.1.7.1. Dietary agents promoting colon cancer

Dietary fat has been the focus of many studies concerning colon cancer. Multiple studies have shown that diets high in saturated fat may increase the risk of colon cancer (Lipkin et al., 1999). Ecological studies have shown that the incidence in colon cancer in many European countries, the United States and Canada is directly correlated with the consumption of animal fat (Lipkin et al., 1999). The review by Lipkin et al. stated that there are case-control studies that show no correlation between animal fat consumption and colon cancer. Based on the vast number of studies that have shown an inverse relationship between animal fat and colon cancer, individuals who are already at a high risk of colon cancer due to family history or medical history are encouraged to reduce the amount of animal fat intake in order to reduce the risk of developing colon cancer.

2.1.7.2. Dietary agents inhibiting colon cancer

Other studies have shown that diets rich in polyunsaturated fats, such as omega-3 fatty acids found in fish may decrease the risk of colon cancer. There have been many fewer studies done on these types of fats as compared to animal fat studies; however, the studies that have been completed have shown an inverse relationship between the consumption of fish and colon cancer incidence (Caygill et al., 1995). Other studies have also shown that fish and fish oil consumption have the same inverse relationship with
colon cancer even in populations with overall high fat intake, suggesting that the effect of polyunsaturated fats on colon cancer overcome the effects of saturated fats (Caygill et al., 1996).

There have also been studies that have looked at the effect of dietary fiber on colon cancer. These studies have shown that the risk of colon cancer in populations with high consumption of total fat can be decreased by also having high consumption of total fiber, fibrous foods and specific whole grain foods (Lipkin et al., 1999). In the Lipkin et al. review, they stated that out of 19 case-control studies, three studies showed dietary fiber having no affect and two studies showing an adverse affect. The other thirteen studies showed that dietary fiber reduced colon cancer risk.

2.2. Tea

2.2.1. Types of Tea

Tea is one of the most commonly consumed beverages worldwide. There are many types of tea, all classified based on the way that they are produced. Green tea and black tea are two of the major commercial types of tea. Green tea makes up approximately 10% of the world’s tea. Green tea is produced by steaming fresh tea leaves and then drying them. The steaming process heats inactivated oxidative enzymes. Green tea has a greenish-yellowish color. Black tea is the most common type of tea worldwide. Black tea is produced by fermentation. This process involves crushing fresh
tea leaves and allowing enzyme-mediated oxidation to occur. The process allows the caffeine amount to triple. Black tea has a reddish-brown color. Oolong tea is the most expensive type of tea. It is produced similar to black tea, but is allowed to oxidize only half way through the fermentation process. This gives the tea leaves a reddish color. White tea is the rarest of all tea types. It is produced the same as green tea, but uses the buds of the tea plant instead of the leaves. The buds are white in color and therefore result in a colorless liquid. Due to the difference in preparation, there is a difference in catechin levels between the tea types.

2.2.2. Introduction to catechins

Catechins are naturally occurring polyphenols found in green tea, red wine, chocolates and many fruits. They belong to the flavonoid group and are considered flavan-3-ols. Common catechins found in tea are (-)epigallocatechin-3-gallate (EGCG), (-)epigallocatechin (EGC), (-)epicatechin-3-gallate (ECG), and (-)epicatechin (EC) (Figure 2.2.). Green tea contains 30-40% catechins while the black tea contains 3-10% catechins (Geyat et al., 2001). Fermentation of black tea converts catechins to oligomeric theaflavins and polymeric thearubigins (Geyat et al., 2001).
Major components of green tea and black tea. Figure obtained from http://www.teatalk.com/science/chemistry1.htm

Figure 2.2. Major components of green and black teas
2.2.3. Health benefits

There has been extensive research in the area of green tea catechins and health benefits. Many studies have associated green tea consumption with a reduced risk of cancer, cardiovascular disease, periodontal disease and obesity (Cabrera et al., 2006; Yang et al., 2000). Other studies have shown that green tea catechins possess antibacterial, antiviral and anti-inflammatory activity, as well as the ability to increase bone mineral density (Cabrera et al., 2006; Yang et al., 2000). Some studies showed that the consumption of green tea had no affect on colon cancer incidence (Marques-Vidal et al., 2006). Marques-Vidal et al. stated that the studies reviewed suggest no relationship between tea consumption and colon cancer risk. Though there are studies that show no affect, a considerable number of studies suggest that consumption of green tea to be beneficial and recommended as a means to reduce many health issues.

2.2.4. Effect of digestion on catechins

Digestion of catechins in the intestine leads to degradation of the compounds. Neilson et al did a study showing the effect of digestion on catechins, and the resulting dimer formation. The study found that EGCG, EGC and ECG significantly degraded during digestions by 71-91%, 72-100% and 60-61%, respectively (Neilson et al., 2007). The study also showed that the loss seen in the intestinal phase of digestion was greater than the loss seen in the gastric phase. This suggests that degradation is mostly correlated to pH levels since the intestinal pH is around 6.0-7.5 and the gastric pH is around 2. EGCG undergoes autooxidation when in a high pH environment, such as the colon. It
forms homocatechin dimers of theasinensin (THSN) A, THSN D and P-2. Other studies have also reported that catechins are stable in acidic environments and unstable in near-neutral or greater pH conditions (Green et al., 2007; Record et al., 2001). Green et al, found that catechin losses of about 80% were seen during digestion of tea compounds (Green et al., 2007).

The Green lab is currently looking at the addition of other common food additives to tea in order to increase the stability of catechins during digestion (Green et al., 2007). They have found that formulating teas with 50% bovine, soy and rice milk increase recovery of catechins as well as the addition of 30mg of ascorbic acid. They also found that the most significant increase in catechin recovery was by the addition of citric juices. Citrus juices significantly increase the recovery of tea catechins after digestion, with EGCG having a 56-76% recovery (Green et al., 2007).

2.3. Tea and cancer – General mechanisms of action

Tea catechins have been suggested to prevent cancer by a variety of different mechanisms. Some studies have used animal models while others have used in vitro models. Tea has been shown to exhibit chemopreventative activities again ultraviolet light, chemically induced and genetic models of carcinogenesis in animal models (Lambert et al., 2003). In the these studies, the lung, skin, oral cavity, esophagus, stomach, liver, pancreas, bladder, small intestine, colon and prostate were all positively affected by tea in terms of chemopreventative activity.
Other studies have used in vitro models to show the anticancer activity of tea and tea catechins. However, these studies have examined the effect of undigested tea and tea catechins, therefore the data may not be as relevant in vivo. Studies looking at digested tea and tea catechins are needed to determine the chemopreventative abilities that would be more applicable to human consumption.

There have been many proposed mechanisms of action for tea and tea catechin chemopreventative activities. One proposed mechanism is antioxidant/pro-oxidant activity (Lambert et al., 2003). Studies have shown that EGCG can inhibit the production of H\textsubscript{2}O\textsubscript{2} in UVB-treated normal human keratinocytes (Katiyar et al., 2001). EGCG has also been shown to inhibit lipid peroxidation in vitro (Erba et al., 1999). Along with that, EGCG has also been shown to induce apoptosis in colon cells by the production of H\textsubscript{2}O\textsubscript{2} (Yamamoto et al., 2004; Yang et al., 1998).

Tumor angiogenesis is the growth of blood vessels leading to and from the tumor cells to supply oxygen and nutrients and remove tumor waste. Vascular endothelial growth factor (VEGF) has been well established as an important angiogenic factor in colon cancer neovascularization (Jung et al., 1999). In stressful a condition such as serum starvation, VEGF has been shown to be significantly increased leading to an increase in tumor angiogenesis for the tumor cell. In vitro studies have shown that extracellular signal related kinases, ERK-1 and ERK-2, are important factors needed for the up-regulation of VEGF mRNA in HT-29 cells. In their next study, Jung et al, investigated the effect of green tea on the activation of ERK-1 and ERK-2 by phosphorylation during serum starvation (Jung et al., 2001). They found that green tea
extract inhibited ERK-1 and ERK-2 activation in a dose-dependent manner.

Another study also showed that EGCG may impart some of its anticancer activity through the inhibition of angiogenesis by reducing VEGF induction via ERK-1 and ERK-2 down regulation (Peng et al., 2006).

Another proposed mechanism is the inhibition of mitogen activated protein kinase (MAPK) signaling. EGCG has been shown to inhibit the activity of numerous transcription factors leading to the deregulation of MAPK pathways (Hwang et al., 2007, Lambert et al., 2003). One previous study showed that EGCG can inhibit the activation of extracellular signal-regulated kinases (ERK1/2), which are involved in functions such as cellular proliferation and neovascularization (Chung et al., 2001).

EGCG has also been shown to inhibit enzymes. One way is that EGCG is able to inhibit the activity of topoisomerase I in human colon cancer cell lines (Berger et al., 2001), which prevents the transcription of many enzymes involved in cellular proliferation and other activities crucial to the development of tumors.

There are many proposed mechanisms for the anticancer activity of EGCG, and many more are still being explored. Most studies have occurred in vitro and will need to be conducted in vivo to determine the physiological relevance. Also, most studies have been done on raw material EGCG. The effect of digestion on catechins has not been taken into consideration, and will need to be studied along with in vivo models.
2.4. Tea and HT-29 Cells

2.4.1. Stability in cell culture

EGCG is stable in acidic conditions, but is autooxidized in neutral and alkaline conditions. HT-29 cells are epithelial-like cells. They were obtained from a forty-four year old female with grade II adenocarcinoma of the colon. HT-29 cells are grown in McCoy’s 5A media, which is pH 7.4. Therefore, EGCG is unstable and has a half life of 30 minutes. However, in the presence of HT-29 cells in McCoy’s 5A media, the half life of EGCG is increased to 130 minutes as opposed to 30 minutes in McCoy’s 5A media without HT-29 cells (Hong et al., 2002). This increase in stability allows time for absorption of EGCG by the cells.

2.4.2. Absorption by HT-29 cells

Absorption of EGCG into HT-29 cells has been studied by many groups. It is suggested that EGCG is absorbed via passive diffusion into HT-29 cells. This suggestion was made due to the uptake of EGCG into HT-29 cells occurring in a dose dependent manner without plateauing (Hong et al., 2002; Lambert et al., 2006). In one study, the group used EGCG in concentrations ranging from 20 to 600 µM with HT-29 cells and found that the cytosolic concentration showed a linear increase as the dose increased (Lambert et al., 2006). A graph of the concentration dependent absorption is shown in Figure 2.3.
2.4.3. Anticarcinogenic mechanisms of tea in HT-29 cells

Chen et al. determined the inhibitory effect of EGCG on colon cancer cell line HT-29 (Chen et al., 2003). They treated the HT-29 cells with varying concentrations of EGCG. They determined that EGCG inhibits the growth of HT-29 cells in a dose dependent manner. They also determined that while HT-29 cells were inhibited by EGCG, EGCG did not exhibit a significant amount of cytotoxicity. This suggests that EGCG inhibited the colon cancer cell growth by a mechanism other than by a cytotoxic manner.
Tea catechins are widely known to be very potent antioxidants. They are able to scavenge reactive oxygen species (ROS) before ROS can cause damage to the cell. ROS are small molecules that include oxygen ion, free radicals and peroxides. They are very reactive due to unpaired valence shell electrons. Some ROS occurring in cells are the hydroxyl radical, hydrogen peroxide and superoxide anion. Some of the harmful effects ROS can cause include DNA damage, oxidation of fatty acids in lipids and oxidation of amino acids in proteins. Damages to DNA can cause mutations and possibly lead to cancer. Antioxidants prevent damage caused by ROS by removing free radical intermediates and inhibiting other oxidation reactions by allowing themselves to be oxidized. The antioxidant ability of tea catechins have been shown to help protect many cells from the ROS damage inhibiting progression to cancer. However, some studies suggest that tea catechins can impose oxidative stress in many tumor cell lines, leading to cytotoxicity and apoptosis through the induction of ROS (Yamamoto et al., 2004).

Yamamota et al found that high concentrations of EGCG damaged tumor cells, but not normal cells. Another study showed that EGCG significantly generated ROS in HT-29 cells (Hwang et al., 2007). The study suggests that the generation of ROS activates AMPK, inhibiting the tumor promotion enzyme cyclooxygenase-2 (COX-2) (Hwang et al., 2007). This could be one mechanism of anticancer activity of tea catechins which could be altered by digestion.
CHAPTER 3

THE EFFECT OF DIGESTIVE MODIFICATION ON THE ANTICANCER ACTIVITY OF TEA CATECHINS IN THE HT-29 HUMAN COLON CANCER CELL LINE

3.1. Objectives

The hypothesis of this study is that digestive modification will affect the chemopreventative properties of tea and tea catechins. The first part of the study will show the effect of digestion of tea catechins on tumor cell viability. The second part of the study will look at mechanisms which could be altered by digestive modification. The first aim is to determine the effect digestive modification has on colon cancer cell viability. It is not known whether the digestion of tea catechins changes the ability to inhibit cancer cell viability that the native compound has been shown to possess. The second aim is to determine the effect digestion has on intracellular oxidative stress. It has not been shown if the amount of intracellular oxidative stress induced by tea catechins is altered by digestion.

3.2. Introduction

Colorectal cancer is the 3rd most common cancer for men and woman worldwide. There will be approximately 108,000 new cases in the year 2008, and it is expected that
approximately 49,000 will die from colon cancer in 2008, accounting for 9% of all cancer deaths (American Cancer Society, 2008). This type of cancer affects men and women equally in which 51% of new cases annually are occurring in men and 49% are occurring in women (Boyle et al., 2000; Hong et al., 2002). Colon cancer is preventable and is curable if detected at an early stage. The development of colon cancer is a slowly evolving process, and typically starts with one or more genetically altered cells (Lipkin et al., 1999).

Colon cancer starts in the epithelial cell lining of the intestinal walls. It is slow growing and usually shows no symptoms until the tumor is large in size. A colon polyp is a fleshy growth that occurs on the lining of the colon. They can be detected by a colonoscopy and removed. Untreated colon polyps are almost always a precursor to colon cancer, but not all polyps will progress into cancer.

Tea is one of the most commonly consumed beverages in the world. There are many types of tea, all classified based on the way that they are produced. Green tea and black tea are two of the major commercial types of tea. Green tea makes up approximately 10% of the world’s tea. Green tea is produced by steaming fresh tea leaves and then drying them. The steaming process heats inactivated oxidative enzymes. Green tea has a greenish-yellowish color. Black tea is the most common type of tea worldwide. Black tea is produced by fermentation. This process involves crushing fresh tea leaves and allowing enzyme-mediated oxidation to occur. The process allows the caffeine amount to triple. Black tea has a reddish-brown color. Oolong tea is the most expensive type of tea. It is produced similar to black tea, but is allowed to oxidize only
half way through the fermentation process. This gives the tea leaves a reddish color.

White tea is the rarest of all tea types. It is produced the same as green tea, but uses the buds of the tea plant instead of the leaves. The buds are white in color and therefore result in a colorless liquid.

Due to the difference in preparation, there is a difference in catechin levels within the two tea types. Common catechins found in tea are (-)epigallocatechin-3-gallate (EGCG), (-)epigallocatechin (EGC), (-)epicatechin-3-gallate (ECG), and (-)epicatechin (EC). Green tea contains 30-40% catechins while the black tea contains 3-10% catechins (Lambert et al., 2003). Fermentation of black tea converts catechins to oligomeric theaflavins and polymeric thearubigins (Lambert et al., 2003).

Digestion of catechins leads to degradation of the compounds. Neilson et al did a study showing the effect of digestion on catechins, and the resulting dimer formation. The study found that EGCG, EGC and ECG significantly degraded during digestions by 71-91%, 72-100% and 60-61%, respectively (Neilson et al., 2007). The study also showed that the loss seen in the intestinal phase of digestion was greater than the loss seen in the gastric phase, therefore suggesting that degradation is mostly correlated to pH levels since the intestinal pH is around 6.0-7.5 and the gastric pH is around 2. EGCG undergoes autooxidation when in a high pH environment. It forms homocatechin dimers of theasinensin (THSN) A, THSN D and P-2 (Neilson et al., 2007). Other studies have also reported that catechins are stable in acidic environments and unstable in near-neutral or greater pH conditions (Green et al., 2007; Record et al., 2001). Green et al, found that
catechin losses of about 80% were seen during digestion of tea compounds (Green et al., 2007).

Research has suggested that tea consumption positively affects cancer risk and outcomes in the gastrointestinal (GI) tract. Tea catechins, such as EGCG, have been shown to exhibit chemopreventative activities. Chen et al., performed a proliferation assay using MTS to determine the antiproliferation ability that EGCG has on colon cancer cell line HT-29 (Chen et al., 2003). In this study it was determined that EGCG inhibited cell proliferation of the HT-29 cells in a dose dependent manner (Chen et al., 2003).

Tea catechins are widely known to be very potent antioxidants. The antioxidant ability of tea catechins have been show to help protect many cells from the ROS damage inhibiting progression cancer. However, some studies suggest that tea catechins can impose oxidative stress in many types of tumor cell lines, leading to cytotoxicity and apoptosis through the induction of ROS (Yamamoto et al., 2004).

Tea catechins have been shown to possess chemopreventative ability in vitro as a raw material form. Whether or not tea catechins possess this same ability once the digestive process has occurred has not been determined.

3.3. Study Design and Methodology

3.3.1. Cell culture

The cells that were used in this study are the colon cancer cell line HT-29. These cells were purchased from the American Type Culture Collection (Rockville, MD). HT-
29 cells were cultured in McCoy’s 5A medium (GIBCO Laboratories, Gran Island, NY) supplemented with 10% fetal bovine serum (FBS). The cell line was incubated in humidified atmosphere (5% CO₂, 95% O₂) at 37°C.

### 3.3.2. Tea and catechin extracts

Different tea extracts were: green tea (GT), black tea (BT) and EGCG. Undigested (raw) compounds and digested samples were evaluated. The Mario Ferruzzi and Rodney Green lab group of Purdue University, Food Science Department, West Lafayette, Indiana, prepared the raw and digested extracts. The digestive modification process included simulation of the gastric and intestinal phases of digestion. In the gastric phase, the raw extracts were exposed to porcine pepsin and pH level of 2. The extracts were incubated for one hour. The intestinal phase of digestive simulation involved the adjustment of pH levels to 5. The extracts were treated with pancreatin, lipase, bile and the pH was adjusted to 7.2. They were incubated for 2 hours and the catechin levels were determined by using high performance liquid chromatography (HPLC).

The tea extracts were applied at a concentration of 3, 1.5, 0.75 and 0.375 mg of extract per mL of media for the specific aims of the study. Table 3.1 shows the catechin profiles for green tea, black tea and EGCG raw and digested extracts. It shows the amount in micrograms of catechin present in 3 mg of extract per mL of media. Figure 3.1 shows HPLC chromatograms of catechin levels in each of the tea extracts.
Table 3.1. Catechin concentrations of tea extracts

<table>
<thead>
<tr>
<th></th>
<th>Green Tea</th>
<th></th>
<th></th>
<th>Black Tea</th>
<th></th>
<th></th>
<th>EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>D</td>
<td>R</td>
<td>D</td>
<td>R</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>EGCG</td>
<td>275</td>
<td>50</td>
<td>54</td>
<td>0</td>
<td>204</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>EGC</td>
<td>188</td>
<td>13</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ECG</td>
<td>46</td>
<td>39</td>
<td>45</td>
<td>23</td>
<td>8</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>EC</td>
<td>31</td>
<td>39</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Caffeine</td>
<td>105</td>
<td>102</td>
<td>200</td>
<td>204</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.1.: Concentration of catechins in ug/mL media in each extract. R = raw extract, D = digested extract.
Figure 3.1. HPLC Chromatograms of extract digestions (Continued)
Figure 3.1.: Continued

b.
Figure 3.1.: Continued

c.

Figure 3.1. a-c are HPLC chromatograms of a. green tea, b. black tea, and c. EGCG pre and post digestion showing the phenolic content of each extract. RM = Raw material, DG = Digested material. EGCG = (-)epigallocatechin-3-gallate, EC = (-)epicatechin, EGC = (-)epigallocatechin, ECG = (-)epicatechin-3-gallate. They were sent from the Mario Ferruzzi and Rodney Green lab group of Purdue University, Food Science Department, West Lafayette, IN.

Figure 3.1. a-c shows the high performance liquid chromatography (HPLC) chromatograms for a. green tea, b. black tea and c. EGCG. The HPLC analysis was performed by Mario Ferruzzi’s Food Science Department lab group at Purdue University of West Lafayette, IN. The chromatograms show that the catechin content of the extracts is significantly reduced during digestion. EGCG is the most affected while ECG is the least affected by digestion. The ability of the tea catechins to still produce anticancer activity after the loss seen during digestion will be examined in the following sections.
3.3.3. Cell viability

The first aim of the study is to determine the effect that digestion of tea phenols has on viability of colon cancer cell line HT-29. The experiment used to establish cell viability would be an MTT assay. This assay determines the relative amount of viable cells remaining after treatment with a specific agent. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow compound that when taken up by the mitochondria of living cells is reduced to a purple formazan by mitochondrial reductase enzymes. A solubilization solution containing hydrochloric acid (HCl) in isopropanol is added to dissolve the insoluble purple formazan crystals. The absorbance of the purple solution is then run on a spectrophotometer. The amount of purple formazan produced by cells treated with the agent is then compared to the amount of purple formazan produced by control cells, which are untreated. The comparison is an indication of the effectiveness of the treatment. In performing this assay, raw material and digested green tea, black tea and EGCG were used to examine the effect of digestion on these compounds. These results were then compared to determine if there are digestive end products contributing to the chemopreventative activity of tea or if digestion impairs the ability to prevent cancer cell viability. The cells were plated on 96-well plates containing 1x10^4 cells per well. They were incubated for thirty-six hours. The cells were then treated with raw material and digested end products of green tea, black tea and EGCG. A plate of cells treated with distilled water is to act as a control to determine if anything used for the digestion of the compounds causes cytotoxicity to the cells. After a period of incubation the MTT assay was performed. MTT was added directly to the wells and
incubated for three hours. HCl in isopropanol was then added and the amount of purple formazan was determined by the spectrophotometer at a wavelength of 610 nm.

### 3.3.4. Intracellular Oxidation

The second aim of this study is to determine the effect that digestion has on intracellular oxidative stress. The 2’,7’-dichlorofluorescein (DCF) assay was used to determine the relative amount of intracellular oxidation induced by the treatment of specific extracts in combination with 2,2’-azobis-(2-aminopropane)dihydrochloride (AAPH). 2’,7’-Dichlorofluorescein-diacetate (DCFDA) is a nonfluorescent probe that is cell permeable. It is taken up by the cell and hydrolyzed into the active probe 2’,7’-dichlorohydofluorescein (DCFH) by intracellular esterases (Tampo et al., 2003). The cells are then treated with AAPH, a radical generator which then oxidizes DCFH to the fluorescent oxidation product 2’,7’-dichlorofluorescein (DCF). For this study, the cells were plated on a 96-well plate at a concentration of 1.5 x 10^5 cells/mL. The cells were treated with test media prepared with predetermined amounts of tea extract along with DCFHDA, and incubated for one hour. AAPH was added to each well and plates were read on a spectrophotometer immediately. They were read for one hour at an excitation emission filter of 485/525 nm at 37°C with data points being taken every 90 seconds to determine the amount of fluorescence emitted by the oxidation product of DCFH, DCF.
3.4. Results

3.4.2. Cell viability

Figure 3.2. IC50

The concentration of gallic acid equivalents required to reduce HT-29 cell viability by 50% (IC50) is given in Figure 3.2. HT-29 cells treated with raw green tea extract reduced HT-29 cell viability with 43% of GAE of the digested green tea extract (raw IC50 = 38 µg/ml, digested IC50 = 88 µg/ml). The effect of treatment with black tea extract on HT-29 cell viability was similar between digested and raw extracts, with the raw extract having an IC50 of 69 µg/ml, which is approximately 95% of the extract needed for the digested extract (IC50 of 72 µg/ml). The raw EGCG extract reduced HT-
29 cell viability to a greater extent than digested EGCG. Treatment of HT-29 cells with raw EGCG needed 64% of GAE of the digested extract to reduce viability to 50%. The raw EGCG had an IC$_{50}$ of 14 µg/ml and the digested had an IC$_{50}$ of 22 µg/ml.

Figure 3.3. Percent Viability (Continued)
Figure 3.3.: Continued

b.

% Viability - Black Tea

Raw Material
Digested Material

(Continued)
The effect of green tea, black tea and EGCG raw versus digested on HT-29 cell viability is illustrated in Figures 3.3 a, b and c. Treatment of HT-29 cells with 3mg/mL of green tea or black tea raw and digested extracts significantly reduced cell viability for HT-29 cells. HT-29 cells treated with 3mg/mL of raw green tea reduced cell viability to 14% and 3mg/mL of digested green tea reduced cell viability to 16% for HT-29 cells (Figure 3.3.a.). The black tea treated HT-29 cells showed that the raw extract had 16% of cells that were viable versus the control and the digested black tea showed 16% viable
Treatment of HT-29 cells with raw and digested EGCG reduced cell viability in HT-29 at 3mg solids/ml; however, the raw material was more effective than the digested (Figure 3.3.c.). The raw EGCG compound decreased HT-29 cell viability to 20% and the digested EGCG showed that 40% of the HT-29 cells were viable versus the control.
3.4.3. Intracellular Oxidation

a.

**Figure 3.4. Intracellular Oxidation**

<table>
<thead>
<tr>
<th>Raw Material</th>
<th>Digested Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCF</td>
<td>+</td>
</tr>
<tr>
<td>AAPH</td>
<td>+</td>
</tr>
<tr>
<td>GT (mg/mL)</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Raw Material</th>
<th>Digested Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.375</td>
<td>+</td>
</tr>
<tr>
<td>0.75</td>
<td>+</td>
</tr>
<tr>
<td>1.5</td>
<td>+</td>
</tr>
<tr>
<td>3.0</td>
<td>+</td>
</tr>
</tbody>
</table>

**Intracellular Oxidation - Green Tea**

Arbitrary Units

- DCF: +
- AAPH: +
- GT (mg/mL): - 0.375 0.75 1.5 3.0

(Continued)
Figure 3.4.: Continued

b.

Intracellular Oxidation - Black Tea

![Graph showing the comparison between raw material and digested material with different concentrations of DCF, AAPH, and BT (mg/mL). The x-axis represents the concentration of BT (mg/mL) and the y-axis represents Arbitrary Units. The graph indicates a significant increase in intracellular oxidation with increasing concentrations of BT.](image-url)
The effect of digested and raw green tea, black tea and EGCG extracts on intracellular oxidation in HT-29 is given in Figures 3.4. a-c.

HT-29 cells treated with 3mg/mL of raw and digested green tea or black tea extracts increased intracellular oxidation in HT-29 cells when in combination with AAPH. Raw green tea and AAPH produced a 40% increase over the untreated HT-29
control cells and the digested produced at 46% increase (Figure 3.4.a.). HT-29 cells that were treated with black tea along with AAPH showed a 42% increase over the control for the raw extract and the digested showed a 48% increase in intracellular oxidation (Figure 3.4.b.). Though there was an increase in intracellular oxidation in HT-29 cells above the extract-free controls for both raw and digested green and black compounds with AAPH, there was no significant difference between raw and digested extracts (p-value = 0.50 for both).

Treatment of HT-29 cells with 3mg/mL EGCG and AAPH also increased intracellular oxidation for both raw and digested extract. HT-29 cells treated with digested extract and AAPH produced a 37% increase over the untreated HT-29 cell control while the HT-29 cells treated with raw extract and AAPH showed a 48% increase (Figure 3.4.c.). Again, there was no significant difference of the increase in intracellular oxidation between the two EGCG extracts (p-value = 0.10).

3.5. Discussion

Digestive modification of green tea and EGCG reduces the ability to decrease cell viability in the HT-29 cell line. Raw green tea had an IC\textsubscript{50} with approximately 43% of GAE concentration that was needed for digested green tea to do the same and the EGCG raw extract only needed 64% of GAE concentration of the digested extract for an IC\textsubscript{50}. Black tea was not affected by digestive modification in terms of cell viability. The two black tea extracts had similar GAE concentrations to produce 50% cell viability. EGCG showed a much lower concentration of GAE to reach IC\textsubscript{50} than the green tea and black
tea for raw and digested material. This could suggest that EGCG is the most effective of
the other gallic acids in green tea and black tea.

Both raw and digested extracts decreased cell viability for green tea, black tea and
EGCG. However, the raw material for green tea and EGCG reached a low viability with
lower concentrations than the digested material needed. Black tea showed no difference
between raw material and digested material. The difference between raw and digested
green tea and EGCG suggests that digestion of tea catechins inhibits the anticarcinogenic
properties seen in previous studies with raw compounds, and this difference could be due
to the alterations in catechins for the extracts.

Intracellular oxidation was increased by both raw and digested compounds for
green tea, black tea and EGCG in HT-29 cells. Although intracellular oxidation was
increased, after t-test statistical calculations, there was no significant difference between
the amounts of increase for raw material versus digested material for green tea, black tea
or EGCG. They all showed similar effects regardless of whether they were raw material
or digested compounds. These results provide evidence that the induction of intracellular
oxidation is not the mechanism that is altered during digestion of tea catechins, which
leads to the decrease in anticarcinogenic activity of these compounds post-digestion.
Further work is required to determine which mechanism of anticancer activity is being
altered during digestion in the HT-29 cells which causes digested material to be less
effective in decreasing cell viability than raw material.
4.1. Summary

The current study showed that the digestion of tea and tea catechins affects the chemopreventative ability in the colon cancer cell line, HT-29. This was shown by the higher catechin concentration needed to reach IC$_{50}$ for green tea and EGCG. Black tea showed no difference between the extracts. This could be attributed to the significantly lower amount of catechins in black tea as opposed to green tea due to the production process. This suggests that the reduction of anticancer activity in green tea is due to the decrease in catechin levels during digestion.

Intracellular oxidation was increased by all raw material and digested material for each of the three extracts. However, there was no significant difference between raw material and digested material for any of the extracts. This suggests that the mechanism of anticancer activity induced by green tea catechins is not the induction of intracellular oxidation because the same increase was seen in raw material and digested extracts.
4.2. Future studies

Data from these studies suggest that digestion affects the chemopreventative ability of green tea catechins. However, the mechanism which is altered is not yet known. Intracellular oxidation was examined in this study, but was eliminated due to no difference between raw material and digested extracts. Future studies will need to be done on other possible mechanisms which could be altered by digestion.

One possible study could be to look at the affect of digestion on extracellular signal-regulated kinases 1 and 2 (ERK1 and 2). Extracellular signal related kinases, ERK-1 and ERK-2, are important factors needed for the up-regulation of VEGF mRNA in the HT-29 cell line. Vascular endothelial growth factor (VEGF) has been well established as an important angiogenic factor in colon cancer neovascularization, leading to tumor growth and development (Jung et al., 1999). Studies have suggested that catechins can inhibit ERK-1 and ERK-2, ultimately reducing angiogenesis (Peng et al., 2006). This is a possible mechanism that could be altered by digestion, reducing the chemopreventative ability of catechins.
LIST OF REFERENCES


