THE EFFECT OF TEA POLYPHENOLS ON CHRONIC DISEASE: OBESITY, THE METABOLIC SYNDROME, AND COLON CANCER

by

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ABSTRACT OF THE DISSERTATION

The Effect of Tea Polyphenols on Chronic Disease:

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The prevalence of obesity and the metabolic syndrome has more than doubled in the last 30 years. Colon cancer is the second leading cause of cancer-related death in the United States. Many studies show beneficial effects of certain dietary constituents, including green tea, in the prevention of these diseases. In the first set of experiments, I determined the effect of (-)-epigallocatechin-3-gallate (EGCG) on weight gain and related factors in a high-fat diet-induced mouse model for obesity and the metabolic syndrome. I found that l6-week treatment of 0.32% EGCG in the diet (w/w) significantly reduced body weight gain (by >50%) and body fat percentage (by 10%) in comparison to control mice. Visceral fat weight was significantly decreased by EGCG treatment. Biochemical measurements revealed that EGCG treatment significantly attenuated insulin resistance and plasma cholesterol. Liver pathologies were significantly lessened by EGCG, as indicated by decreased liver

weight, triglyceride content, and transaminase release into plasma. Plasma monocyte chemoattractant protein (MCP-1) levels were also decreased by EGCG, suggesting reduction of inflammation by EGCG.

In the next set of experiments, I determined the effect of a combination of EGCG (0.16% in the drinking fluid) and fish oil in the diet (12% w/w), on intestinal tumorigenesis in *Apc*$^{Min/+}$ mice. Combination treatment reduced tumor number by 53% compared to controls; at the doses used, neither agent alone had a significant effect. β-catenin nuclear positivity in intestinal adenomas from the combination group was lower than control mice, indicating modulation of *Wnt* signaling. Fish oil and the combination significantly reduced prostaglandin E_2 levels in adenomas compared to controls, suggesting modulation of aberrant arachidonic acid metabolism. Akt phosphorylation in adenomas was significantly reduced in all treatment groups, which may have contributed to the observed increase in apoptosis. The results from these two studies indicate that long-term treatment with EGCG decreases risk for obesity and conditions associated with the metabolic syndrome, and that a combination of EGCG and fish oil can inhibit intestinal tumorigenesis in *Apc*$^{Min/+}$ mice. These effects may be mediated through multiple mechanisms and should be addressed in future studies in the prevention of chronic disease.

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DEDICATION

I would like to dedicate this dissertation to my family, especially my mother and father, who gave me their unwavering support throughout graduate school; my cousin Suhel (aka “Carl”), who is a very loving and nurturing older brother; my cousins Kaustav (aka “Shubs”) and Preshona (aka “Mums”), who always had faith in their “Bordi”; and my sister Mahua (aka “Monster”), who made me laugh when no one else could. I would like to dedicate my dissertation to my husband of almost 6 years, Andrew Betzer, who has given me the strength to complete this part of my life. I look forward to sharing the rest of our life together.

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ABBREVIATIONS

ACF	Aberrant crypt foci
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AOM	Azoxymethane
APC	Adenomatous polyposis coli
AST	Aspartate aminotransferase
ATP III	Adult Treatment Panel III
BMI	Body mass index
COMT	Catechol-*O*-methyltransferase
COX	Cyclooxygenase
CRP	C-reactive protein
DEXA	Dual-energy x-ray absorptiometry
DHA	Docosahexaenoic acid
DMH	Dimethylhydrazine
EC	(-)-Epicatechin
ECG	(-)-Epicatechin-3-gallate
EGC	(-)-Epigallocatechin
EGCG	(-)-Epigallocatechin-3-gallate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIA	Enzyme immunoassay

ABBREVIATIONS (cont.)

EPA	Eicosapentaenoic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular regulated kinase
FAP	Familial adenomatous polyposis
GLUT4	Glucose transporter 4
G6P	Glucose-6-phosphatase
HDL	High-density lipoprotein
HF	High fat diet
HFE	High fat plus 0.32% EGCG diet
HOMA-IR	Homeostasis model assessment of insulin resistance
IGF	Insulin-like growth factor
IRS	Insulin receptor substrate
LDL	Low-density lipoprotein
LF	Low fat diet
LNA	α-Linolenic acid
MCP-1	Monocyte chemoattractant protein-1
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NCEP	National Cholesterol Education Program
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PGE_2	Prostaglandin E_2

ABBREVIATIONS (cont.)

PI3K	Phosphoinositide-3-kinase
PKC	Protein kinase C
TIIDM	Type II diabetes mellitus
TNF-α	Tumor necrosis factor alpha
ω-3 PUFA	Omega-3 polyunsaturated fatty acids

CHAPTER 1

INTRODUCTION AND SPECIFIC AIMS

1.1.1. Obesity and the metabolic syndrome

Obesity is a health condition that is characterized by a body mass index (BMI) that is 30 or higher [1]. This increase in BMI is usually attributed to an accumulation of excess adipose tissue, and is a result of a positive energy balance, where energy intake is greater than energy expenditure. The prevalence of obesity has increased dramatically in the last several decades. The National Health and Examination Survey reported that the prevalence of obesity among adults in the United States was more than 2 times higher in the 2003-2004 survey in comparison to the 1976-1980 survey [2]. Similar trends were observed for children and adolescents [3]. According to the World Health Organization, in 2005, there were 400 million obese adults worldwide [4]. Obesity has become an issue of national and global concern because of its rapidly increasing rates and its co-morbidities, such as type II diabetes and cardiovascular disease. [5-7]. Obesity is also associated with increased risk of various cancers, including endometrial, breast, and colon cancer [8-13].

The metabolic syndrome is a clustering of pathologies associated with obesity that includes excess abdominal fat (central adiposity), insulin resistance (which can lead to type II diabetes), and hyperlipidemia (which can lead to cardiovascular disease) [5]. According to the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III), the metabolic syndrome is diagnosed in patients when they exhibit 3 of the 5 following symptoms: waist circumference > 40 inches, serum triglycerides $\geq$ 150

mg/dl, high-density lipoprotein (HDL) cholesterol < 50 mg/dl, blood pressure > 135/85 mg Hg, and fasting blood glucose > 110 mg/dl. Insulin resistance is also usually present in the metabolic syndrome. Other symptoms associated with the metabolic syndrome include endothelial dysfunction, hepatic steatosis, and increased inflammation (local and systemic) [14]. The prevalence of the metabolic syndrome has increased concomitantly with the prevalence of obesity in the United States [15]. In 2000, there were approximately 47 million men and women in the United States who were diagnosed with metabolic syndrome [16].

Consumption of a high-fat diet is a strong risk factor for the development of obesity and metabolic syndrome [17]. Epidemiological studies have shown that obesity is generally more prevalent in societies that consume a Western-style diet, which, in addition to being deficient in several nutrients such as calcium and vitamin D, is also high in fat (30-40% of kcal in diet) [18-20]. Dietary, pharmacological, and surgical strategies have been developed in the last decade to prevent the metabolic effects of a high-fat diet. These methods control food intake, increase energy expenditure, promote fat oxidation in the body, or inhibit fat absorption into the body. Pharmacological treatment for the metabolic syndrome often consists of separate drugs targeted at the individual symptoms of the disease [15]. Despite these advances, there are still several risks associated with pharmacological and surgical intervention of obesity and the metabolic syndrome, suggesting that dietary modification may be the safest and most cost-effective option for those who are moderately obese [21-24]. To date, nutritional intervention is still the

preferred method of treatment and prevention for obesity and the metabolic syndrome [14, 15].

1.1.1.1. Dietary prevention of weight gain by tea and tea constituents

Tea (*Camellia sinensis,* Theaceae*)* is one of the most widely consumed beverages in the world, second only to water in popularity. There are four major types of tea made from this plant. White tea and green tea are both prepared from either steaming or pan-frying tea leaves to inactivate oxidative enzymes. Oolong and black tea are crushed and allowed to undergo oxidation (fermentation). The two major components of tea are caffeine and polyphenols, and it is believed that the health benefits of drinking tea are attributable to its polyphenol content. Although all types of tea are rich in polyphenolic compounds, the processing of tea dictates the types and quantities of polyphenols that are found in each specific beverage. Whereas oolong and black tea contain oligomeric compounds called theaflavins and thearubigens, green tea contains mainly monomeric polyphenols known as catechins. A typical brewed green tea beverage (2.5 g green tea in 250 ml hot water) contains about 240-320 mg catechins including (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and epigallocatechin-3-gallate (EGCG) (Figure 1.1). EGCG is the most abundant catechin present in green tea, and it is believed to be the most biologically active component of green tea [25].

Among its many proposed health benefits, tea is believed to have hypolipidemic and potential weight loss effects. Although epidemiological studies have observed an inverse relationship between green tea consumption and blood lipid levels [26-28], only one major epidemiological study has reported a clear association between green tea

intake and body fat percentage [29]. Human intervention trials have offered more insight as to how tea can promote weight loss, but many factors have affected the outcome of each study. Since 1999, 12 human intervention trials concerning the effects of tea ingestion on weight loss and energy metabolism have been published.

In 1999, Dulloo *et al.*, in a randomized, crossover trial with 10 young males, showed that green tea extract (containing 90 mg EGCG and 50 mg caffeine, administered 3 times daily over 5 days) increased energy expenditure and fat oxidation, whereas the same doses of caffeine alone did not produce such effects [30]. This finding was followed by a study by Rumpler *et al.* using a similar crossover design with 12 men [31]. Ingestion of oolong tea (containing 48.8 mg EGCG and 54 mg caffeine, administered 5 times daily) for 3 days increased 24-hour energy expenditure and fat oxidation in comparison to that of control subjects given only water. Subjects who consumed the corresponding amount of caffeine showed an increase in 24-hour energy expenditure similar to the effects found in the tea group [31]. This finding suggested that the effects on energy expenditure and fat oxidation were due mainly to the caffeine, in contrast to the findings of Dulloo *et al.* In 2005, Berube-Parent *et al.* administered different amounts of green tea extract (containing 90-400 mg EGCG) mixed with Guarana extract (to maintain caffeine levels at 200 mg) 3 times daily to 14 males in a randomized crossover study [32]. They observed increased 24-hour energy expenditure and decreased carbohydrate oxidation with all doses of treatment. The most significant effect was observed in the lowest dose (90 mg EGCG and 200 mg caffeine, 3 times daily). Higher doses of EGCG did not result in significantly larger increases in energy expenditure. A recent crossover trial by Hsu *et al*.

showed that subjects who consumed 38 g lipids and ingested polyphenol-enriched oolong tea (750 ml containing 250 mg polyphenols, 3 times daily) for 10 days had increased fecal lipid excretion in comparison to subjects consuming the same amount of lipids with a placebo beverage, suggesting that tea polyphenols inhibit fat absorption [33].

Longer trials were also conducted to determine the effects of green tea on decreases in body weight or body fat. Most of these studies ran for 10-12 weeks, and the results of these studies varied. In 2004, Tokimitsu *et al.* showed that consumption of a beverage containing 588 mg tea catechins daily for 12 weeks significantly decreased body weight and total body fat area in a population of healthy males and females compared to daily consumption of a control beverage containing 126 mg tea catechins [34]. Caffeine content of the beverages was not reported, and it may have been a confounding factor. This study was followed by Nagao *et al.*, who found that healthy men (n=17) who consumed oolong tea enriched with tea catechins (690 mg tea catechins total and 22 mg caffeine) daily for 12 weeks showed significantly greater body weight loss, BMI reduction, and reductions in body fat mass and total fat area than subjects who consumed a control oolong tea beverage (that contained 22 mg tea catechins plus 23 mg caffeine, n=18) [35]. A study by Westerterp-Plantenga *et al*. showed that, when compared to a placebo-treated group, green tea consumption (45 mg EGCG and 25 mg caffeine 6 times daily) for 3 months following weight loss (by a low energy diet) prevented body weight and fat mass gain in 78 overweight male and female subjects [36]. This effect, however, was only observed in subjects who were low caffeine consumers at baseline (<300 mg/day). That is, during the weight maintenance phase after weight loss, ingestion

of green tea extract resulted in significantly less body weight regain in low caffeine consumers than in subjects who consumed more than 300 mg caffeine/day (at baseline) or in subjects from the placebo group. This decrease was accompanied by an increase in fat oxidation and energy expenditure in the low-caffeine consumers treated with green tea. However, another 10-week study by Diepvens *et al.* showed that among 46 overweight female subjects on a low-calorie diet, treatment with 134 mg green tea catechins (66.2 mg EGCG and 26.3 mg caffeine), 9 times daily, produced no added effect on weight loss [37]. It should be noted, however, that the subjects for this study were originally moderate caffeine consumers (300-312 mg caffeine/day).

In all of the above studies, caffeine was present to some extent in the treatment. The caffeine found in tea beverages may play a role in promotion of weight loss. In the studies that attempted to distinguish the effect of tea catechins from that of caffeine [30-32, 35], tea catechins seemed to have a slight additive effect on energy expenditure or weight loss compared to caffeine alone. It appears that the length of treatment and an individual's prior caffeine consumption may influence the role of green tea and green tea catechins in promoting weight loss. The possible synergistic effect of catechins and caffeine on weight loss remains to be determined. In addition, the differences in the human populations studied (*i.e.* dietary lifestyle, genetics of the population) may also play a role as to whether tea consumption affects weight gain or body fat accumulation.

Since 1999, 14 papers have reported the effect of tea on weight loss in animals. Most studies with rodent models have observed a consistent effect of tea and tea polyphenols on weight reduction. These studies have also suggested biological

mechanisms by which tea catechins exert their metabolic effects. In these investigations, rodents were either treated with green tea extract, which contained caffeine and catechins, or individual catechins, particularly EGCG. Kao *et al.* (2000) reported that daily treatment of EGCG (*i.p.*) to obese and lean Zucker rats (92 and 81 mg EGCG/kg body weight, respectively) significantly reduced body weight after 7 days, but the change in body weight was mainly attributed to the decrease in food intake by the treated mice [38]. In another study, obese Zucker rats receiving 50-125 mg EGCG/kg body weight, *i.g.*, showed no significant reduction of body weight gain [39].

Several subsequent studies in rodents given green tea or EGCG perorally have demonstrated an inhibitory effect of treatment on body weight gain. Murase *et al.* showed that in C57BL/6J mice on a high-fat diet (30% w/w), administration of 0.2%-0.5% green tea catechins in the diet for 11 months significantly reduced body weight and visceral fat weight compared to control mice. This same study also showed that short-term treatment (1 month) with 0.5% green tea catechins increased hepatic lipid β-oxidation activity and expression of several enzymes associated with β-oxidation, such as acyl CoA oxidase and medium-chain acyl-CoA dehydrogenase [40]. Another study by Shimotoyodome *et al.* confirmed these findings on the reduction of body weight and fat. They also showed that green tea catechin treatment increased muscle endurance capacity during exercise [41].

In 2005, Ikeda *et al*. showed that administration of a diet containing 1% THEAFLAN 90S (a decaffeinated green tea powder containing 44.1% EGCG) for 23 days significantly reduced visceral body fat (as a percentage of body weight) in Sprague-Dawley rats [42]. Ikeda *et al*. found that an oral dose of green tea catechins (100 mg

THEAFLAN 90S) followed by a lipid emulsion dose delayed lymphatic recovery of postprandial triacylglycerol, suggesting decreased lipid absorption as a possible mechanism by which tea catechins mediate their weight loss effects [43].

Other groups have investigated the dietary effects of EGCG on weight loss in rodents. Klaus *et al.* observed significantly decreased body weight gain, total body fat gain, and epididymal body fat weight in New Zealand Black mice treated with a high-fat diet (15% w/w) containing 0.5-1% TEAVIGO (a green tea extract containing ≥94% EGCG and ≤0.1% caffeine) for 4 weeks compared to control mice [44]. New Zealand mice that were orally administered TEAVIGO (500 mg/kg body weight, *i.g.*) for three days showed a significantly decreased respiratory quotient compared to mice not given TEAVIGO, suggesting increased fat oxidation by the treatment. Wolfram *et al.* also observed that TEAVIGO supplementation (1% in the diet) to high-fat (20% w/w) fed C57BL/6J mice for 5 months significantly decreased body weight and the subcutaneous and epididymal adipose tissue weights [45].

Tea catechins may cause body weight loss by multiple mechanisms, including inhibition of lipid absorption, enhancement of fat oxidation, and inhibition of fatty acid synthesis [30-33, 40-45]. Some studies performed *in vitro* have found modulation of adipocyte differentiation, proliferation, and cellular uptake of fatty acids by EGCG [46-48]. Other studies have suggested that EGCG may increase energy expenditure by inhibiting the activity of catechol-*O*-methyl transferase (COMT), which mediates the degradation of norepinephrine [49]. Inhibition of this enzyme may prolong the effect of norepinephrine in promoting the sympathetic nervous system to increase energy

expenditure. To this effect, Dulloo *et al.* showed an increase in norepinephrine excretion with green tea catechin treatment in a population of young males [30]. More *in vivo* studies are warranted to determine whether these effects contribute to the body weight reduction effects of green tea catechins.

1.1.1.2. Dietary prevention of conditions associated with metabolic syndrome by tea catechins

There are very few studies on the effect of tea on risk for metabolic syndrome. A recent epidemiological study reported no clear association between green tea consumption and metabolic syndrome in a Japanese population [50]. A recent animal study showed that EGCG treatment (200 mg/kg body weight, *i.g.*) for 3 weeks significantly reduced blood pressure, and significantly increased insulin sensitivity and adiponectin levels in spontaneously hypertensive rats, a rodent model of the metabolic syndrome [51]. Despite the small number of reports on the effects of tea on metabolic syndrome, there have been several studies that have observed the effects of tea on individual components of the metabolic syndrome.

1.1.1.3. Insulin resistance and type II diabetes

Type II diabetes mellitus (TIIDM) is generally characterized by a failure of the body to appropriately respond to insulin (insulin resistance), in contrast to type I diabetes, when the body cannot produce enough insulin [17]. As a result of either condition, glucose disposal to key organs such as skeletal muscle is decreased and blood glucose levels remain high. If uncontrolled, this can lead to blindness, nerve damage, kidney disease, cardiovascular disease, and other potentially fatal complications [52]. In 2005,

the total prevalence for either type of diabetes was 7% in the United States. Currently, TIIDM is more common than type I diabetes in the U.S. [17].

There are three major organs in the body that are affected by insulin resistance: the muscle, the liver, and the adipose tissue. These are the tissues that, under normal conditions, produce a response to elevated levels of blood glucose, via the action of insulin [53]. When blood glucose is elevated, insulin is released into the bloodstream from the β-cells in the pancreas. In the muscle and the adipose tissue, the major response of insulin binding to cell surface receptors is the activation of phosphoinositide-3-kinase (PI3K) signaling cascade, which activates the protein AKT. The activation of AKT contributes to the translocation of the glucose transporter GLUT4 from the cytosol to the cell surface, so that glucose can enter the cell [54]. The uptake of glucose in muscle and adipose tissue plays an important role in returning blood glucose to normal levels. In the liver, insulin binding also triggers the PI3K cascade, resulting in an inhibition of gluconeogenic enzymes and suppression of hepatic glucose output [53]. In insulin-resistant individuals, insulin is released upon elevation of blood glucose; however, there is a diminished response from the muscle, adipose tissue, and liver to increase glucose uptake or decrease glucose output. The pancreas initially responds by producing more insulin to maintain normal blood glucose levels. Nevertheless, these individuals usually remain hyperglycemic and are also hyperinsulinemic [53]. Type II diabetes is characterized by both insulin resistance and the eventual development of pancreatic β-cell dysfunction [17].

1.1.1.4. Dietary prevention of TIIDM and insulin resistance by tea polyphenols

Several human and animal studies have suggested that tea catechins also help to improve glucose homeostasis independent of their effects on fat metabolism. Iso *et al.* reported in 2006 that TIIDM risk was inversely associated with green tea consumption in a population of Japanese females and overweight males [55]. This trend was irrespective of body mass index. Since 2003, there have been 4 major human intervention trials published describing the effects of tea ingestion on the risk of TIIDM and insulin resistance.

In 2003, a randomized, crossover study by Hosoda *et al.* showed that healthy males and females (n=20) administered oolong tea (containing 77.2 mg EGCG and 70.5 mg caffeine, 5 times daily) for 4 weeks had significantly lower fasting blood glucose and fructosamine (a marker of long-term glucose homoeostasis) levels, but the treatment did not affect body weight or body mass index [56]. Tsuneki *et al.* showed that consumption of a 1500-ml green tea beverage (0.1% green tea, containing 84 mg EGCG and 64.5 mg caffeine) significantly improved glucose tolerance in healthy volunteers compared to volunteers consuming water (n=22) [57]. However, a study by Fukino *et al.* reported no effect of 2-month tea catechin treatment (456 mg/day) on fasting glucose and insulin levels in a population of diabetic or pre-diabetic Japanese men and women [58]. Recently, Zhong *et al.* found that when healthy subjects (n=20, crossover design) were given an extract of green, black, and mulberry tea (containing 300 mg EGCG, 100 mg theaflavins, and 5 mg deoxynojirimycin-type compounds), concurrently with a high

starch and lipid meal, carbohydrate absorption (as measured by breath hydrogen) was significantly blunted [59].

In 1995, Gomes *et al.* showed the blood glucose lowering effect of green tea and black tea extract in Sprague-Dawley rats [60]. Since then, 5 animal studies have been performed to further elucidate the effect of tea catechins on diabetes and insulin resistance. Tsuneki *et al.* showed that insulin-resistant transgenic *db/db* mice that were given an oral dose (300 mg /kg body weight) of green tea powder (containing 5.6% EGCG and 4.3% caffeine) had significantly lower fasting blood glucose levels 2 hours after treatment compared to mice treated with saline [57]. Wu *et al.* showed that fructose-fed (insulin resistant) Sprague-Dawley rats treated with 0.5% green tea (as drinking fluid) for 12 weeks exhibited improved glucose tolerance and significantly decreased plasma insulin levels following an oral glucose tolerance test [61]. A recent study by Wolfram *et al.* showed that *db/db* mice treated with 0.25-1% TEAVIGO for 7 weeks improved glucose tolerance and insulin sensitivity. TEAVIGO-treated mice displayed higher plasma insulin levels in the fed state, in contrast to the previous study by Wu *et al* [62]. A similar finding was described in this study from insulin-resistant ZDF transgenic rats, implying that EGCG increased hyperinsulinemia. It was suggested that this effect might be due to EGCG improving pancreatic β-cell function. It cannot, however, be ruled out that fed state measurements introduce confounding variables such as the effects of individual food intake on insulin levels.

Several mechanisms by which green tea catechins improve glucose homeostasis have been proposed. Animal studies have suggested that increased insulin sensitivity

from tea catechin treatment may be due to increased affinity of insulin to its receptor in insulin-responsive tissues and increased level of glucose transporters in these tissues [61, 63]. The findings of Zhong *et al.* that showed decreased carbohydrate absorption by tea treatment in humans are supported by previous reports that treatment with tea catechins inhibited the activity of the sodium-glucose transporter in intestinal cells and brush border membrane vesicles [64-66]. It has also been shown *in vivo* and *in vitro* that tea catechins, particularly EGCG, inhibited expression of hepatic gluconeogenic enzymes, which subsequently suppressed liver glucose output [62, 67, 68]. Whether these mechanisms apply to the human situation remains to be investigated.

1.1.1.5. Hepatic steatosis and nonalcoholic fatty liver disease

Hepatic steatosis (fatty liver) is a condition that is defined by fat accumulation within hepatocytes that exceeds 5% of the liver by weight [69]. Initially, it was believed that this condition was mainly attributable to excess alcohol consumption, but studies in the last several decades have also linked obesity and diabetes to the presence of fatty liver, coining the term nonalcoholic fatty liver disease (NAFLD) [70]. Other characteristics of NAFLD include liver inflammation and fibrosis [71].

There are several proposed grades of NAFLD that characterize the severity of the condition. Grades 1-2 describe simple steatosis, with mild inflammation, whereas grades 3-4 describe florid steatosis, chronic and acute inflammation throughout the liver, and fibrosis [72]. The elevated condition found in grades 3-4 is referred to as nonalcoholic steatohepatitis (NASH). The incidence of NASH is strongly associated with the development of liver disorders, such as cirrhosis [69].

Currently, NAFLD is the most common form of liver disease [73, 74]. However, accurate representation of the prevalence of NAFLD and NASH has been challenging, as only a liver biopsy would truly determine the presence of either condition. It is estimated that the prevalence of NAFLD in U.S. adults ranges from 6-14% [75]. A study of 351 patients at autopsy revealed that while 35% of lean subjects had some type of steatosis, 70% of the obese subjects possessed steatosis. NASH was present in 18.5% of the obese subjects while only 2.5% of the lean subjects exhibited NASH [76]. Other reviews have suggested that 75% of NASH patients are obese or have TIIDM [69, 71]

It is now widely accepted that NASH is the hepatic component of the metabolic syndrome; risk factors for the disease include obesity, insulin resistance, and hypertriglyceridemia [71, 77-79]. NASH contributes to liver dysfunction, as indicated by elevated liver enzymes, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in blood from patients with NASH [79]. Blood ALT and AST levels are also associated with obesity and type II diabetes. [80, 81]. However, it should be noted that not all patients with NAFLD exhibit elevated liver enzymes in the blood, nor does this measurement represent the degree of the condition [82].

1.1.1.6. Dietary prevention of fatty liver and liver dysfunction by tea constituents

To date, there is only one major epidemiological study that has observed the effect of tea consumption on liver dysfunction. In 1995, a study in Japanese men (ages 51.2-55.7 years) found that green tea consumption was associated with a trend for

decreased serum ALT and AST levels [83]. However, these trends did not reach statistical significance.

There are several studies that have reported the effect of tea or tea catechins on hepatic steatosis in animal models. Only two studies, however, have studied the effect of tea constituents on high-fat diet-induced liver pathologies. Murase *et al.* showed that 0.5% green tea extract in the diet for 11 weeks significantly decreased liver lipid accumulation caused by a high-fat diet in C57BL/6J mice [40]. Another study showed that treatment with 0.2-0.4% tea catechins for 35 weeks significantly decreased inflammation due to fatty liver in low density lipoprotein (LDL)-receptor deficient mice on a hypercholesterolemic diet [84]. Other studies have reported the reduction of hepatic steatosis and liver toxicity (as measured by elevated plasma ALT) by tea catechins by rodents administered ethanol [85-87], tamoxifen [88], endotoxins [89], and liver ischemia/reperfusion injury [90].

Accumulation of lipid in the liver can be caused by several pathologies, including an increased availability of free fatty acids for uptake, deregulation of fatty acid oxidation, or increases in *de novo* lipogenesis [79]. Dysfunction in lipoprotein metabolism may also play a role in the development of hepatic steatosis. Treatments with tea in animal models have shown to modulate several of these conditions. Two studies have found decreased plasma free fatty acid levels with green tea extract treatment in mice fed a high-fat diet when compared to mice without tea treatment [40, 41]. Murase *et al.* also showed increased fatty acid oxidation in the liver in mice treated with green tea extract [40]. Ikeda *et al.* showed that treatment with THEA-FLAN 90S decreased the

activity of fatty acid synthase (the rate-limiting enzyme for *de novo* fatty acid synthesis) in the liver of rats on a high-fat diet compared to rats with no tea treatment [42]. More studies need to be conducted on the specific components of green tea that mediate its benefits on liver function. EGCG and other catechins decrease fatty acid synthase in cells and cell-free studies [91-93], but effects by specific tea catechins need to be verified *in vivo.*

1.1.1.7. The role of inflammation in the metabolic syndrome

In the last decade, there have been increasing reports showing that the development of obesity is often accompanied by chronic, low-grade inflammation. This is characterized by an accumulation of macrophages in the adipose tissue and the liver, increased release of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) from these macrophages, and increased levels of these cytokines in the blood. These changes were found in both studies with obese human subjects and also animal models of obesity [94-98]. It is now widely accepted that metabolic disorders such as insulin resistance and cardiovascular disease are influenced by the presence of local and systemic inflammation. In addition, the metabolic syndrome is characterized by inflammation. Inflammatory markers, such as C-reactive protein (CRP), are predictors of metabolic syndrome and related events in humans [99-103]. Several studies suggest that inflammation is the link between obesity and these related pathologies [104-109]. Reduction of inflammation may be an important strategy in reducing the risk of obesity-induced metabolic syndrome.

1.1.1.8. Prevention of diet-induced inflammation by tea polyphenols

There is one epidemiological study that has recently reported a negative association between tea consumption and plasma CRP and serum amyloid A, another well-established inflammatory marker [110]. An earlier study did not support this finding [111]. Two clinical trials found that tea ingestion decreased plasma CRP levels or inflammatory cell adhesion molecule levels [112, 113], but results from other reports have conflicted with these findings [114, 115]. These discrepancies may be due to the difference in treatment doses, time of treatment, or the kinds of tea used for the studies.

Although there have been numerous animal studies that have discussed the effect of tea constituents on inflammation in the context of cancer prevention [116], there are few papers published that address the effect of tea and tea polyphenols on diet-induced inflammation. Suzuki *et al.* found that 0.2-0.4% tea catechins treatment (35 weeks) significantly decreased liver inflammation in LDL-receptor deficient mice fed a high-cholesterol diet when compared to mice without catechin treatment [84]. A recent study showed that green tea treatment for 6 weeks (1 g tea/kg diet) significantly reduced the mRNA expression of TNF-α and the pro-inflammatory prostaglandin E_2 (PGE_2) in the skeletal muscle of rats fed a high-fructose diet in comparison to rats without treatment. Tea treatment also reduced TNF-α expression in the liver of these rats. These decreases in gene expression were accompanied by increases in tristetraprolin expression in the rat and liver by tea treatment [117]. Tristetraprolin is an anti-inflammatory protein that prevents that transcription of several cytokines, including TNF-α and PGE_2. These studies suggest that tea may attenuate diet-induced inflammation; this inhibition may be a means by which tea can decrease the risk of obesity-induced metabolic syndrome.

1.1.1.9. The C57BL/6J high-fat fed mouse, an appropriate model for obesity and the metabolic syndrome

Over the past 30 years, several transgenic and knockout rodent models of obesity, diabetes, and the metabolic syndrome have been introduced and these models have provided much needed insight into the pathogenesis of these conditions [118-121]. However, most human cases of obesity and the metabolic syndrome are primarily due to diet [5]. Therefore, a diet-induced model of obesity and the metabolic syndrome may be a more appropriate model to accurately determine the efficacy and mechanism(s) of action of putative preventive agents against these conditions. The C57BL/6J mouse has been documented to be susceptible to diet-induced obesity, diabetes, and related metabolic disorders. With dietary fat content at 24% and higher (w/w), these mice showed significantly increased body weight and adiposity and higher fasting glucose and insulin levels than C57BL/6J mice on a low-fat diet after 12 weeks [105, 122, 123]. High-fat diet also significantly increased hepatic steatosis and liver triglyceride content in this strain of mice [124, 125]. The above-mentioned studies suggest that the high-fat fed mouse model will exhibit the distinct phenotype of diet-induced obesity, diabetes, and the metabolic syndrome, so that we can clearly identify the effect of dietary intervention on prevention of these conditions.

1.1.2. Colorectal cancer

Colorectal cancer is the fourth most prevalent type of cancer in the United States and the second leading cause of death. The incidence and mortality rates of colorectal cancer are the second highest worldwide [126]. It is estimated that in 2007, there will be 153,000 new cases of colorectal cancer in the United States and there will be 55,000 deaths due to colorectal cancer [127]. The 5-year survival rates for colorectal cancer in the United States across different ethnic groups range from 52-62% [126].

While 5% of reported colorectal cancers worldwide are caused by inherited germline mutations, the majority of the cases are sporadic [126]. Somatic mutation of the *Adenomatous Polyposis Coli* (APC) gene is a frequent genetic occurrence associated with colon cancer. Mutation of APC is an early event, and usually results in a nonfunctional truncated APC protein [128]. One important function of wildtype APC is that it forms a trimer in the cell with the proteins axin and glycogen synthase kinase, which targets the cytoskeletal protein β-catenin for ubiquitin-mediated degradation. Nonfunctional APC protein inhibits the formation of this trimer, allowing β-catenin to travel to the nucleus where it can interact with the transcription factor TCF-4, resulting in the transcription of genes associated with cell proliferation [128].

As the majority of reported cases of colon cancer are sporadic, it appears that environment plays a key role in determining individual risk for colon cancer. Indeed, epidemiological studies have shown that individuals who emigrate to countries with higher rates of colorectal cancer usually have a higher risk for developing the disease than individuals who remain in their country of origin [126]. Obesity is associated with

increased risk for colon cancer [8, 10-12], and consumption of a high-fat diet and red meat is positively correlated with incidence of colon cancer in epidemiological studies [129-133]. Conversely, fruit and vegetable intake are inversely correlated to risk for colon cancer [129, 134, 135]. Studies have suggested that polyphenolic compounds in fruits, vegetables, and beverages such as red wine and tea may be related to decreased colon cancer risk [136].

1.1.2.1. Dietary prevention of colon cancer by tea catechins

Many epidemiological studies have investigated the effects of tea consumption on risk for colon cancer. Although several studies have suggested a protective effect of tea consumption, results are not conclusive. A meta-analysis published in 2006 surveyed several epidemiological studies on tea and colon cancer risk [137]. The results varied from no association to an inverse association between green tea consumption and colon cancer in the 8 studies included in the analysis, with the population gender, diet, and type of study (case-control *vs.* cohort) cited as confounding variables. There was an overall significant reduction (18%) in colon cancer risk with high green tea consumption (>5 cups per day) throughout all of the studies. However, a recent epidemiological study also found no effect of green tea consumption in a large Japanese cohort [138].

Very few human intervention trials have studied the effect of tea on colon cancer risk and related parameters. A study in 1999 studied the effect of a one-time green tea treatment (0.6-1.8 g) on PGE_2 levels in patients undergoing surgery related to colon cancer or inflammatory bowel disease [139]. PGE_2 is an inflammatory metabolite of arachidonic acid that is associated with increased risk for colon cancer [140]. The authors

found that all doses of green tea significantly reduced PGE_2 levels (compared to baseline levels) in rectal mucosa of these patients 4-8 hours after treatment, but PGE_2 levels returned to baseline levels 24 hours after administration [139].

Tea chemoprevention studies in animal models of colon cancer have yielded conflicting results. Two early studies found a significant inhibitory effect of green tea extract on tumor incidence in dimethylhydrazine (DMH)- and azoxymethane (AOM)-injected rats, while another found a significantly decreased tumor formation and multiplicity by tea in AOM-treated rats [141, 142]. More recently, 2% green tea treatment for 32 weeks was shown to significantly decrease tumor multiplicity and tumor volume in DMH-treated rats [143]. A study in our laboratory found that 0.1% EGCG in the drinking fluid for 32 weeks significantly inhibited colon tumor incidence (by 49%) in AOM-treated mice (Bose, Chin, Park, Husain, Liao, Vittal, Kopelovich, Huang, Yang, unpublished results). However, other studies in these models have found no effect of tea treatment [144, 145]. One study even reported an increase in tumor diameter in DMH-treated rats by 0.1-1% Polyphenon E treatment (a standardized green tea polyphenol preparation containing about 65% EGCG) [146], and another study showed an increase in tumor multiplicity in AOM-treated rats with 3600 ppm treatment of green tea extract [147].

The *Apc*$^{Min/+}$ mouse is a transgenic model for intestinal tumorigenesis that is widely used for chemoprevention studies. In contrast to the above-mentioned reports, studies in *Apc*$^{Min/+}$ mice show a relatively consistent effect of tea catechins on tumorigenesis. Two studies have reported that green tea or green tea extract decreased

intestinal tumor multiplicity in *Apc*$^{Min/+}$ mice [148, 149]. A recent study in our laboratory by Ju *et al.* found a significant dose-dependent effect of EGCG on intestinal tumor multiplicity in male and female *Apc*$^{Min/+}$ mice; with 0.32% EGCG, the inhibition was ~40% [150].

Other studies have shown the effect of green tea catechins on established biomarkers in animal models of colon cancer. Ju *et al.* found that 0.6% green tea treatment (10 weeks) significantly reduced the formation of colonic aberrant crypt foci (ACF) in AOM-treated mice on a high-fat diet [151], and another study by Jia *et al.* also reported the reduction of ACF by green treatment in DMH-treated rats [152].

Several mechanisms have been suggested based on cell line studies as to how tea and EGCG exert chemopreventive effects. However, *in vivo* studies investigating the mechanisms of how tea constituents prevent colon cancer are limited. Some early studies suggested that tea modulated cytochrome P450 activity and potentially decreased the activation of carcinogens [153], but subsequent studies failed to show a clear effect on this pathway [147, 154]. Ju *et al*. found decreased nuclear levels of β-catenin and increased E-cadherin levels in adenomas of *Apc*$^{Min/+}$ mice treated with EGCG for 8 weeks [150]. E-cadherin is a membrane-bound cytoskeletal protein that binds to β-catenin and prevents translocation to the nucleus. A study by Perl *et al*. showed that loss of E-cadherin is associated with the progression of carcinoma development [155]. The effects of EGCG on E-cadherin levels in tumors may be related to its effects on intestinal tumorigenesis. Ju *et al.* also found that EGCG (75 mg/kg body weight, *i.g.*) decreased PI3K protein levels and extracellular-regulated kinase (ERK) phosphorylation in

adenomas of *Apc*$^{Min/+}$ mice compared to mice without treatment [150]. These effects may be direct or indirect. Recent studies have shown that EGCG treatment inhibits epidermal (EGF) and insulin-like growth factor (IGF) in human colon cancer cells [156, 157]. EGF and IGF mediate the activity of ERK and PI3K, respectively. However, effects on EGF and IGF signaling by tea catechins need to be demonstrated in animal studies.

1.1.2.2. Dietary prevention of colon cancer by omega-3 polyunsaturated fatty acids

Omega-3 polyunsaturated fatty acids (ω-3 PUFA) are distinguished by the presence of more than one carbon-to-carbon double (unsaturated) bond, with the first double bond located 3 carbons away from the methyl end of the fatty acid chain (Figure 1.2). ω-3 PUFA, along with ω-6 PUFA, are considered essential fatty acids, as they are not synthesized in the body. ω-3 PUFA can be found in soybean oil, flaxseed oil, and rapeseed oil, all of which contain α-linolenic acid (LNA). ω-3 PUFA can also be found in fish oil, which contains LNA and the longer chain ω-3 PUFA, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid (Figure 2). Recently, ω-3 PUFA have also become available as fish oil supplements [158].

Several epidemiological studies have suggested an inverse relationship between ω-3 PUFA and colon cancer. Epidemiological studies have shown that colon cancer incidence is lower in populations that consume a large amount of ω-3 PUFA (about 10 g /day) in comparison to populations that maintain a Western diet (<0.25 g ω-3 PUFA /day) [159]. Several studies have shown a negative correlation between fish consumption and colon cancer incidence [158], although other studies did not find a clear relationship

[158, 160]. One study suggested that the ratio of ω-3 PUFA to ω-6 PUFA intake may be a stronger determinant of risk for colon cancer than the ω-3 PUFA intake alone [161].

There have been 3 major human intervention studies that have examined the effect of ω-3 PUFA on endpoints associated with colon cancer. In 1992, Anti *et al*. showed that, in a population of 20 patients with sporadic adenomatous colorectal polyps, 12-week daily treatment with fish oil (containing 4.1 g EPA and 3.6 g DHA per day) significantly reduced markers of cell proliferation in the rectal mucosa compared to placebo treatment [162]. Another study by Huang *et al*. reported the effects of consumption of 9 g ω-3 PUFA/day for 6 months in patients with colon carcinoma or adenomatous polyps. They found that patients treated with ω-3 PUFA had a lower plasma ω-6/ω-3 ratio than patients with placebo treatment, and that this ratio was strongly correlated with markers of proliferation in colonic epithelial cells of these patients [163]. However, these effects of ω-3 PUFA were only significant in patients with high baseline levels of colonic cell proliferation. A recent study by Cheng *et al.* showed that treatment with a fish oil supplement (containing 100 mg EPA and 400 mg of DHA daily) significantly increased markers of apoptosis in colonic mucosa (when compared to no supplementation) in a group of men and women who had previously undergone colonic polypectomy [164]. These studies suggest that dietary supplementation of ω-3 PUFA or fish oil may decrease risk for colon cancer in humans. However, it should be noted that the basal levels of colonic proliferation in a population may influence the extent of its effect.

There have been several studies published regarding the effect of ω-3 PUFA on carcinogenesis in animal models of colon cancer. One study in DMH-treated rats found that a 30-week administration of DHA (0.97 g, 3 times a week, *i.g.*), but not pure EPA or LNA (1 ml, 3 times a week, *i.g.*), significantly reduced tumor incidence and multiplicity in comparison to rats treated with pure oleic acid (1 ml, 3 times a week, *i.g.*) [165]. Another study in the same model found that a 4-week dietary treatment of 0.8% fish oil (containing 19% EPA and 8% DHA) significantly reduced colonic crypt cell proliferation and aberrant crypt foci, and increased apoptosis compared to corn oil-treated controls [166]. Rao *et al*. reported in 2001 that administration of a high-fat diet rich in fish oil (17% fish oil, 3% other mixed lipids, w/w) significantly reduced colon tumor incidence, colon tumor number, and aberrant crypt foci in AOM-treated rats after 38 weeks of treatment in comparison to those administered a high-fat diet (20% w/w) without fish oil [167]. These effects were accompanied by a significant increase in apoptosis. Studies in the *Apc*$^{Min/+}$ mouse model have also shown the beneficial effects of dietary EPA (31 g/kg diet, 7 week treatment), DHA (31 g/kg diet, 7 week treatment) [168], and fish oil concentrate containing 54% EPA and 30% DHA (2.5% in the diet, 8 week treatment) [169] on tumor multiplicity and tumor size. One study in nude mice bearing human colon tumor xenografts showed that dietary fish oil (18 w/w, 53 days of treatment) significantly reduced tumor growth and volume compared to mice on a high-fat diet (20 w/w) without fish oil [170]. Another study using this model found that treatment with dietary fish oil (16% w/w) or dietary golden algae oil (16% w/w), which is rich in DHA, significantly reduced tumor growth and weight compared to mice fed a corn oil diet (24% w/w) [171].

These studies show that administration of ω-3 PUFA, particularly EPA and DHA, is effective in reducing tumorigenesis and related parameters in different animal models of intestinal cancer.

The majority of the animal studies conducted on the effects of ω-3 PUFA have shown that ω-3 PUFA mediate their effect by inhibition of arachidonic acid metabolism. Arachidonic acid is an ω-6 PUFA that is incorporated into cellular membranes and serves as a substrate for the enzyme phospholipase A_2, which gives rise to the intermediate arachidonyl ethanolamide. This metabolite is a substrate for the cyclooxygenase (COX) enzymes, resulting in the production of inflammatory prostaglandins such as PGE_2, which may play a role in cellular growth and differentiation. ω-3 PUFA also serve as substrates for these enzymes. In contrast to arachidonic acid, metabolism of ω-3 PUFA by these enzymes results in the production of anti-inflammatory prostaglandins [158]. One study in the *Apc*$^{Min/+}$ mouse showed that replacement of dietary sources of arachidonic acid with EPA or DHA decreased intestinal tumorigenesis [172]. Another study showed reduction of COX expression in colon tumors by fish oil diet [167]. Conversely, one study in nude mice with xenografts of HCT-116 cells, a human colon tumor cell line that does not express COX, suggested that fish oil mediates its effects through a mechanism independent of its effects on the COX pathway and arachidonic acid metabolism [170]. Some studies in cell lines have supported this finding, citing modulation of gene transcription and intracellular signal transduction pathways such as protein kinase C (PKC) as potential mechanisms [173, 174].

1.1.2.3. Chemoprevention of colon cancer by a combination of dietary constituents

Colon carcinogenesis is a multi-stage process, comprised of a number of genetic changes that contribute to the initiation, promotion, and/or invasion stages of the disease. Although several dietary and pharmacological compounds have shown promise as chemopreventive agents, there are limitations in using a single agent for effective cancer prevention in practice [149, 175-177]. COX inhibitors initially showed promise as effective chemopreventive agents against colon cancer, but it was later revealed that long-term use of these compounds had undesirable side effects [178, 179]. Pre-clinical trials with dietary chemopreventive agents have demonstrated less toxicity than drugs, but have often shown poor bioavailability and lower potency than drugs [180-182], suggesting that large amounts need to be consumed to confer any cancer preventive effect. In addition, there are studies that often conflict with one another as to whether or not a particular agent is effective. This implies that consumption of any one dietary nutrient may not be a practical means of effective cancer chemoprevention. Combination of two or more pharmacological agents has been shown to be effective for cancer chemoprevention, maximizing efficacy by affecting different molecular targets and minimizing toxicity by lowering doses of the individual drugs [183, 184]. Combination of two or more dietary compounds may be a practical and effective approach to cancer chemoprevention for those same reasons.

1.1.2.4. The *Apc*$^{Min/+}$ mouse, a widely used model for colon cancer chemoprevention

The APC gene is frequently mutated in colon cancer [130, 185]. In the molecular pathogenesis of colon cancer, mutation of APC is one of the earliest events in the development of adenomas, the precursor to adenocarcinoma [186]. It has been suggested that this gene acts as a tumor suppressor as well as a gatekeeper for ensuing genetic events associated with the progression to colon cancer [128, 187]. Germline mutation of this gene in humans results in Familial Adenomatous Polyposis (FAP), an inherited disorder characterized by numerous adenomatous polyps along the length of the colon. Individuals with this condition have an increased risk of developing colon cancer [188]. In 1992, Moser *et al*. generated a mouse strain on a C57BL/6J background that possessed a dominant heterozygous mutation at codon 850 in the mouse homologue of the APC gene. These mice exhibited multiple adenomas in the small intestine and were thus named *Apc*$^{Min/+}$ mice (Multiple Intestinal Neoplasia) [189]. By age 4-5 months, these mice bear numerous visible tumors in the intestinal tract (~40-50) and usually die due to intestinal blockage, bleeding, and severe anemia. Despite certain phenotypic differences between characteristics observed in the *Apc*$^{Min/+}$ mouse and those of colon cancer, this model still possesses early genetic alterations, such as mutation of APC and loss of heterozygosity, that have shown to be crucial initial steps in the development of human colon cancer. Since the development of this strain, the *Apc*$^{Min/+}$ mice have been well characterized and have shown to be a sufficient model for imitating human intestinal carcinogenesis. This model has been successfully used in a large variety of

chemoprevention studies, suggesting that the *Apc*$^{Min/+}$ mouse is a useful and well-known model for intestinal cancer.

1.2. HYPOTHESES AND SPECIFIC AIMS

1.2.1. Hypotheses

The objectives of these studies were to determine the effects of dietary EGCG in a mouse model of obesity and the metabolic syndrome and to determine the inhibitory activity of dietary EGCG in combination with fish oil in a mouse model of intestinal cancer. These will be achieved by testing the following hypotheses:

Dietary EGCG can decrease weight gain, insulin resistance, hypercholesterolemia, and hepatic steatosis in a model of obesity. These effects may be mediated by its weight loss effects or by attenuation of local and systemic inflammation. Both EGCG and fish oil have well-known chemopreventive properties and it is expected that a combination of dietary EGCG and fish oil can synergistically inhibit intestinal tumorigenesis. This inhibition may be mediated through multiple mechanisms, including modulation of aberrant arachidonic acid metabolism and β-catenin signaling.

1.2.2. Specific Aims

1. **To determine the effect of dietary EGCG on high-fat diet-induced weight gain and body fat gain in C57BL/6J mice.** I treated high-fat fed male C57BL/6J mice with dietary EGCG for 16 weeks (long-term treatment) or 4 weeks (short-term treatment). I measured body weight and food consumption weekly, and then I measured body fat percentage and visceral fat weight at the end of the study. These experiments are described in Chapter 2.

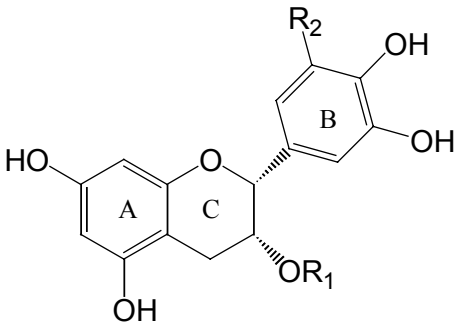
2. **To determine the effect of dietary EGCG on parameters of obesity-associated metabolic syndrome.** I used the mice from Specific Aim 1 and measured fasting blood glucose over the treatment period, and obtained measurements for insulin resistance, liver dysfunction, and systemic inflammation at the end of the study. Livers were also examined biochemically (for triglyceride measurements) and histologically (for lipid accumulation in hepatocytes). These experiments are also described in Chapter 2.

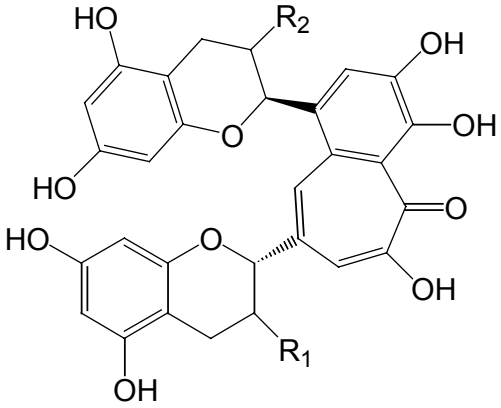
3. **To determine the inhibitory activity of dietary EGCG in combination with fish oil on intestinal tumorigenesis in the *Apc*$^{Min/+}$ mouse model.** I treated high-fat fed female *Apc*$^{Min/+}$ mice with a dietary combination of EGCG and fish oil for 14 weeks (long-term treatment) or 3 weeks (short-term treatment). Adenomas from the long-term study were analyzed for cell proliferation, apoptosis, and related molecular changes (β-catenin signaling and arachidonic metabolism). Combined immunohistochemical and biochemical analyses were used for these studies. These experiments are described in Chapter 3.

These studies will provide useful information as to the effects of EGCG on the risk for chronic diseases, and they will also serve as a foundation for understanding the important mechanisms by which EGCG may help prevent these diseases.

1.3. Figures

Figure 1.1. Structures of the major tea polyphenols





Epicatechin: $R_1 = R_2 = H$

Epigallocatechin: $R_1 = H$; $R_2 = OH$

Epicatechin-3-gallate: R_1 = Galloyl; $R_2 = H$

Epigallocatechin-3-gallate: R_1 = Galloyl; $R_2 = OH$

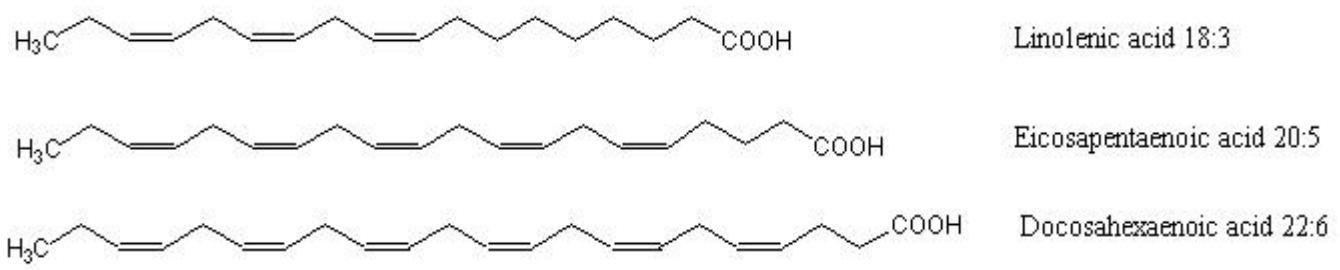
Theaflavin: $R_1 = R_2 = OH$

Theaflavin-3-gallate: R_1 = Galloyl; $R_2 = OH$

Theaflavin-3'-gallate: $R_1 = OH$; R_2 = Galloyl

Theaflavin-3,3'-digallate: $R_1 = R_2$ = Galloyl

Figure 1.2. Structures of omega-3 (ω-3) fatty acids found in fish oil



CHAPTER 2

INHIBITION OF OBESITY AND RELATED PATHOLOGIES BY EGCG IN HIGH-FAT FED C57BL/6J MICE

2.1. Background and Rationale

Obesity is a health condition that is characterized by a body mass index (BMI) that is 30 or higher [1]. The rates of obesity have increased dramatically in the United States in the last 20 years and even more rapidly in the last 10 years [2]. Globally, the prevalence of obesity is currently increasing in developing countries at rates higher than in the United States [4]. Studies in the last 50 years have suggested a strong positive association between obesity and the incidence of diseases such as type II diabetes, cardiovascular disease, and hypertension [5-7]. These associations led to the concept of the metabolic syndrome, a clustering of symptoms including abdominal obesity, insulin resistance, and dyslipidemia. The metabolic syndrome is currently defined by the National Cholesterol Education Program (NCEP) as a condition where an individual exhibits any 3 of the 5 following characteristics: waist circumference > 40 inches in men, >35 inches in women; serum triglycerides $\geq$ 150 mg/dl; high-density lipoprotein (HDL) cholesterol < 50 mg/dl in men, <40 mg/dl in women; blood pressure > 135/85 mg Hg; and fasting blood glucose > 110 mg/dl [5]. The metabolic syndrome is also often characterized by chronic inflammation and by hepatic steatosis, fat accumulation in the liver [14].

Green tea is a beverage consumed worldwide, especially in East Asian countries. Studies in the last several decades have been conducted to elucidate the potential health benefits of drinking green tea. Aside from caffeine, green tea contains polyphenolic

compounds called catechins. The most abundant catechin found in green tea is (-)-epigallocatechin-3-gallate (EGCG), and it is believed that EGCG is responsible for the potential health effects of tea [190].

In 1999, Dulloo *et al.* found that administration of a green tea extract significantly increased energy expenditure and fat oxidation in a population of young males [30]. Since then, several clinical trials have reported the effects of green tea extract on increasing energy expenditure, fat oxidation, weight loss, fat mass, and weight maintenance after weight loss [31, 34, 35, 191]. Several studies in rodent models showed that green tea extract decreased weight gain and body fat gain [40-42]. Recently, Klaus *et al.* reported that 0.5-1% TEAVIGO (a green tea extract containing ≥94% EGCG and ≤0.1% caffeine) in the diet for 4 weeks significantly reduced body weight gain, total body fat gain, and epididymal body fat weight in New Zealand Black mice on a high-fat diet [44]. Another study by the same group found that 1% TEAVIGO in the diet (5 month treatment) significantly reduced body weight and subcutaneous and epididymal adipose tissue weight in high-fat fed C57BL/6J mice [45].

To date, there is one published study that showed an ameliorating effect of EGCG in a model of the metabolic syndrome [51]. Nevertheless, several studies have observed the effects of tea and EGCG on glucose homeostasis and other characteristics of the metabolic syndrome. Several clinical investigations showed the effects of tea extracts on fasting blood glucose, glucose intolerance, and carbohydrate absorption in humans [56, 57, 59]. Studies in rodent models showed that green tea treatment significantly improved fasting blood glucose and insulin sensitivity [57, 61]. A recent study by Wolfram *et al.*

reported that 0.25-1% TEAVIGO treatment significantly improved glucose tolerance and insulin sensitivity in *db/db* mice, a transgenic model of insulin resistance [62].

Hepatic steatosis (or nonalcoholic fatty liver disease) and chronic low-grade inflammation are two conditions that are associated with obesity and the metabolic syndrome [14, 71, 80]. Studies showed green tea consumption is inversely correlated with liver dysfunction (a consequence of progressive hepatic steatosis) and with markers of inflammation in humans [83, 110, 112]. A study in 2002 showed that green tea treatment (0.5% for 11 weeks) significantly reduced liver lipid content in high-fat fed C57BL/6J mice [40]. Recently, a study by Cao *et al.* showed that green tea treatment for 6 weeks (1 g tea/kg diet) significantly reduced the expression of the inflammatory cytokine tumor necrosis factor alpha (TNF-α) in the skeletal muscle and liver of fructose-fed rats [117].

Currently, the effects of physiologically relevant doses of EGCG on body weight, body fat, and fat distribution are not clearly understood. In addition, the effects of EGCG on high-fat induced pathologies associated with the metabolic syndrome are not known. The goals of this study were to determine the effect of dietary EGCG on weight gain, markers of insulin resistance, liver dysfunction, and inflammation in a high-fat diet-induced mouse model for obesity and the metabolic syndrome. I used a high-fat diet that consisted of 35% fat (60% kcal), comprised mainly of lard as the lipid source. I chose this diet because previous studies showed that this diet clearly induces the phenotype of obesity, insulin resistance, hypercholesterolemia, and hepatic steatosis in the C57BL/6J mouse strain [105, 122-125].

I measured weight gain, body fat, fasting blood glucose, insulin, and cholesterol levels. I measured plasma monocyte chemoattractant protein-1 (MCP-1) as a marker of systemic inflammation. I chose this marker because previous studies showed that plasma MCP-1 levels are significantly higher in mice on a high-fat diet in comparison to mice on a low-fat diet [192]. A recent study also showed MCP-1 plays an important role in the development of macrophage accumulation in adipose tissue, insulin resistance, and hepatic steatosis [193]. A dose of 0.32% EGCG in the diet was selected as this dose is the equivalent of 10 cups of green tea (containing 2 g green tea leaves per cup) per day, which is the average amount consumed in cultures that regularly drink green tea [194]. This calculation was based on an allometric scaling conversion [195] conducted in our laboratory. Assuming 12 kcal as the average daily energy requirements for an adult mouse [196], 0.32% EGCG would equal 0.8 mg EGCG per kcal consumed, if mice consume 3 g diet per day. Assuming the energy requirements for the average human as 2000 kcal per day, human EGCG consumption at this dose would be 1600 mg, or 1.6 g per day. EGCG consists of about 50% of all the catechins present in tea; therefore catechin consumption at this dose would be 3.2 g per day. Catechins comprise about 30% of green tea; steeping in hot water would extract about 50% of tea solids, therefore green tea consumption at this dose would be 19.2 g per day. An average green tea bag contains 2 g green tea per bag; therefore, 10 tea bags per day would be necessary to equal the dose of 0.32% in mice.

2.2 Materials and methods

2.2.1. Chemicals and diets

EGCG was a gift from Mitsui Norin (Shizuoka, Japan). Low-fat (5% w/w), high-fat (35%, w/w), and high-fat plus 0.32% EGCG diets (w/w) were purchased from Research Diets, Inc. (New Brunswick, NJ) (Table 2.1).

2.2.2. Diet treatment

Experiment 1

Forty-four male C57BL/6J mice (age 5 weeks) from our breeding colony, established in the animal facility of the Susan Lehman Cullman Laboratory for Cancer Research (Rutgers, The State University of New Jersey, Piscataway, NJ), were given the following diet treatments for 16 weeks: low-fat diet (LF, n=12), high-fat diet (HF, n=16), or high-fat diet plus 0.32% EGCG (w/w) incorporated into the diet (HFE, n=16). Body weight and food intake were measured weekly. Feed efficiency was calculated weekly by dividing average weight gain (in grams) by total food consumption (in grams) from Week 1 of treatment.

Experiment 2

In another experiment, 56 male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). At age 5 weeks, mice were given the same diet treatments as described in Experiment 1 (n=12-22 per group) for 16 weeks. Body weight and food intake were measured weekly. Feed efficiency was calculated weekly by dividing average weight gain (in grams) by total food consumption (in grams) from Week 1 of treatment. During Week 10, cage bedding was changed and feces were collected from the cages the following day for fecal lipid measurements.

Experiment 3

To determine the short-term effects of EGCG on reversal of obesity, 5-6 week old male C57BL/6J mice from our breeding colony were fed either LF (n=12) or HF (n=24) diet for 9 weeks. At 9 weeks of diet treatment, 12 mice from the HF group were randomly chosen and switched to HFE diet. Mice were treated with diets for an additional 4 weeks. Body weight and food intake were measured weekly. Feed efficiency was calculated weekly by dividing average weight gain (in grams) by total food consumption (in grams) from Week 9 of treatment.

Experiment 4

To determine the effect of EGCG on fat absorption, male C57BL/6J mice in metabolic cages (n=3 per group) were fed a high-fat diet (20% w/w, Table 2.2) with 5% sucrose polybehenate (HFSB) or HFSB diet with 0.32% EGCG in the drinking fluid for 5 days. Sucrose polybehenate is a non-absorbablelipophilic marker that can estimate lipid absorption when included in diet. This method has been validated in previous studies [197]. On Days 3 and 4 of treatment, feces from mice were collected and frozen. Samples were subsequently sent to the Mouse Metabolic Phenotyping Center (Cincinnati, OH) for gas chromatography analyses of fatty acid composition.

2.2.3. Fasting blood glucose

On the day of blood glucose measurement, food was removed and the cage bedding was changed (to minimize coprophagy) 7-8 hours before measurement. Blood was sampled from the tail vein and glucose levels were measured by the OneTouch Basic glucose monitor (LifeScan, Inc. Milpitas, CA). Fasting blood glucose was measured 3 times throughout the course of Experiment 1, and 6 times throughout the course of Experiment 2. For Experiment 3, fasting blood glucose was measured 3 times throughout

the course of the study, twice before mice were transferred to HFE diet (at Week 4 and Week 7 of study) and once at the end of the study (at Week 13).

2.2.4. Tissue harvesting

Experiment 1

After 16 weeks of treatment, mice were fasted for 6 hours prior to sacrifice. Mice were weighed and sacrificed by CO_2 overdose. Total body fat percentage was measured postmortem by dual-energy x-ray absorptiometry (DEXA) using a PIXImus densitometer (Lunar GE Medical Systems, Madison, WI). Visceral adipose tissues (mesenteric, epididymal, and retroperitoneal depots) were harvested, rinsed, and weighed.

Experiment 2

After 16 weeks of treatment, mice were fasted for 6 hours prior to sacrifice. Mice were weighed and sacrificed by CO_2 overdose. Whole blood was obtained by cardiac puncture. Visceral adipose tissues (mesenteric, epididymal, and retroperitoneal depots) were harvested, rinsed, and weighed. One segment of the epididymal depot was fixed in 10% formalin. Liver was harvested, rinsed, and weighed. Fatty liver was initially determined by altered coloration (pink color due to lipid accumulation). One lobe of the liver was fixed in 10% formalin, the rest was snap frozen on dry ice. After sacrifice, whole blood was centrifuged at 10,000 rpm for 15 minutes to separate plasma from red blood cells. Plasma was collected and snap frozen on dry ice. All frozen samples were stored at -80° C. Fixed samples were stored at room temperature overnight in 10% formalin, and then transferred to 1X PBS and stored at 4° C.

Experiment 3

After 4 weeks of EGCG treatment to high-fat fed obese mice, all mice from LF, HF, and HFE groups were weighed and sacrificed by CO_2 overdose. Visceral adipose tissue (mesenteric, epididymal, and retroperitoneal depots) were harvested, rinsed, and weighed.

2.2.5. Biochemical plasma measurements

All plasma analyses were from mice in Experiment 2. Fasting plasma insulin was measured by enzyme-linked immunosorbent assay (ELISA) (Linco Research, St. Charles, MO). Fasting plasma cholesterol was measured by a Cholesterol E kit (Wako Diagnostics, Richmond, VA). Plasma MCP-1 levels f were determined by ELISA (R&D Systems, Minneapolis, MN). Plasma alaine aminotransferase (ALT) levels were determined spectrophotometrically with a kit from Catachem, Inc. (Bridgeport, CT).

2.2.6. Calculation of homeostasis model assessment of insulin resistance

The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using fasting glucose and insulin determinations in mice from Experiment 2. This method is widely used to estimate insulin resistance in humans and animal models [80, 198, 199]. The following formula was applied [53]:

$$\frac{\text{Fasting glucose (mmol/L)} \times \text{Fasting insulin (mU/L)}}{22.5}$$

2.2.7. Measurement of liver triglycerides

For determination of hepatic triglyceride content in mice from Experiment 2, liver tissue (50-100 mg) was homogenized in 2 ml isopropanol with a Polytron disrupter. The homogenate was centrifuged at 2,000 *g* for 10 minutes, and the supernatant was collected. The triglyceride content of the supernatant was measured by a Triglyceride H kit (Wako Diagnostics, Richmond, VA).

2.2.8. Fecal lipid analyses

For determination of fecal lipid content, feces collected during Week 10 from mice in Experiment 2 were weighed, added to 3-4 ml deionized H_2O, and allowed to sit at 4^oC overnight. The following day, the feces mixture was homogenized by vortexing. Following vortexing, 7.5 ml methanol:chloroform (2:1) were added to the mixture and shaken at room temperature for 30 minutes. After shaking, 2.5 ml each of chloroform and deionized H_2O were added to the mixture and shaken at room temperature for 30 minutes. The mixtures were then centrifuged at 2000 x *g* for 15 minutes. The bottom lipophilic chloroform layer was collected from the tubes and dried under vacuum. Total lipids were measured gravimetrically.

2.2.9. Liver and adipose tissue histopathology

Embedded liver and adipose tissue blocks were cut into 6-micron segments. Slides were stained with H&E. Mounting medium and cover slips were placed on the slides and left to dry overnight.

2.2.10. Statistical analyses

One-way analysis of variance (ANOVA) with Tukey's post-hoc test were used for statistical analysis of body weight gain, body fat, and biochemical measurements (GraphPad software, San Diego, CA) for comparison among multiple groups. For analysis of body weight and fasting blood glucose over the 16-week treatment, a repeated-measures ANOVA was used. A chi-squared test was performed for analysis of the incidence of hepatic steatosis.

2.3. Results

2.3.1. Effect of EGCG on body weight, body fat, and fat absorption

In these experiments, mice were fed LF, HF, or HFE diets for 16 weeks (Experiments 1 and 2), or treated with LF or HF diets for 9 weeks, and then half of the mice on the HF diet were randomized to receive HFE diet for 4 more weeks (Experiment 3). There was no significant difference in the amount of daily food intake between all groups in each of the separate experiments (Figure 2.1).

Experiment 1

In Experiment 1, HF mice were significantly heavier than LF mice by week 3 of treatment (27.5±0.8 *vs.* 24.0±0.3 g, Figure 2.2). This trend remained throughout the course of the treatment period (At 16 weeks, 48.5±0.8 *vs.* 30.2±0.6 g, Figure 2.2). By week 5 of treatment, HFE mice weighed significantly less than the HF mice, and HFE mice weighed 11% less than the HF mice at sacrifice (Figure 2.2). Body weight gain was 41% lower in HFE mice compared to HF mice (Table 2.3). A significant effect of EGCG on feed efficiency was also observed (Figure 2.3).

Results from the DEXA analysis showed that HF mice exhibited significantly increased body fat percentage (2.5 fold) compared to LF mice. Treatment with EGCG mice had a small but significantly decreased body fat percentage (10%) compared to HF mice (Figure 2.4).

HF mice had significantly increased total visceral adipose weight compared to LF mice (5.8±0.8 *vs.* 1.5±0.4 g, respectively, Table 2.3). EGCG treatment reduced total visceral adipose weight by 9% compared to HF mice, but this reduction was not significant. Nevertheless, HFE showed significant reductions in the mesenteric depot (19% decrease) and retroperitoneal depot (21% decrease) compared to HF mice (Table 2.3).

Experiment 2

In Experiment 2, HF mice were significantly heavier than LF mice by week 4 of treatment (24.1±0.3 *vs.* 22.1±0.4 g, Figure 2.2). This trend remained throughout the course of the treatment period (at 16 weeks, 47.9±0.8 *vs.* 27.7±0.5, Figure 2.2). At the end of the 16-week period, HFE mice weighed 21% less than the HF mice (Figure 2.2). Body weight gain over the 16-week treatment was reduced by 33% on HFE mice compared to HF mice (Table 2.3). A significant effect of EGCG on feed efficiency was also observed (Figure 2.3).

HF mice showed significantly increased total visceral adipose tissue weight in comparison to LF mice (5.5±0.6 *vs.* 1.2±0.4 g, respectively, Table 2.3). EGCG treatment reduced total visceral adipose tissue weight by 37% compared to HF mice (Table 2.3). This was attributable to significant weight decreases in the mesenteric (48% decrease), epididymal (28% decrease) and retroperitoneal (34% decrease) depots (Table 2.3).

Experiment 3

I measured the body weight of LF mice, HF mice, and mice that were switched to HFE diet (for 4 weeks) after 9 weeks of HF diet. After 9 weeks of LF or HF diet, HF mice were significantly heavier than the LF mice (45.6±1.8 *vs.* 29.5±1.2 g, Figure 2.2). EGCG treatment of the HF-fed mice for 4 weeks thereafter did decrease the rate of body weight gain compared to mice that remained on HF diet; by the end of the 4-week treatment, HFE mice were 5% lighter than HF mice, although this decrease was not significant (Figure 2.2). Body weight gain over the 4-week treatment period was 38% lower in HFE mice compared to HF mice, but this reduction was also not significant (Table 2.3). There was, however, a significant effect of the EGCG treatment on feed

efficiency over the 4-week treatment period in comparison to the HF-fed mice (Figure 2.3), suggesting a decrease in weight gain had the study continued longer.

After 9 weeks of LF or HF diet, HF mice showed increased total visceral fat weight compared to LF mice (5.69±1.0 *vs*. 1.47±0.6 g, Table 2.3). EGCG treatment for 4 weeks produced a 21% decrease in total visceral fat weight, but the reduction was not significant. There was, however, a significant effect of 4-week EGCG treatment on the weight of the mesenteric depot (36% decrease, Table 2.3).

Experiment 4

For Experiment 4, mice were fed a HFSB diet or HFSB plus 0.32% EGCG in the drinking fluid. Mice on HFSB plus EGCG consumed very little drinking fluid in comparison to the HFSB group throughout the 5-day period (data not shown). As a result, there was no effect of EGCG on lipid absorption as determined by this method (99.2±0.19% *vs*. 99.4±.06%, Figure 2.5).

2.3.2. Effect of EGCG treatment on fecal lipids

In Experiment 2, feces from mice were collected at Week 10 of treatment. There was an increase (~51%) in fecal lipid content in HF-treated mice compared to LF mice (Figure 2.6), but the increase was not significant. EGCG treatment significantly increased (1.5-fold) fecal lipid content compared to HF mice (43.2±10.8 *vs*. 17.7±1.4 mg lipids/g feces, $p<0.05$, Figure 2.6).

2.3.3. Effect of EGCG treatment on fasting blood glucose, fasting plasma insulin, and HOMA-IR index

Experiment 1

For Experiment 1, I measured fasting blood glucose in all treatment groups 3 times throughout the 16-week treatment. After 6 weeks of treatment, HF mice showed significantly higher fasting blood glucose levels compared to LF mice (173.1±3.8 *vs.* 125.0±3.9 mg/dl, respectively, Figure 2.7). This trend remained throughout the entire treatment period (by week 16, 189.6±6.7 *vs.* 146.3±6.6 mg/dl, Figure 2.7). By week 12, HFE-treated mice showed significantly lower fasting blood glucose levels than HF mice (14% decrease). By week 16, HFE mice had 7% lower fasting blood glucose than HF mice, but the trend for decreased fasting blood glucose by EGCG was not significant (Figure 2.7).

Experiment 2

I measured fasting blood glucose at 6 time points throughout the 16-week treatment period of Experiment 2. HF diet significantly increased fasting blood glucose levels compared to LF mice after 3 weeks of treatment (124.3±5.5 *vs.* 102.6±3.8 mg/dl, Figure 2.7). This trend remained throughout the course of the study (by week 16, 198.3±5.5 *vs.* 123.6±3.9 mg/dl, Figure 2.7) By week 10, HFE mice showed 24% lower fasting blood glucose levels than HF mice (Figure 2.7). This trend remained throughout the course of the study. At the end of the 16-week treatment period, HFE mice had 25% lower fasting blood glucose than HF mice, (Figure 2.7).

Fasting plasma insulin was measured at the end of the 16-week study. HF mice showed significantly increased plasma insulin levels compared to LF mice (5.23±2.7 *vs.* 0.43±0.1 ng/ml, Table 2.4). HFE mice had reduced plasma insulin levels compared to HF mice (61% reduction, Table 2.4). HOMA-IR calculations (using the final fasting blood glucose and insulin measurements) showed that HF diet significantly increased the

HOMA-IR index compared to LF mice (74.5±11.2 *vs.* 3.1±0.6, Table 2.4). HFE mice exhibited a 76% decrease in HOMA-IR index compared to HF mice (Table 2.4).

For Experiment 3, fasting blood glucose was measured twice before HF mice were separated into HF and HFE groups. By week 4 of the study, HF showed significantly elevated blood glucose levels in comparison to LF mice (145.8±5.0 *vs.* 119.9±7.1 mg/dl, Figure 2.7). At week 13 (after 4 weeks of EGCG treatment), HF mice still showed higher fasting blood glucose levels than LF mice (209.0±11.2 *vs.* 123.8±7.0 mg/dl, respectively, Figure 2.7), but the HFE-treated mice had significantly lower blood glucose levels compared to HF mice (22% decrease, Figure 2.7).

2.3.4. Effect of 16-week EGCG treatment on plasma lipids

In Experiment 2, I studied the effect of EGCG on fasting plasma cholesterol in high-fat fed mice. HF mice showed significantly higher cholesterol levels than LF mice (215.0±22.2 *vs.* 101.6±19.5 mg/dl, Table 2.4). EGCG treatment significantly attenuated the elevation in cholesterol by 24% (Table 2.4).

2.3.5. Effect of 16-week EGCG treatment on biochemical liver measurements

In Experiment 2, I determined the effect of EGCG treatment on liver size, triglyceride content, and the levels of liver enzymes (ALT) in the plasma. Mice on HF diet showed significantly increased liver size (relative to body weight) in comparison to LF mice. These mice also showed a significantly higher incidence of fatty liver (95% *vs.* 0%,), higher triglyceride content (189.9±66.1 *vs.* 47.0±22.5 mg/g tissue,), and elevated plasma ALT levels (105.2±60.3 *vs.* 13.2±5.0 U/L, Table 2.5, Figure 2.8). EGCG treatment significantly reduced liver size (by 22%), incidence of fatty liver (HFE 18% *vs*.

HF 95%), liver triglyceride content (by 69%), and plasma ALT level (by 67%,) compared to HF mice (Table 2.5, Figure 2.8).

2.3.6. Effect of 16-week EGCG treatment on liver and adipose issue histopathology

Histopathological analyses showed increased lipid content within the liver of HF mice compared to those of LF mice (Figure 2.9). Microvesicular fat was present throughout the liver; however, it had a central lobular distribution. The zone surrounding the portal triad was relatively free of accumulated lipids. Livers from HFE mice showed a marked decrease in liver lipid accumulation compared to HF mice.

In the adipose tissue, there appeared to be little to no morphological markers of inflammation and macrophage accumulation (Figure 2.10). Therefore, the effects of EGCG on inflammation in the adipose tissue could not be determined by this method.

2.3.7. Effect of 16-week EGCG treatment on markers of systemic inflammation

In Experiment 2, I looked at the effect of EGCG treatment on plasma MCP-1 levels as a markers of inflammation. HF mice had significantly elevated and MCP-1 levels compared to LF mice (57.0±37.7 *vs.* 7.3±3.8 pg/ml, Table 2.6). MCP-1 levels were 69% lower in HFE mice than in HF mice ($p<0.05$, Table 2.6).

2.4. Discussion

In my studies for Specific Aim 1, I observed the effect of dietary EGCG on body weight and body fat. I found that 16-week EGCG treatment significantly decreased body weight in mice fed a high-fat diet. In obese mice, 4-week EGCG treatment decreased body weight, although this decrease was not significant. Although previous studies have shown that EGCG treatment reduced body weight in mice fed a high-fat diet, the dose used in the current study is the lowest reported thus far. Previous studies reported doses

of 0.5-1% EGCG (in the form of TEAVIGO) decreasing weight gain in high-fat fed mice [44, 45]. Based on allometric scaling, dietary administration of 0.32% EGCG in mice is the equivalent of 10 cups of green tea (containing 2 g tea leaves per cup) per day, assuming a human energy requirement of 2000 kcal per day. The dose used in my studies may be a more attainable dose than those previously reported. Future studies will determine if there is a dose-response effect of EGCG on reduction of weight gain.

Long-term EGCG treatment (16-weeks) also significantly decreased total body fat percentage and visceral body fat weight. The decrease in visceral body fat by EGCG was apparent in the mesenteric, epididymal, and retroperitoneal depots. These decreases in body fat may be due to EGCG inhibiting lipid absorption or increasing fat oxidation. The results from Experiment 2 showed increased fecal lipid content with EGCG treatment compared to high-fat fed control mice, supporting the idea that inhibition of lipid absorption may be one mechanism by which EGCG decrease body fat accumulation. The results from Experiment 4, however, do not support this hypothesis. This lack of effect may be explained by the finding that mice that were treated with EGCG drank very little fluid during this treatment period.

The largest decrease in visceral body fat accumulation was observed in the mesenteric depot, followed by the decrease in the retroperitoneal depot. Although there was no significant change in visceral fat weight in diet-induced obese mice treated with 0.32% EGCG for 4 weeks, there was significant decrease in the weight of the mesenteric depot of visceral fat. Previous studies showed that EGCG significantly decreased total body percentage, subcutaneous fat weight, and epididymal fat weight in high-fat fed mice [44, 45]. This is the first report to show the effects of EGCG on the mesenteric and

retroperitoneal adipose depots in high-fat fed mice. Weight loss studies in humans have shown that visceral adipose tissue is more metabolically active than subcutaneous adipose tissue [200, 201]. This finding is supported by studies that found that visceral fat reduction was more effective at decreasing risk for metabolic syndrome than reduction of the subcutaneous depot in obese humans [202, 203]. A recent study in high-fat fed mice found that mesenteric adipose tissue produced significantly higher levels of the MCP-1 compared to the subcutaneous, epididymal, or renal depots in obese mice [204]. Another study found significantly increased TNF-α levels in the retroperitoneal adipose tissue depot, but not mesenteric or epididymal depot, in rats fed a high-fat diet for 1 week or 5 weeks [205]. These findings, in addition to my data showing significantly lower plasma MCP-1 levels by EGCG treatment in high-fat fed mice, suggest that EGCG-mediated decreases in mesenteric and the retroperitoneal adipose tissue weight may play a role in the effects of EGCG on diet-induced inflammation and the development of metabolic syndrome. I did not, however, observe adipose tissue inflammation in high-fat treated mice. This may be due to the method used to determine macrophage accumulation in adipose tissue. Previous studies have used antibodies specific to macrophages to measure the degree of macrophage infiltration in adipose tissue caused by a high-fat diet [96, 193]. Future studies using this method may determine whether EGCG affects local inflammation in adipose tissue.

The metabolic syndrome is a grouping of pathologies related to obesity. Insulin resistance is at the center of these pathologies. For Specific Aim 2, I tested the effects of EGCG on risk factors for metabolic syndrome. I found that EGCG significantly decreased fasting blood glucose, fasting insulin, and insulin resistance in high-fat fed

mice. To date, this is the first report on the effects of EGCG on fasting glucose, insulin, and insulin resistance in a high-fat diet-induced mouse model of insulin resistance. A previous study showed that 1% EGCG (in the form of TEAVIGO) reduced insulin levels in high-fat fed C57BL/6J mice [62]; however, these measurements were taken in the fed state, therefore an effect of food intake on insulin levels cannot be ruled out. This same study also showed that 1% EGCG significantly decreased fasting blood glucose, fed-state insulin levels, and improved glucose tolerance and insulin sensitivity in the *db/db* mouse, a transgenic model of insulin resistance [62]. The effects observed in my studies are likely to be attributable to the observed decrease in weight gain and body fat; however, there may also be some direct effects of EGCG on improvement of glucose homeostasis. Green tea supplementation increased muscle glucose transporter content in insulin-resistant rats [61]. A study by Koyama *et al.* found that EGCG treatment (0.15% in the diet) for 7 days to mice significantly decreased the expression of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P) in the liver [67]. PEPCK is a rate-limiting step in gluconeogenesis, converting oxaloacetate to phosphoenolpyruate. G6P removes phosphates from glucose at the 6-carbon position, so that newly formed glucose can leave hepatocytes and enter the bloodstream. Studies have also shown decreased expression of PEPCK and G6P by EGCG in rat hepatoma cells [68, 206]. These studies suggest that green tea and EGCG improves insulin sensitivity and glucose homeostasis, in part by directly increasing glucose disposal into the muscle, and decreasing gluconeogenesis in the liver.

Hepatic steatosis is a condition that is associated with the metabolic syndrome. In these studies I found that 0.32% EGCG decreases hepatic steatosis induced by a high-fat

diet. This was demonstrated histologically and by biochemical measurement of liver triglycerides. I also found that 0.32% EGCG significantly reduced plasma ALT levels induced by a high-fat diet. Elevated ALT in the plasma is an indication of hepatocyte injury. These results suggest that EGCG prevents liver dysfunction that is caused by a high-fat diet. To date, this is the first study on the effects of EGCG on hepatic steatosis and associated liver dysfunction in a diet-induced model of the metabolic syndrome.

The effect of EGCG on hepatic steatosis may explain the effects of EGCG on insulin resistance. The liver is an insulin-sensitive organ, which responds to insulin by suppressing hepatic gluconeogenesis [53]. Hepatic steatosis is associated with insulin resistance in humans and studies in rodent models have shown that suppression of hepatic steatosis improved hepatic insulin sensitivity [77, 80, 207, 208]. Studies showed that the effect of hepatic steatosis on insulin resistance may be mediated through the activation of protein kinase C (PKC), which is stimulated by the metabolism of fatty acids, and contributes to the phosphorylation of the insulin receptor substrate (IRS) at serine 307 [208, 209]. Serine phosphorylation inhibits tyrosine phosphorylation of IRS, an important event in normal insulin signaling [210]. EGCG has been shown to both inhibit PKC and increase tyrosine phosphorylation of IRS in cell culture studies [68, 211, 212].

Conversely, the effects of EGCG on reducing high-fat diet-induced hepatic steatosis may be downstream of the effects of EGCG on insulin resistance. Insulin resistance causes increased free fatty acid levels in the blood, due to deregulation of lipolysis, fat oxidation, and lipoprotein metabolism. The elevation in free fatty acid levels enhances the uptake of fatty acids into the liver, which are stored as triglycerides [79]. In addition, deregulated lipoprotein metabolism also causes accumulation of lipid in the

liver [213]. It is possible that EGCG improves insulin sensitivity, and, as a consequence, reduces the risk for hepatic steatosis. Further studies are needed to determine the causal relationship between insulin resistance and hepatic steatosis, and the role that EGCG serves in attenuating both of these conditions.

In these studies, I also observed that EGCG decreased plasma MCP-1 levels, which were elevated by a high-fat diet. A previous study found that EGCG treatment reduced MCP-1 expression in endothelial cells exposed to phorbol 12-myristate 13-acetate, a chemical inducer of inflammation [214], but this is the first report on the *in vivo* effects of EGCG on MCP-1 levels. A recent study by Kanda *et al.* showed that MCP-1 is involved in the recruitment of macrophages into the adipose tissue [193]. Macrophage accumulation in adipose tissue results in chronic inflammation, characterized by the release of inflammatory cytokines, such as TNF-α [96]. It is now widely accepted that obesity-induced inflammation plays a key role in the development of metabolic syndrome [215, 216]. A seminal study by Hotamisligil *et al.* found that downstream signaling of TNF-α results in aberrant serine phosphorylation of IRS in insulin-responsive tissues from obese rats [217], and another study from this group also showed that TNF-α is involved in the proteasomal degradation of IRS [218]. Both of these actions mediated by TNF-α contribute to insulin resistance. Kanda *et al.* reported that MCP-1 contributed to the development of insulin resistance [193]. The results from my studies suggest that attenuation of inflammation may be one mechanism by which EGCG reduces insulin resistance.

Inflammation is also important in the development of atherosclerosis. Macrophage recruitment to endothelial lesions provides the foundation for atherosclerotic

plaques and also contributes to the instability of the plaque, which result in myocardial infarction [17]. Increased plasma cholesterol is another risk factor for atherosclerosis. I found that EGCG significantly reduced total plasma cholesterol that was elevated by a high-fat diet. Studies in rodents showed that orally administered EGCG decreased plasma cholesterol and inhibited cholesterol absorption from the intestine, possibly by decreasing micellar solubilization of cholesterol and subsequent absorption in the intestine [219, 220]. It is possible that EGCG reduces risk for cardiovascular disease by decreasing inflammation and by preventing cholesterol absorption.

The studies completed in Specific Aims 1 and 2 show that a physiological relevant dose of dietary EGCG decreases body weight, body fat accumulation, hyperglycemia, insulin resistance, hypercholesterolemia, and hepatic steatosis in a high-fat diet-induced mouse model of obesity and metabolic syndrome. The effects observed in insulin resistance, cardiovascular risk factors, and liver lipid accumulation may be related to anti-inflammatory effects mediated by EGCG. A recent study showed that resveratrol, another polyphenolic compound, decreased insulin resistance and hepatic steatosis, and increased lifespan in high-fat fed mice [221]. Future studies will determine whether the effects of EGCG that were observed in my studies influence the lifespan of high-fat fed mice.

These studies provide a foundation for future studies that will identify the mechanistic bases for the metabolic effects of EGCG. Regarding the effects of EGCG on weight loss and reduction of hepatic steatosis, these studies suggest that EGCG may mediate some of its effects by inhibiting lipid absorption from the intestine. It will be important in futures studies to confirm these findings, and also to clearly determine in if

EGCG influences weight loss by other mechanisms, including increasing fat oxidation, decreasing fatty acid synthesis, or a combination of any or all of these events. With respect to the effects of EGCG on restoring glucose homeostasis, it will be important to determine which steps in the process of insulin signaling and glucose metabolism that EGCG affects. It will also be essential to determine whether restoration of inflammation blunts the metabolic effects of EGCG. Finally, it will be important to clearly determine whether these effects occur in humans, and whether EGCG or green tea consumption can be used as a tool to prevent the development of obesity and its co-morbidities.

2.5. Tables

Table 2.1. Composition of diets used for obesity and metabolic syndrome studies

	LF		HF		HFE	
Macronutrient	gram %	kcal %	gram %	kcal %	gram %	kcal %
Protein	19.2	20.0	26.2	20.0	26.1	20.0
Carbohydrate	67.3	70.0	26.3	20.0	26.3	20.1
Fat	4.3	10.0	34.9	60.0	34.8	59.9
Total		100.0		100.0		100.0
Kcal/gram		3.8		5.2		5.2
Ingredient	gram	kcal	gram	kcal	gram	kcal
Casein	200.0	800.0	200.0	800.0	200.0	800.0
L-Cystine	3.0	12.0	3.0	12.0	3.0	12.0
Corn starch	315.0	1260.0	0.0	0.0	0.0	0.0
Maltodextrin	35.0	140.0	125.0	500.0	125.0	500.0
Sucrose	350	1400	68.8	275.2	68.8	275.2
Cellulose	50.0	0.0	50.0	0.0	50.0	0.0
Lard	20.0	180.0	245.0	2205.0	245.0	2205.0
Soybean oil	25.0	225.0	25.0	225.0	25.0	225.0
Mineral mix*	10.0	0.0	10.0	0.0	10.0	0.0
Vitamin mix*	10.0	40.0	10.0	40.0	10.0	40.0
Choline Bitartrate	2.0	0.0	2.0	0.0	2.0	0.0
EGCG	0.0	0.0	0.0	0.0	2.48	0.0
Total	1055.1	4057.0	773.9	4057.0	776.4	4057.0

* Research Diets, Inc. mineral and vitamin mixture for Product #D12450B and D12451 (New Brunswick, NJ)

Table 2.2. Composition of diets used for lipid absorption studies (Experiment 4)

	HFSB	
Macronutrient	gram %	kcal %
Protein	24.2	20.8
Carbohydrate	46.4	40.0
Fat	20.2	39.2
Total		100.0
Kcal/gram	4.7	3.8
Ingredient	gram	kcal
Casein	200.0	800.0
DL-Methionine	3.0	12.0
Corn starch	203.0	812.0
Maltodextrin	100.0	400.0
Sucrose	0.0	0.0
Dextrose	77.0	308.0
Cellulose	50.0	0.0
Lard	17.0	153.0
Butter fat, anhydrous	20.4	184
Soybean oil	25.0	225.0
Sucrose polybehenate	8.5	77.0
Mineral mix*	5.0	0.0
Vitamin mix*	10.0	40.0
Calcium phosphate, dibasic	3.9	0.0
Potasssium phosphate, monobasic	13.6	0.0
Sodium chloride	2.6	0.0
Choline Bitartrate	2.0	0.0
Total	1055.1	4057.0

*AIN-76A mineral and vitamin mixtures (American Institute of Nutrition 1977 and 1980)

Table 2.3. The effect of 0.32% EGCG on body weight gain and visceral fat weight in high-fat fed C57BL/6J mice

Experiment 1

Diet (n)	BW gain (g)	Visceral fat weight (g)			
		Mesenteric	Epididymal	Retroperitoneal	Total
LF (12)	6.80±0.8[a]	0.48±0.1[a]	0.73±0.3[a]	0.25±0.1[a]	1.46±0.4[a]
HF (15)	23.80±0.2[b]	1.95±0.3[b]	2.41±0.6[b]	1.41±0.4[b]	5.77±0.8[b]
HFE (12)	14.02±5.3[c]	1.57±0.6[c]	2.63±0.7[b]	1.11±0.3[c]	5.31±1.3[b]

Experiment 2

Diet (n)	BW gain (g)	Visceral fat weight (g)			
		Mesenteric	Epididymal	Retroperitoneal	Total
LF (12)	10.41±0.1[a]	0.39±0.2[a]	0.62±0.2[a]	0.14±0.1[a]	1.15±0.4[a]
HF (22)	30.48±2.2[b]	2.23± 0.5[b]	2.49± 0.5[b]	0.79±0.2[b]	5.51±0.6[b]
HFE (22)	19.21±5.1[c]	1.15±0.9[c]	1.80±0.9[c]	0.52±0.3[c]	3.47±1.8 [c]

Experiment 3

Diet (n)	BW gain* (g)	Visceral fat weight (g)			
		Mesenteric	Epididymal	Retroperitoneal	Total
LF (12)	2.20±0.7	0.49±0.1[a]	0.77±0.3[a]	0.21±0.1[a]	1.47±0.6[a]
HF (22)	4.16±3.0	1.64±0.5[b]	2.90±0.5[b]	1.16±0.2[b]	5.69±1.0[b]
HFE (22)	2.57±0.6	1.05±0.5[c]	2.46±0.7[b]	1.02±0.4[b]	4.52±1.3[b]

* During final four weeks of study. Data are presented as mean ± S.D. Different letters indicate statistical significance at $p<0.05$ (One-way ANOVA).

Table 2.4. Effect of 0.32% EGCG (16-week treatment) on fasting plasma insulin, HOMA-IR index, and plasma cholesterol in high-fat fed C57BL/6J mice

Diet (n)	Insulin (ng/ml)	HOMA-IR	Cholesterol (mg/dl)
LF (11)	0.43±0.1[a]	3.12±0.6[a]	101.59±19.5[a]
HF (21)	5.23±2.7[b]	74.47±11.2[b]	214.96±22.2[b]
HFE (21)	2.05±2.3[a]	17.41±21.6[a]	162.51±27.9[c]

Data are presented as mean ± S.D. Different letters indicate statistical significance at $p<0.05$ (One-way ANOVA).

Table 2.5. Effect of 0.32% EGCG (16 week treatment) on liver pathologies in high-fat fed C57BL/6J mice

Diet (n)	Liver wt/BW	Fatty liver incidence	Liver trigylcerides (mg/g tissue)	Plasma ALT (U/L)
LF (11)	0.036±0.002[a]	0/12[a]	47.01±22.5[a]	13.23±5.0[a]
HF (21)	0.045±0.011[b]	21/22[b]	189.92±66.1[b]	105.21±60.3[b]
HFE (21)	0.035±0.005[a]	4/22[a]	58.06±88.3[a]	35.25±24.0[a]

Data are presented as mean ± S.D. Different letters indicate statistical significance at $p<0.05$ by one-way ANOVA or chi-squared test (for fatty liver incidence).

Table 2.6. Effect of 0.32% EGCG (16 week treatment) on plasma MCP-1 levels in high-fat fed C57BL/6J mice

Diet (n)	MCP-1 (pg/ml)
LF (11)	7.27±3.8[a]
HF (21)	56.97±37.7[b]
HFE (21)	17.41±12.9[a]

Data are presented as mean ± S.D. Different letters indicate statistical significance at $p<0.05$ by one-way ANOVA.

2.6. Figure legends and figures

Figure 2.1. Effect of 0.32% EGCG on food intake in high-fat fed C57BL/6J mice. Mice were administered LF, HF, or HFE diet for 16 weeks (Experiments 1 and 2), or administered LF or HF diet for 9 weeks, and then the HF group was randomized to either HF or HFE for 4 weeks thereafter (Experiment 3). Food intake was measured weekly on a per cage basis. All data are presented as mean ± S.E.

Figure 2.2. Effect of 0.32% EGCG on body weight in high-fat fed C57BL/6 mice. Mice were administered LF, HF, or HFE diet for 16 weeks (Experiments 1 and 2), or administered LF or HF diet for 9 weeks, and then the HF group was randomized to either HF or HFE for 4 weeks thereafter (Experiment 3). Body weight was measured weekly. All data are presented as mean ± S.E. LF was significantly different from HF by Week 4 ($p<0.05$, one-way ANOVA), HFE was significantly different from HF by Week 6 ($p<0.05$, one-way ANOVA) Different letters indicate statistical significance at $p<0.05$ (by repeated measures ANOVA).

Figure 2.3. Effect of 0.32% EGCG on feed efficency in high-fat fed C57BL/6 mice. Mice were administered LF, HF, or HFE diet for 16 weeks (Experiments 1 and 2), or administered LF or HF diet for 9 weeks, and then the HF group was randomized to either HF or HFE for 4 weeks thereafter (Experiment 3). Feed efficiency for each was reported as the ratio of weight gain (in grams) to the amount of food consumed (in grams) from Week 1 of treatment (Experiments 1 and 2) or from Week 9 of treatment (Experiment 3).

All data are presented as mean ± S.E. Different letters indicate statistical significance at $p<0.05$ (by repeated measures ANOVA).

Figure 2.4. Effect of 0.32% EGCG (16-week treatment) on body fat percentage in high-fat fed C57BL/6J. Mice from Experiment 2 were analyzed postmortem for body fat percentage (by DEXA) at 16 weeks of treatment. All data are presented as mean ± S.E. Different letters indicate statistical significance at $p<0.05$ (by one-way ANOVA).

Figure 2.5. No effect of 0.32% EGCG (5-day treatment) on lipid absorption in mice on HFSB diet. Mice from Experiment 4 were fed HFSB diet or HFSB diet plus EGCG (in drinking fluid) for 5 days. Feces samples were collected on Days 3 and 4 and measured for fatty acid composition by gas chromatography. Data are presented as mean ± S.E.

Figure 2.6. Effect of 0.32% EGCG (10-week treatment) on fecal lipid content in high-fat C57BL/6J mice. Feces were collected from mice during Week 10 in Experiment 2 and measured for lipid content (after lipid extraction) gravimetrically. Data are presented as mean ± S.E. Different letters indicate statistical significance at $p<0.05$ (by one-way ANOVA).

Figure 2.7. Effect of 0.32% EGCG on fasting blood glucose of high-fat fed C57BL/6J mice. Mice were administered LF, HF, or HFE diet for 16 weeks (Experiments 1 and 2), or administered LF or HF diet for 9 weeks, and then the HF group was randomized to either HF or HFE for 4 weeks thereafter (Experiment 3). Fasting

blood glucose was measured 3 times throughout the course of Experiment 1, 6 times throughout the course of Experiment 2, and 3 times throughout the course of Experiment 3. All data are presented as mean ± S.E. Different letters indicate statistical significance at $p<0.05$ (by repeated measures ANOVA).

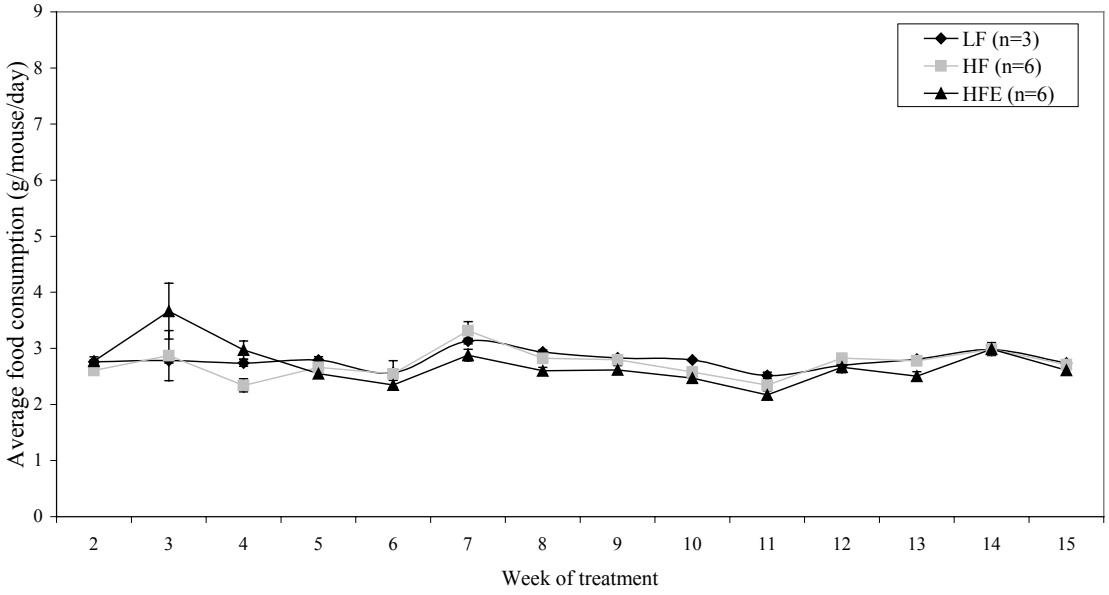
Figure 2.8. Fatty liver in LF- (A), HF- (B), and HFE- (C) treated C57BL/6J mice (gross examination). Liver samples from Experiment 2 were harvested, rinsed, and weighed. HF mice had increased liver size and altered coloration due to increased lipid deposition compared to LF or HFE mice.

Figure 2.9. Liver lipid accumulation in LF- (A), HF- (B), and HFE- (C) treated C57BL/6J mice (histological examination, 5X and 10X magnification). Liver samples were fixed, embedded, cut into slides and stained with H&E. HF mice showed increased microvesicular lipid deposition compared to LF or HFE mice. Lipid deposition was present mainly in the centrilobular hepatocytes, rather than in the periportal hepatocytes.

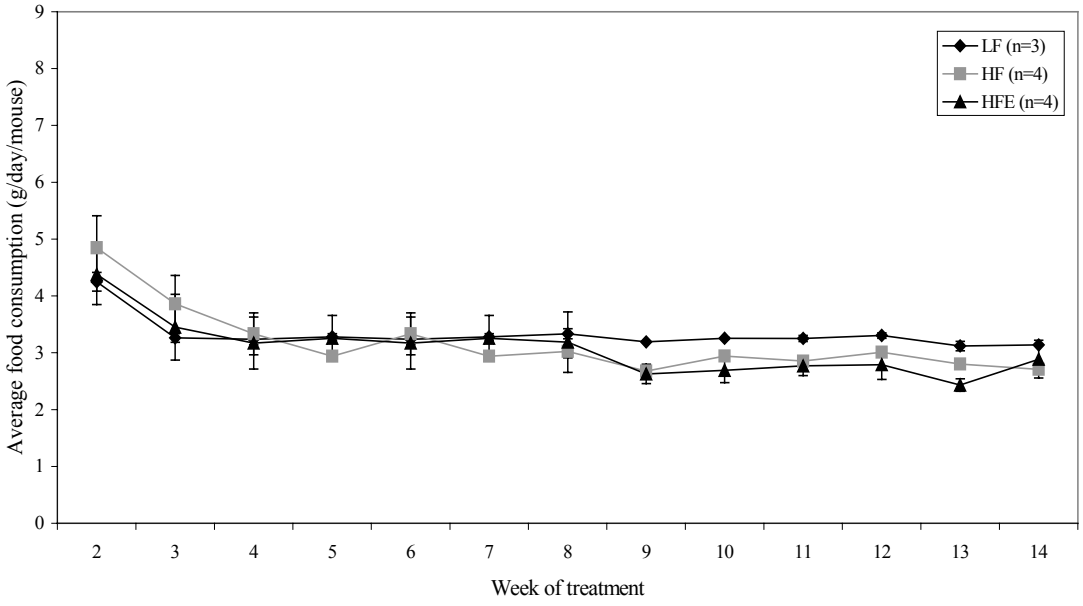
Figure 2.10. No inflammation in adipose tissue of LF- (A), HF- (B), and HFE- (C) treated C57BL/6J mice. Epididymal adipose tissue samples. fixed, embedded, cut into slides and stained with H&E. No macrophage infiltration was visible with H&E staining. Samples are shown with 10X magnification.

Figure 2.1

Experiment 1



Experiment 2



Experiment 3

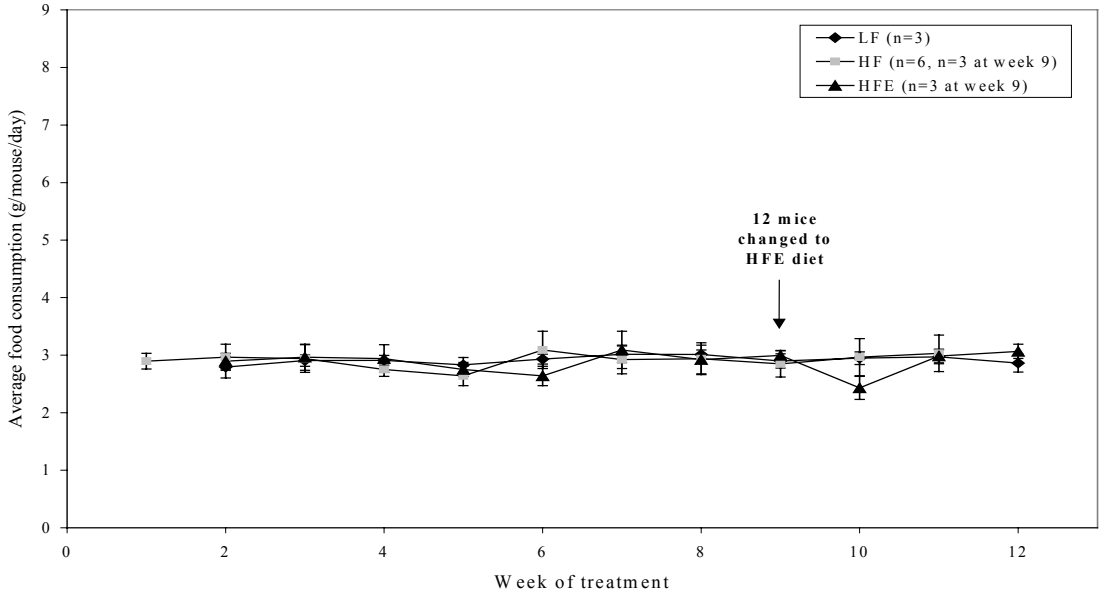
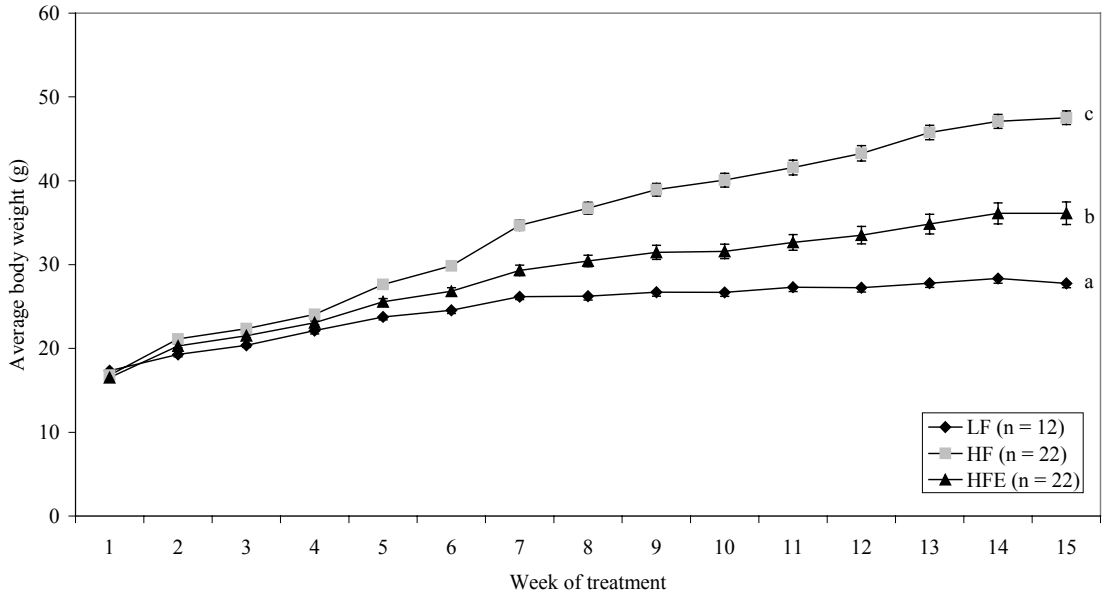
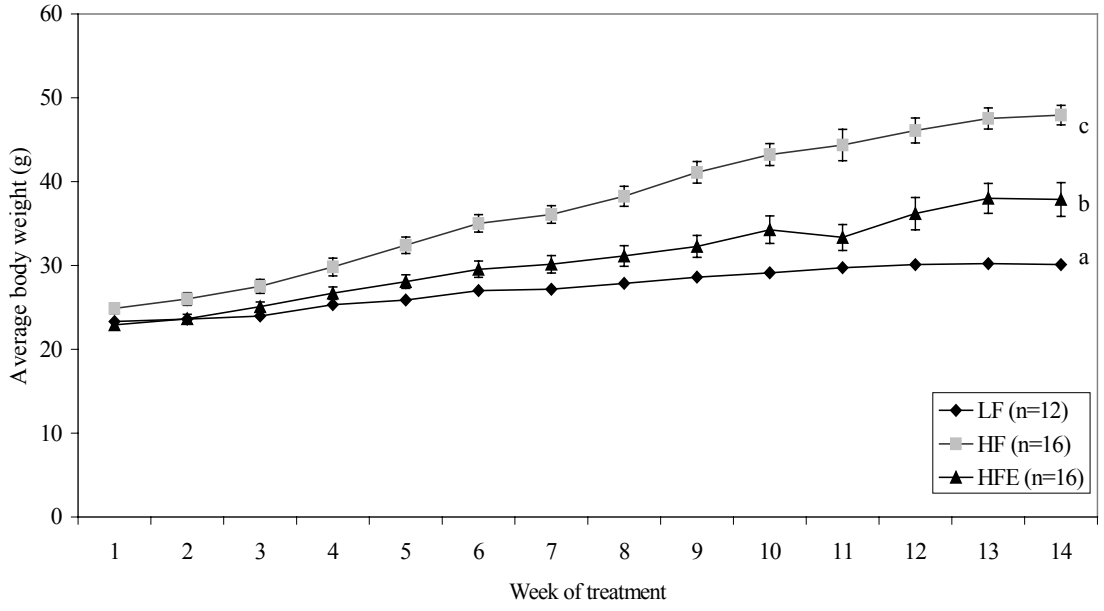


Figure 2.2

Experiment 1



Experiment 2



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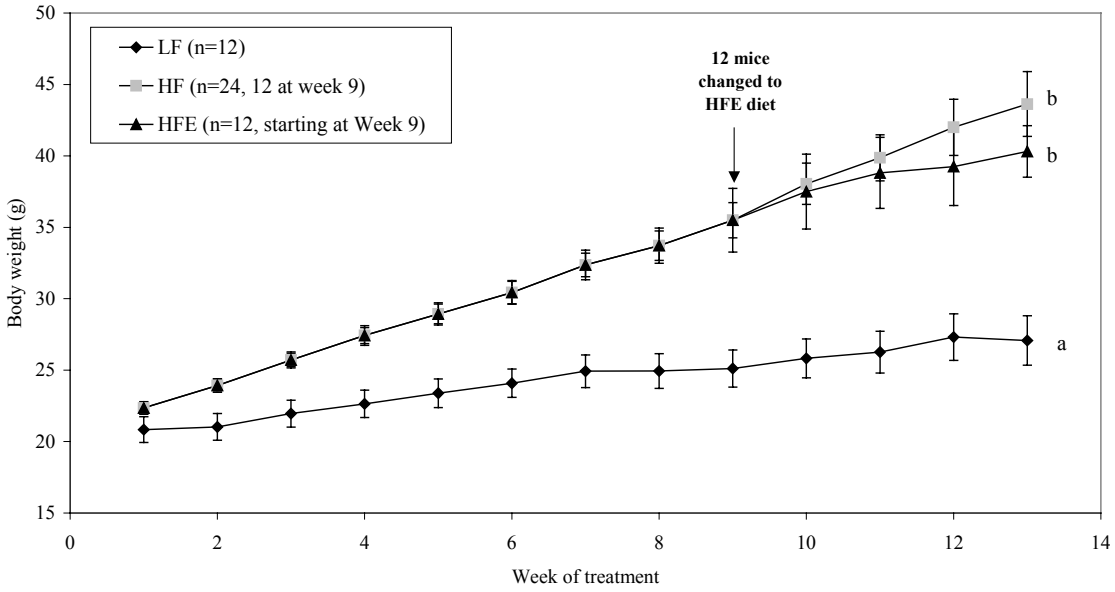
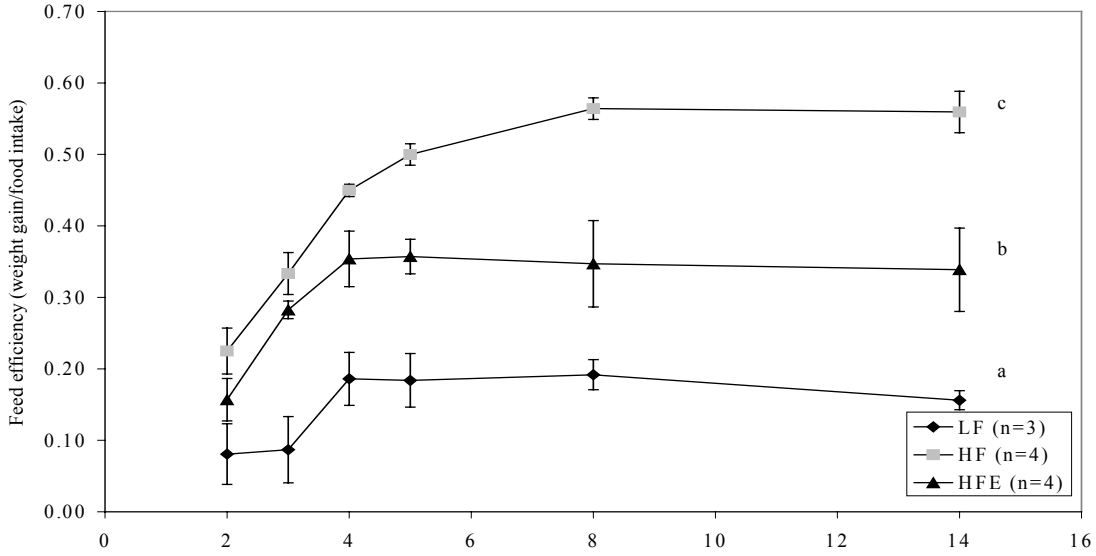


Figure 2.3

Experiment 1



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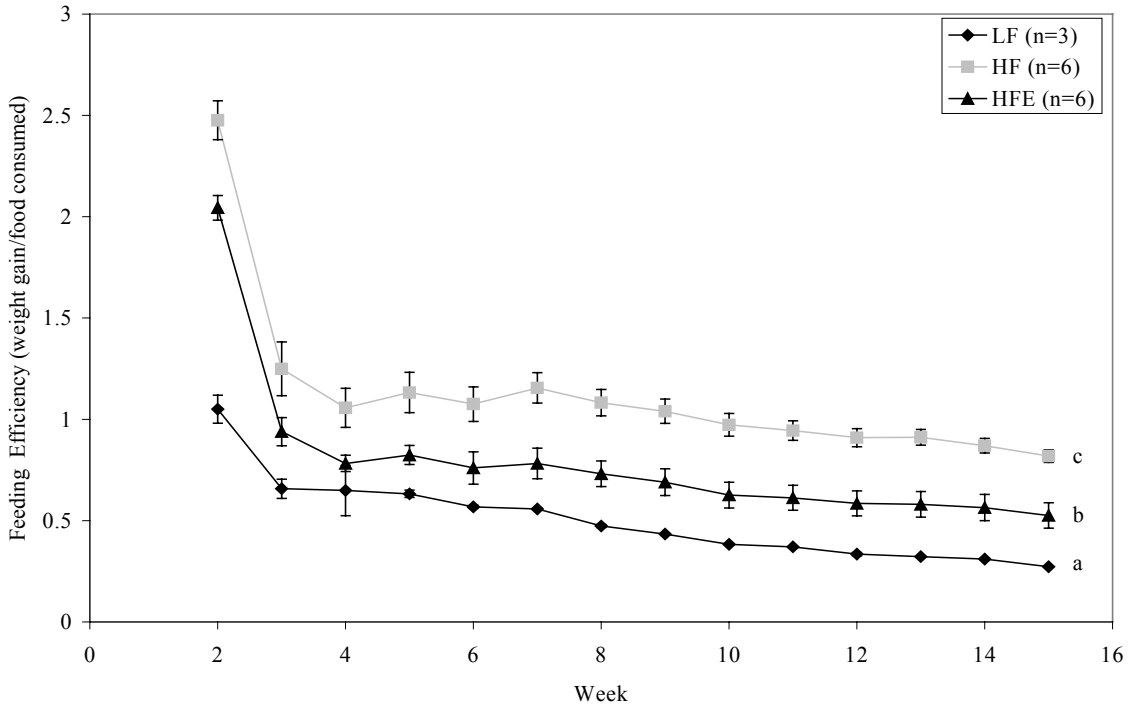

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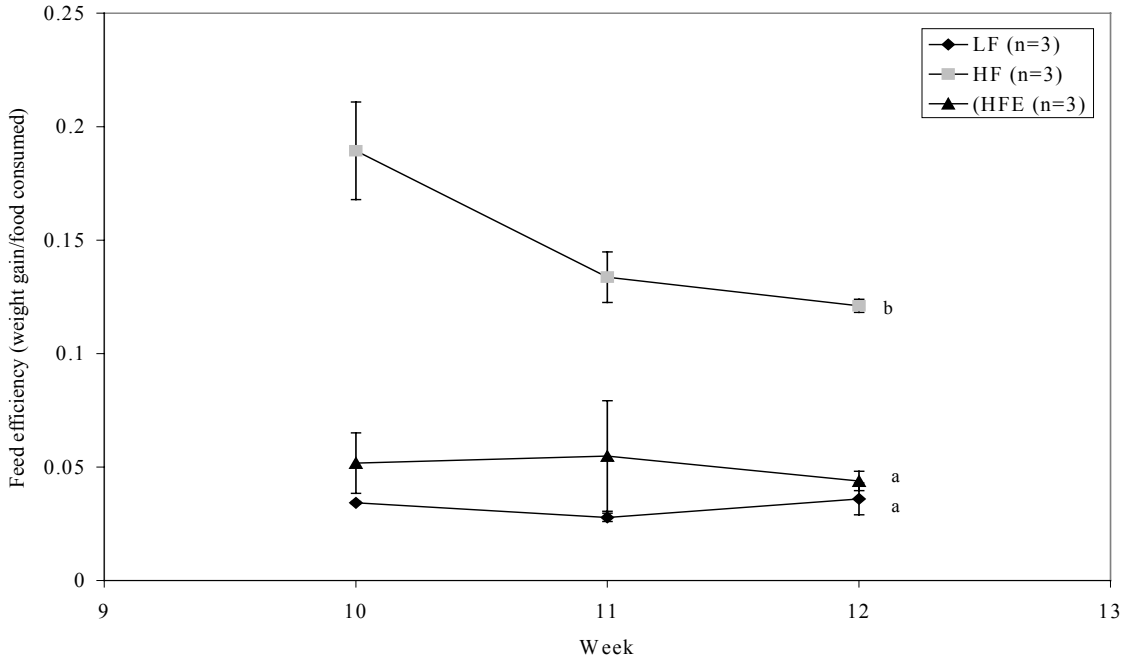

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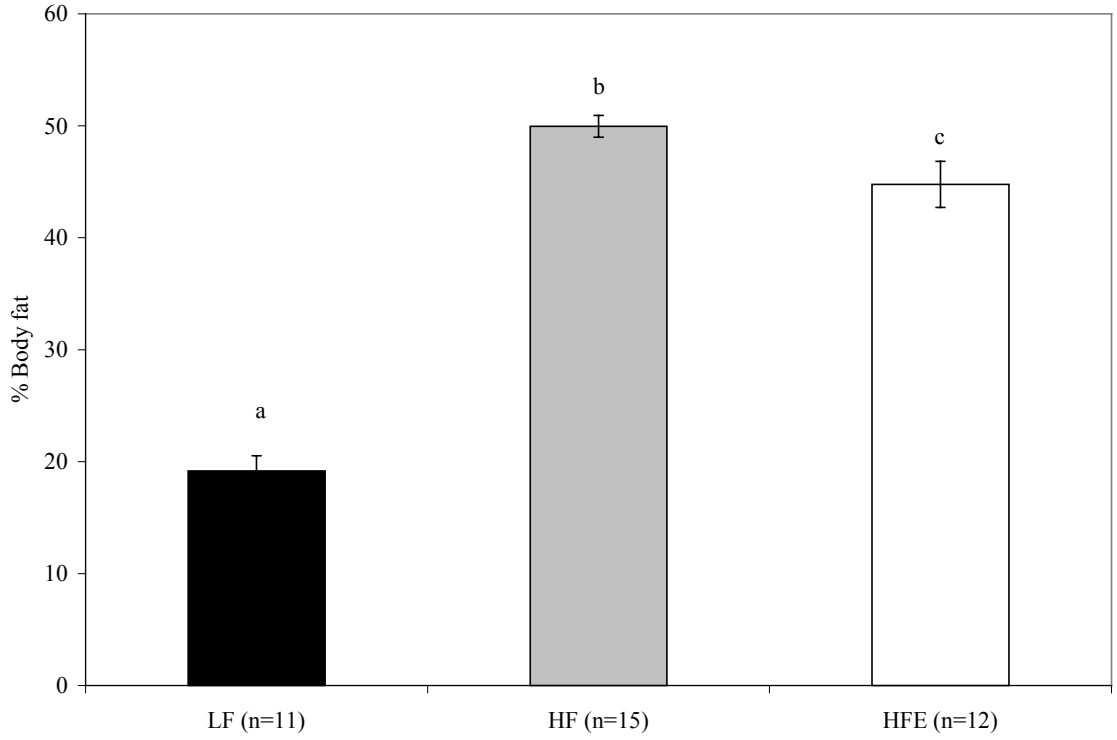

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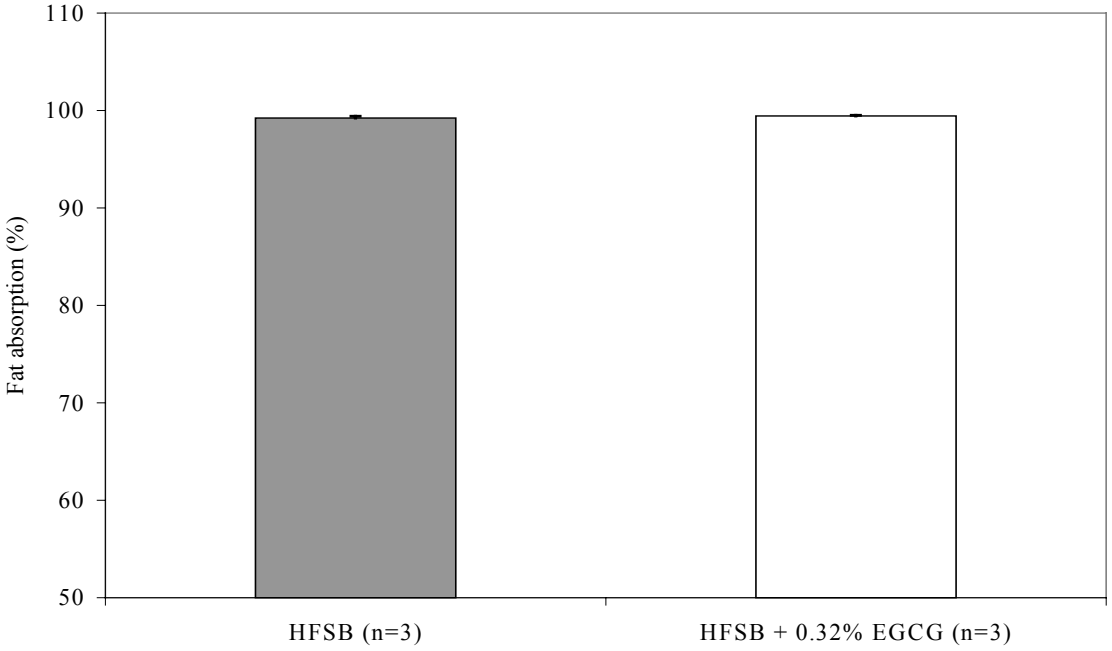


Figure 2.6

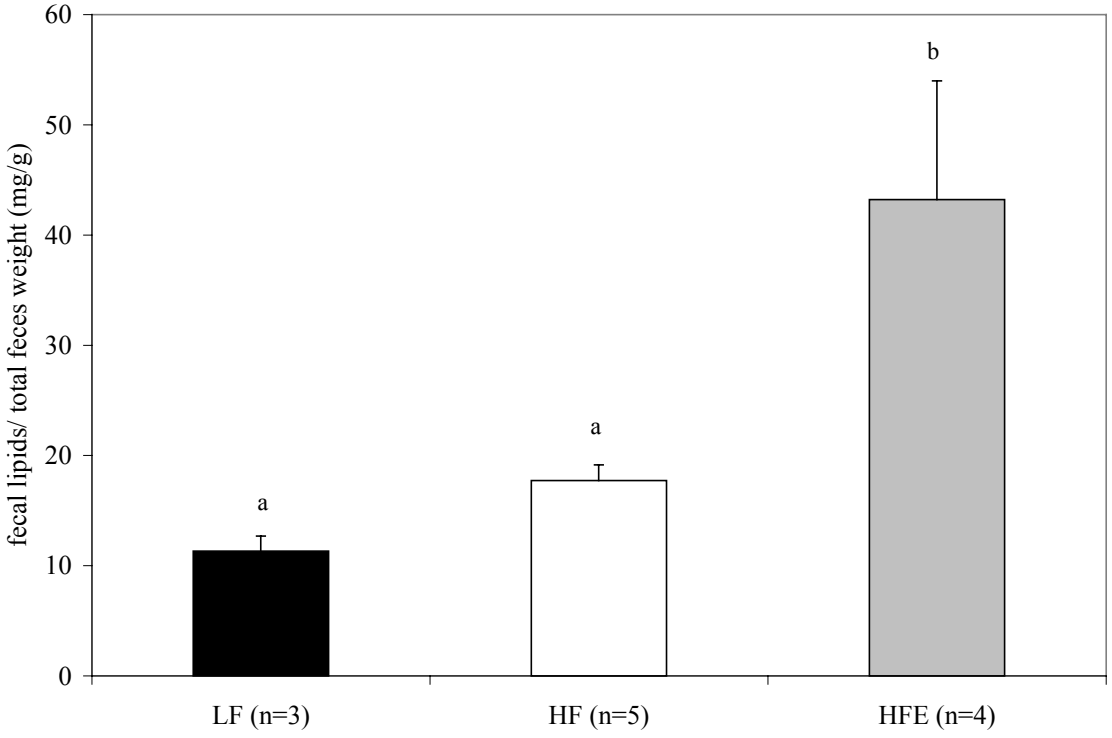
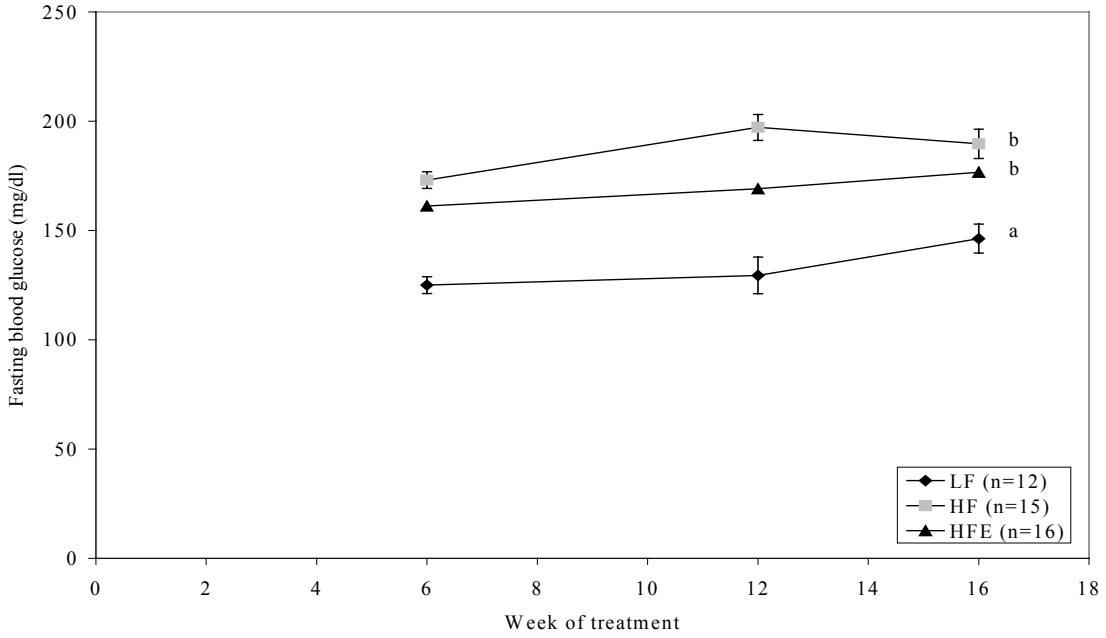


Figure 2.7

Experiment 1



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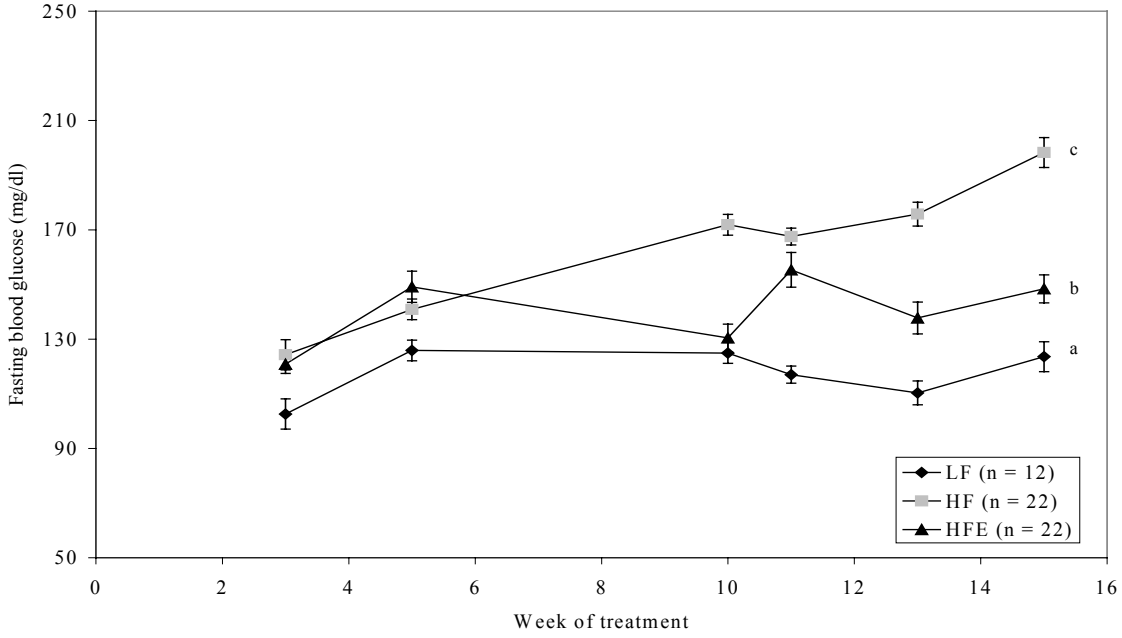

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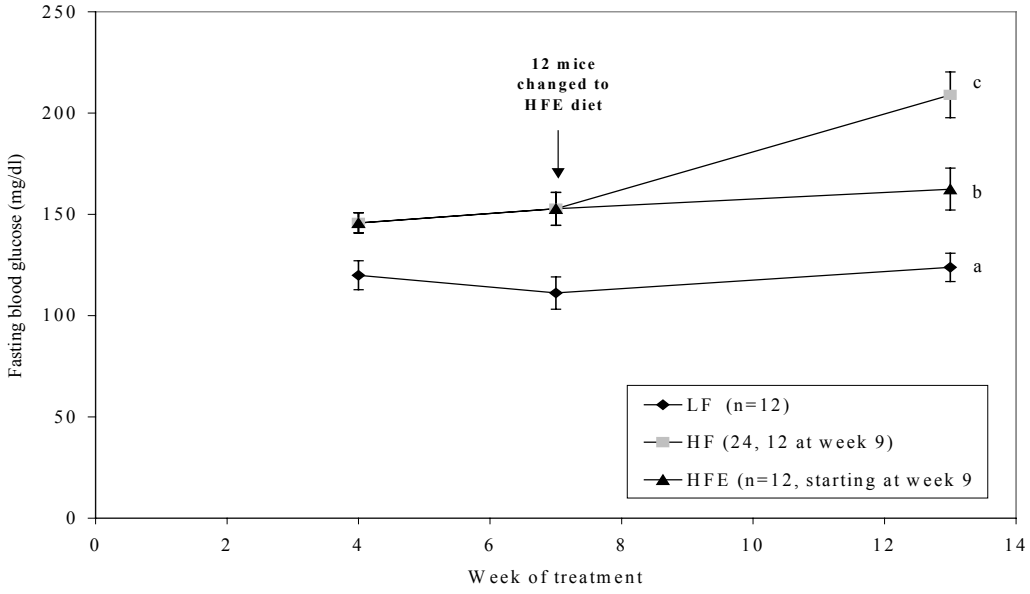

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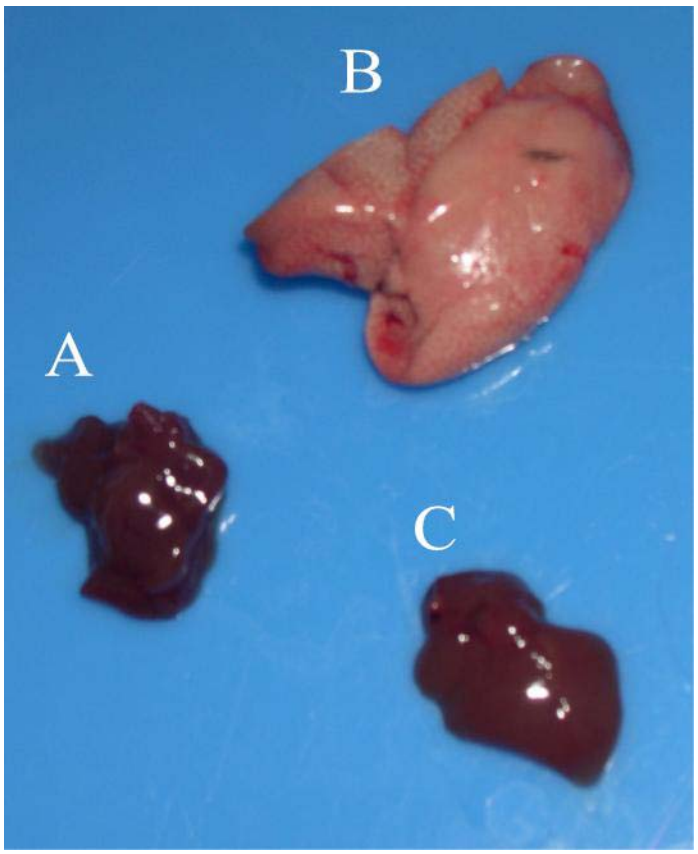
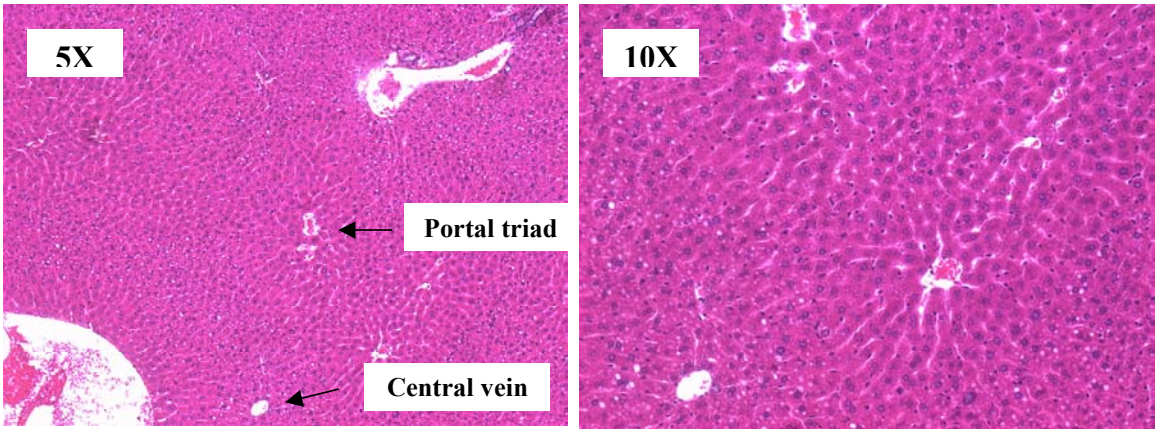


Figure 2.9

A



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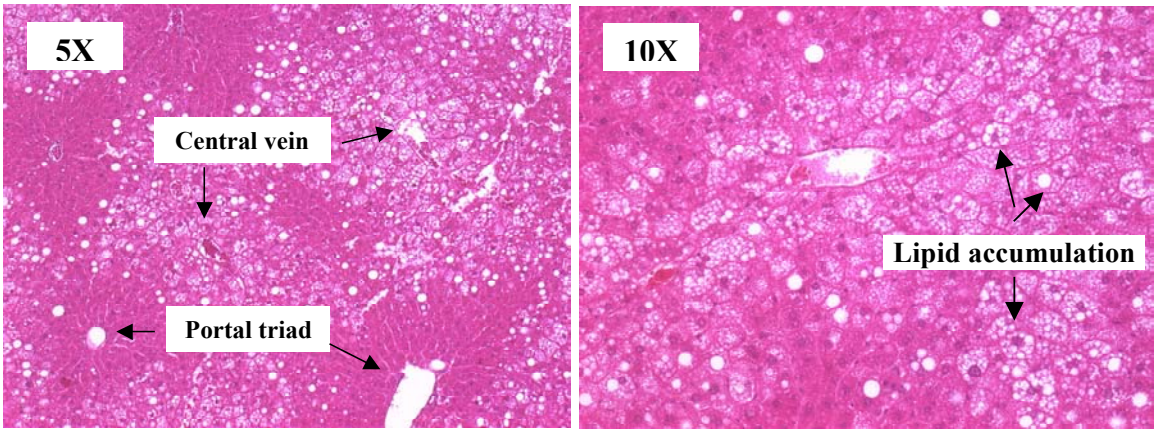


C

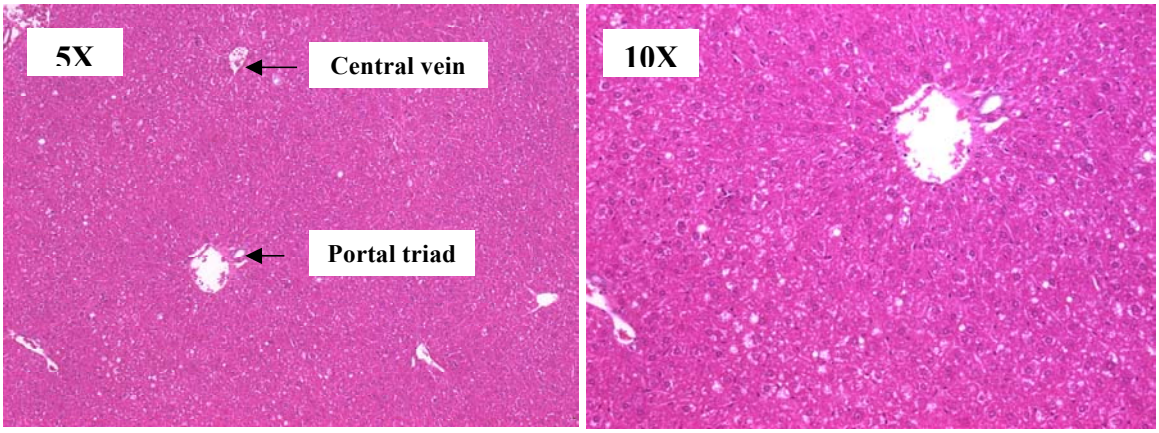
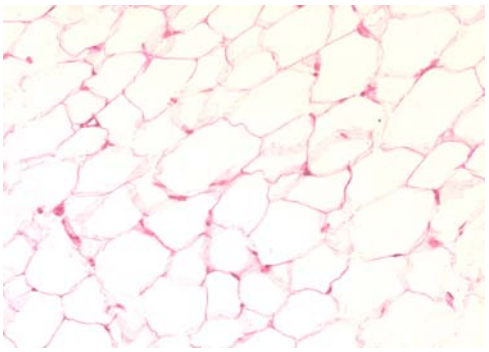


Figure 2.10

(A)



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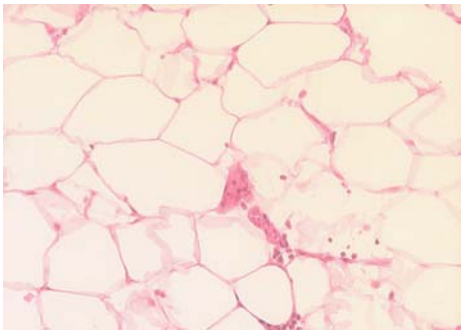

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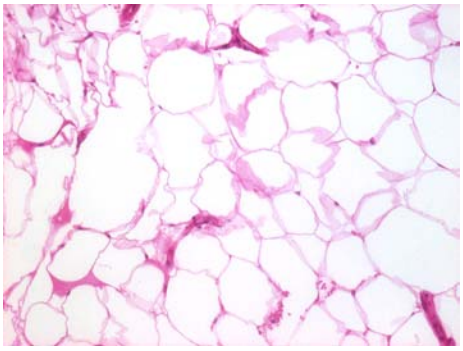


CHAPTER 3

INHIBITION OF INTESTINAL TUMORIGENESIS BY EGCG AND FISH OIL IN *APC*$^{MIN/+}$ MICE

3.1. Background and rationale

Colon cancer is the second leading cause of death in the United States [127]. Epidemiological studies have suggested that consumption of certain dietary constituents, including green tea polyphenols and ω-3 polyunsaturated fatty acids (PUFA) may decrease colon cancer risk [222-224]. Many studies have suggested that (-)-epigallocatechin-3-gallate (EGCG), the most abundant and biologically active polyphenol in green tea, is responsible for the majority of the chemopreventive activities of green tea [25, 224, 225]. Animal studies using these dietary constituents have been performed to evaluate the potential chemopreventive activities of these agents.

Several studies in mice have shown that green tea can reduce intestinal and colon cancer risk. Orner *et al.* showed that green tea extract reduced tumor multiplicity in the *Apc*$^{Min/+}$ mouse, a widely used model for intestinal tumorigenesis [148]. A study in our lab demonstrated that green tea extract significantly reduced the occurrence of aberrant crypt foci in azoxymethane (AOM)-treated mice on a high-fat diet [151]. We also found that 0.1% EGCG in the drinking fluid can inhibit colon tumor incidence in AOM-treated mice (Bose, Chin, Park, Husain, Liao, Vittal, Kopelovich, Huang, Yang, unpublished results). Recent studies in our laboratory showed that EGCG in the drinking fluid dose-dependently decreased intestinal tumor multiplicity in the *Apc*$^{Min/+}$ mouse; at a dose of 0.32% EGCG, the inhibition was ~40% [150]. EGCG has been shown to inhibit aberrant *Wnt* signaling by inhibiting translocation of the *Wnt* mediator β-catenin to the nucleus

[150]. This inhibition may prevent β-catenin from interacting with the transciption factor TCF-4, which suppresses the transcription of genes associated with the cell cycle and cell proliferation (as reviewed in [226].

ω-3 PUFA, found predominantly in fish oils, have been shown in different animal models to inhibit intestinal tumorigenesis [167, 171, 175]. A study using *Apc*$^{Min/+}$ mice showed that K85, a fish oil concentrate of ω-3 PUFA (2.5% w/w in the diet) reduced tumor multiplicity by 66% in comparison to untreated mice; a similar reduction in tumor growth by a fish oil diet (16% w/w in the diet) was observed in nude mice bearing human colon carcinoma xenografts [169, 171]. Rao *et al.* observed a 50% decrease in colon tumor number in AOM-treated rats on a high-fat diet composed of mainly fish oil compared to similarly treated rats on a high-fat diet consisting of mixed lipids [167]. Studies in colon cancer cell lines have shown that the inhibition of arachidonic acid metabolism by ω-3 PUFA is related to the proliferation and tumorigenicity of these cells. This suggests that the reduction of inflammatory prostaglandins, such as prostaglandin E_2 (PGE_2), produced by arachidonic acid may be one way by which ω-3 PUFA mediate their chemopreventive effect [227].

Although these and other compounds have shown promise as chemopreventive agents, there are limitations in using a single agent for cancer prevention in practice [149, 176]. This appears to be the case for both drugs and dietary agents. Cyclooxygenase inhibitors initially showed promise as effective chemopreventive agents against colon cancer, but long-term use of these compounds was later found to have undesirable side effects, such as increased cardiovascular risk [178, 179]. Pre-clinical trials with dietary chemopreventive agents demonstrate less toxicity than the drugs but often have poor

bioavailability and lower potency than drugs [180-182]. These findings imply that consumption of any one dietary nutrient may not be a practical means of effective cancer chemoprevention. Combination of two or more pharmacological agents has been shown to be effective for cancer chemoprevention, maximizing efficacy by affecting different molecular targets and minimizing toxicity by lowering doses of the individual drugs [183, 184]. Combination of two or more dietary compounds may be a practical and effective approach to cancer chemoprevention for those same reasons.

Currently, there are no published studies on the effect of a dietary combination of EGCG and fish oil on intestinal cancer risk. Preliminary results in our laboratory showed that 0.08% EGCG in combination with 12% fish oil for 8 weeks non-significantly reduced intestinal tumor multiplicity by >33% in female *Apc*$^{Min/+}$ mice on a high-fat diet, and EGCG alone had very little effect (7% reduction). As a result, I conducted a 9-week study to determine the effect of 0.16% EGCG in combination with 12% fish oil on intestinal tumorigenesis in female *Apc*$^{Min/+}$ mice on a high-fat (20% mixed lipid) diet. I also conducted another study to determine the effects of a short-term (3 weeks) treatment of this combination on intestinal tumorigenesis. The mixed lipid content is aimed to represent the fat composition of the average American diet [228].]. A dose of 0.16% EGCG was used, which is the equivalent of 4-5 cups of green tea (containing 2 g green tea leaves per cup) per day. This calculation was based on an allometric scaling conversion [195] conducted in our laboratory. Assuming 12 kcal as the average daily energy requirements for an adult mouse [196], 0.16% EGCG would equal 0.3 mg EGCG per kcal consumed, if mice consume 2 ml fluid per day. Assuming the energy requirements for the average human as 2000 kcal per day, human EGCG consumption at

this dose would be 600 mg per day. EGCG consists of about 50% of all the catechins present in tea; therefore catechin consumption at this dose would be 1.2 g per day. Catechins comprise about 30% of green tea; steeping in hot water would extract about 50% of tea solids, therefore green tea consumption at this dose would be 7.5 g per day. An average green tea bag contains 2 g green tea per bag; therefore, 4 tea bags per day would be necessary to equal the dose of 0.16% in mice. I chose the dose of EGCG for this study so as not to saturate the dose of agents. Additionally, preliminary data showed that a very low dose (0.08%) of EGCG is not effective at inhibiting tumorigenesis in the *Apc*$^{Min/+}$ mouse model on high-fat diet. The dose of fish oil was based on studies using other models of colon carcinogenesis. These studies showed that 17% fish oil (w/w) in the diet effectively inhibited colon tumor formation and incidence of aberrant crypt foci in AOM-injected rats on a high-fat diet [167]. I chose a dose of 12% so as to not saturate the dose for the combination group.

3.2. Materials and methods

3.2.1. Chemicals and Diets

EGCG (Mitsui Norin, Japan) solution was prepared in deionized H_2O containing 0.5% citric acid and used as drinking fluid. Fish oil was a gift from the Menhaden Oil Refinery of Omega Protein, Inc. (Reedville, VA). Menhaden oil contains about 1.7% α-linolenic acid, 13% eicosapentaenoic acid (EPA), and 12% docosahexaenoic acid (DHA) [229]. The control high-fat diet (Table 3.1) consisted of a 20% fat content (w/w), formulated using a minor modification of the American blend fat developed by the Institute of Shortening and Edible Oils [beef fat (16%), lard (10%), butter fat (12%), hydrogenated soybean oil (30%), peanut oil (5%), and corn oil (27%)] [228]. The fish oil

diet (Table 6) also had a 20% fat content (w/w), however, it consisted of 12% fish oil and 8% of the lipid mixture. Diets were purchased from Research Diets, Inc. (New Brunswick, NJ).

3.2.2. Breeding and genotyping of *Apc*$^{Min/+}$ mice

Male C57BL/6J-*Apc*$^{Min/+}$ and female wild-type littermate mice were initially purchased from The Jackson Laboratory (Bar Harbor, ME) as founders and a breeding colony was established in the animal facility of the Susan Lehman Cullman Laboratory for Cancer Research (Rutgers, The State University of New Jersey, Piscataway, NJ). Pups were produced from the colony and weaned at 3 weeks of age. Genotyping was performed by routine polymerase chain reaction (PCR) assays of DNA from tail snips using the DNEasy Tissue DNA Extraction Kit (Qiagen, Valencia, CA) and a commercially available master mix (Bio-Rad, Hercules, CA). An *Apc*^{Min} nonsense mutation–specific primer (*Apc*-mutant: 5'-TTCTGAGAAAGACAGAAGTTA-3'), together with a complementary 3'-end primer (*Apc*-common: 5'-TTCCACTTTGGCATAAGGC-3'), detected the mutant *Apc* allele (313 bp), which is only present in *Apc*$^{Min/+}$ mice. The *Apc*$^{+}$ allele–specific primer (*Apc*-wild-type: 5'-GCCATCCCTTCACGTTAG-3') with the *Apc*-common primer detected the wild-type allele (619 bp).

3.2.3. Diet Treatment and Tissue Harvesting

Experiments with mice were carried out according to a protocol approved by the Institutional Review Board for the Animal Care and Facilities Committee at Rutgers University. After genotyping, female C57BL/6J-*Apc*$^{Min/+}$ mice (5-6 weeks old, 15 mice per group) were treated as follows: 1) high-fat diet, 2) high-fat diet plus 0.16% EGCG (as

sole source of drinking fluid), 3) high-fat fish oil diet, or 4) high-fat fish oil diet plus 0.16% EGCG (as sole source of drinking fluid). Female mice were chosen as there were more female mice produced from this generation of breeding than male mice. Body weight, food and fluid consumption were measured weekly. After 9 weeks of treatment, mice were euthanized by CO_2 asphyxiation. The entire intestinal tract was harvested, flushed thoroughly with cold 0.9% saline, cut open longitudinally and flattened on filter paper to expose tumors in the lumen. The flattened tissues on filter paper were placed on dry ice briefly to facilitate scoring the visible tumors. All tumors were excised from 5 females per group and snap frozen on dry ice for biochemical analyses. A 150 mm segment from the jejunal portion of the small intestine from another 5 females in each group was fixed in a 10% formalin solution for 24 hours and then transferred to 1X PBS for tissue processing and immunohistochemical analyses.

In a separate experiment, 12 week old female C57BL/6J-*Apc*$^{Min/+}$ mice were administered the same treatments for 3 weeks (n=7-10/group). After this short-term treatment, mice were euthanized and the intestinal tract was harvested as described before

3.2.4. Immunohistochemistry

Embedded tissue blocks were cut serially for at least 30 slides and labeled numerically. Slides 1, 10, 20 and 30 were stained for H&E for histopathological evaluation and the remaining were used for immunohistochemistry. A standard ABC method was used for immunohistochemistry as previously described [230]. Briefly, tissue sections were de-paraffinized in xylene and rehydrated in distilled water. The endogenous peroxidase was quenched with 0.3% hydrogen peroxide in methanol for 30 min. Subsequently, sections were subjected to antigen retrieval by heating the slides in sodium

citrate buffer (0.01 M, pH 6.0) in a pressure cooker for 3 min after reaching full pressure. Non-specific staining was blocked with either 10% normal horse or goat serum. Antibodies diluted to appropriate concentrations were applied to tissue sections and the slides were incubated in a humidified chamber overnight at room temperature. After rinsing with PBS, the sections were incubated with the appropriate biotinylated secondary antibody, and then stained using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) for 30 min. 3-3' Diaminobenzidine (Vector Laboratories, Burlingame, CA) was used as the chromogen.

Proliferative cells were identified by staining with antibodies against Ki-67 (1:100 dilution, TEC-3, Dako, Carpinteria, CA). Quantification of the total number of cells and the number of Ki-67 positive cells in adenomas was performed using the Image-Pro Plus system (Silver Spring, MD). The color image containing tumor cells was converted into a black and white image. The area of tumor cells was selected manually and circled with green on the black and white image. The selected cells were highlighted with red and adjusted according to cell intensity to ensure that the highlighted cells were matched well with that in the color image. The number of Ki-67 positive cells and the total number of tumor cells were counted automatically. Proliferation index was defined as the percentage of Ki-67 positive cells in the total number of tumor cells. Apoptotic cells were stained with an antibody against cleaved-caspase 3 (1:200 dilution, rabbit polyclonal Asp175, Cell Signaling Technology, MA) and were quantified similarly. Positivity of nuclear staining for β-catenin and phospho-Akt (1:3000 and 1:100 antibody dilution, respectively, Cell Signaling, MA) was determined manually and expressed as the

percentage of positive-staining cells in the total number of tumor cells. All of the tumor cells in the adenomas were counted.

3.2.5. PGE_2 levels in small intestinal tumors

Small intestinal tumors were homogenized in tissue lysis buffer containing protease inhibitors and 10 μM indomethacin (a cyclooxygenase inhibitor). The indomethacin was added to maintain the steady state levels of PGE_2. Homogenates were acidified with 0.1 N HCl, and extracted with 1 ml ethyl acetate. The ethyl acetate fraction was dried under vacuum and re-dissolved in 500 μl enzyme immunoassay (EIA) buffer. The levels of PGE_2 were measured using an EIA kit (Cayman Chemical, Ann Arbor Michigan) according to the manufacturer's protocol.

3.2.6. Statistical analyses

One-way analysis of variance (ANOVA) with appropriate post-hoc tests were used for statistical analysis of tumor multiplicity, PGE_2 levels (GraphPad software, San Diego, CA), and immunohistochemistry results [231] for comparison among multiple groups. Two-way ANOVA was used to determine interaction of treatments on tumorigenesis. Significance was assigned at $p<0.05$.

3.3. Results

3.3.1. Effect of EGCG in combination with fish oil on intestinal tumorigenesis in *Apc*$^{Min/+}$ mice

In this experiment, I investigated the effects of EGCG in combination with fish oil on intestinal tumorigenesis in *Apc*$^{Min/+}$ mice fed a high-fat diet. The average fluid intake in the EGCG groups was significantly lower (about ~50%) than the groups without EGCG (Figure 3.1A). The average food consumption and body weight in the EGCG

groups was lower than the other groups (on average, by 19% and 8%, Figure 3.1B-C)), although the differences were not statistically significant.

The combination of fish oil and 0.16% EGCG significantly reduced total tumor multiplicity in female *Apc*$^{Min/+}$ mice (53% decrease compared to control group, $p<0.05$ by one-way ANOVA) (Table 3.2). There appeared to be no effect of fish oil on total tumor multiplicity. The inhibition by the combination of agents was greater than that observed in mice treated with EGCG alone (18.5% decrease), although analysis by 2-way ANOVA showed no significant interaction of the two agents on inhibition of tumorigenesis. The inhibition of tumor multiplicity by the combination was mainly attributable to the decrease in tumor number in the distal portion of the small intestine (58% decrease compared to control group, $p<0.01$ by one-way ANOVA). Combination treatment significantly decreased the number of large-sized tumors (94%, $p<0.05$ by one-way ANOVA), as did the fish oil treatment (55%, $p<0.05$).

In the second study, I treated 12-week-old female *Apc* $^{Min/+}$ mice with these agents for a period of 3 weeks. As expected, I found that there was no effect of the treatments on total tumor multiplicity; however, the combination did significantly reduce the number of large-sized tumors (66% decrease, $p<0.05$ by one-way ANOVA, Table 3.3) in comparison to the control group. Two-way ANOVA analysis showed no significant interaction of the two agents on inhibition of large-sized tumors.

3.3.2. Effects of 9-week treatment on cell proliferation and apoptosis

Antibodies against Ki-67 and cleaved caspase-3 were used to immunohistochemically determine the treatment effect on cell proliferation and apoptosis. Both antibodies showed positive staining in the nucleus (Figure 3.2). The Ki-

67 staining was decreased in tumors of all treatment groups, but not in the normal crypts, in comparison to the control group. There was a significant decrease in proliferation index in adenomas from the EGCG group (by 53%) and the combination group (by 27%) in comparison to that of the control group (Table 3.4). The apoptotic index was significantly higher in the tumors of all treatment groups (about 4-fold) in comparison to the control group (Figure 3.2, Table 3.4). The apoptotic index was low in the normal epithelia, and no appreciable change was observed as a result of the treatment.

3.3.3. Effects of 9-week treatment on β-catenin expression and levels of phosphorylated Akt

All adenomas from the control group had enhanced nuclear and cytoplasmic β-catenin staining, but reduced membranous staining (Table 3.4). A significant reduction in nuclear staining positivity was observed in the adenomas from the groups treated with EGCG (by 44.8%), fish oil (by 62.5%) and the combination of the two agents (by 62.7%) in comparison to the control group (Table 3.4). In adenomas from the treated groups, the intensity of cytoplasmic staining was reduced to moderate expression levels while membranous staining was increased or even restored totally (Figure 3.2). In the adenomas from untreated *Apc*$^{Min/+}$ mice, phospho-Akt staining was observed in both the nucleus and cytoplasm (Figure 3.2). All treatment groups showed reduced nuclear staining of phospho-Akt in the adenomas, both in terms of staining intensity and number of positive-staining cells. The percent of phospho-Akt nuclear positive-staining cells in the adenomas was significantly decreased in these treatment groups compared to the control group (by 47-59%, Table 3.4).

3.3.4. Effects of 9-week treatment on PGE_2 levels in tumors

In the long-term study, I examined the effects of the different treatments on PGE_2 levels in the small intestinal tumors. Treatment with fish oil alone and in combination with EGCG decreased PGE_2 levels by 89% or 81%, respectively (Table 3.4).

3.4. Discussion

In Specific Aim 3, I studied the effect of EGCG and fish oil on intestinal tumorgenesis in the *Apc*$^{Min/+}$ model. I found that a combination of low doses of EGCG and fish oil can inhibit tumor formation and size in high-fat fed *Apc*$^{Min/+}$ mice, whereas the single agents did not produce a significant effect. The effects on decreasing tumor size can also be observed after only 3 weeks of treatment. To our knowledge, this is the first report to investigate the combination effects of EGCG and fish oil in an animal model of cancer.

Although previous studies have shown that both EGCG and fish oil alone can inhibit tumor multiplicity [150, 168, 169, 172], under our experimental conditions, neither single agent had a significant effect on tumor multiplicity. This may be partially due to the large standard deviation in tumor multiplicity in each group and the variability between experiments often associated with this model [150]. The former factor may have also affected our analysis on the interaction of EGCG and fish oil on tumor multiplicity.

β-catenin translocation from the cell membrane to the nucleus is a key event in colon carcinogenesis that results in increased transcription of genes involved with cell proliferation. My results show that β-catenin translocation from the cell membrane to nucleus is significantly reduced in adenomas of all treatment groups compared to the control group. My findings with the effects of EGCG on β-catenin localization is consistent with previous findings from our laboratory that show reduced nuclear protein

expression of β-catenin in adenomas from *Apc*$^{Min/+}$ mice treated with 0.32% EGCG in the drinking fluid [150]. To our knowledge, this is the first study to report decreased nuclear β-catenin localization of tumors in an animal model treated with fish oil.

I found that adenomas of EGCG- and fish oil-treated *Apc*$^{Min/+}$ mice show decreased Akt phosphorylation in comparison to adenomas of control mice. This is the first study to show the effect of fish oil on Akt activation in an *in vivo* model for colon cancer. Other studies in colon cancer cell lines and *in vivo* models have also shown the inhibitory effect of EGCG on Akt phosphorylation [150, 157, 232]. Recent studies by Schley *et al.* have shown that breast cancer cells incubated with EPA and DHA showed decreased Akt phosphorylation [233].

The observed decrease in phospho-Akt levels in adenomas of mice treated with fish oil and the combination may be related to the decrease in PGE_2 levels in the tumors of these mice. Previously Moran *et al.* have shown that PGE_2 can activate epidermal growth factor receptor (EGFR) in *Apc*$^{Min/+}$ small intestinal tissues, which can activate phosphoinositide-3-kinase (PI3K) and subsequently Akt [234]. It is possible that fish oil decreased the availability of arachidonic acid and PGE_2 levels in tumors, and this led to lower levels of Akt phosphorylation. Previous studies have shown that EGCG inhibits EGFR phosphorylation, which also results in reduced levels of Akt phosphorylation [157, 235]. EGCG has also been shown to inhibit Akt phosphorylation by a non-EGFR mediated pathway [236]. The decrease in Akt phosphorylation by EGCG, fish oil, and the combination may contribute to the observed increase in caspase-3 activation, since Akt phosphorylation has been shown to inhibit apoptosis [237, 238].

The studies conducted for Specific Aim 3 shows that a combination of low doses of EGCG and fish oil is effective at inhibiting intestinal tumorigenesis in the *Apc*$^{Min/+}$ mouse model. The combination treatment also decreased cell proliferation, PGE_2 formation, nuclear localization of β-catenin, and the level of phosphorylated Akt in the nucleus of the small intestinal tumors, as well as enhanced apoptosis. Most of the changes in these parameters were also caused by individual agents, except that cell proliferation was not significantly reduced by fish oil and PGE_2 levels were not significantly reduced by EGCG. All of these changes could contribute to the inhibition of tumorigenesis, but the present observed changes could not be quantitatively correlated with the tumor yield due to the high standard deviation in the tumor multiplicity.

3.5. Tables

Table 3.1. Diet composition of high-fat mixed lipid and high-fat fish oil diets

	High-Fat		High-fat fish oil	
Macronutrient	gram %	kcal %	gram %	kcal %
Protein	23.9	20.8	23.9	20.8
Carbohydrate	45.9	40.0	45.9	40.0
Fat	20.0	39.2	20.0	39.2
Total		100.0		100.0
Kcal/gram	4.6		4.6	
Ingredient	gram	kcal	gram	kcal
Casein	200.0	800.0	200.0	800.0
DL-Methionine	3.0	12.0	3.0	12.0
Corn starch	203.0	812.0	203.0	812.0
Maltodextrin	100.0	400.0	100.0	400.0
Dextrose	77.0	308.0	77.0	308.0
Cellulose	50.0	0.0	50.0	0.0
Corn oil	46.0	414.0	18.5	166.5
Fish oil[#]	0.0	0.0	102.0	918.0
Beef fat	27.2	244.8	11.0	99.0
Lard	17.0	153.0	6.8	61.2
Butter fat, anhydrous	20.4	183.6	8.0	72.0
Soybean oil	51.0	459.0	20.4	183.6
Peanut oil	8.5	76.5	3.4	30.6
Mineral mix*	35.0	0.0	35.0	0.0
Vitamin mix*	10.0	40.0	10.0	40.0
Choline Bitartrate	2.0	0.0	2.0	0.0
Total	850.2	3903.0	850.2	3903.0

*AIN-76A mineral and vitamin mixtures (American Institute of Nutrition 1977 and 1980)

[#] Fish oil contains 1.7% α-linolenic acid, 13% eicosapentaenoic acid (EPA), and 12% docosahexaenoic acid (DHA) [229].

Table 3.2. Effect of 0.16% EGCG and 12% fish oil on tumor multiplicity and size in *Apc*$^{Min/+}$ mice after treatment for 9 weeks*

Diet (n)	Small Intestine Tumor Number						
	Region			Size			Total
	Proximal	Middle	Distal	< 1 mm	1-2 mm	> 2 mm	
Control (15)	7.13±4.5	15.60±11.5	23.93±13.3^{a}	18.00±9.4	20.73±16.0	7.93±5.7^{a}	46.67±26.5^{a}
Control + 0.16% EGCG (15)	6.20±4.2	13.73±9.8	18.07±11.6^{ab}	21.33±12.6	12.53±12.5	4.13±3.3^{ab}	38.00±23.0^{ab}
Fish oil (15)	5.73±3.4	16.13±12.0	26.27±16.5^{a}	27.07±15.1	17.47±15.4	3.60±4.7^{b}	48.13±29.7^{a}
Fish oil + 0.16% EGCG (15)	3.60±207	8.20±10.7	10.13±9.1^{bc}	13.13±8.6	8.33±15.0	0.47±0.7^{b}	21.93±20.3^{b}

*Age 5 weeks at beginning of treatment. Data are presented as mean ± S.D. Different superscripted letters indicate statistical significance ($p<0.05$, one-way ANOVA)

Table 3.3. Effect of 0.16% EGCG and 12% fish oil on tumor multiplicity and size in *Apc*$^{Min/+}$ mice after treatment for 3 weeks*

Diet (n)	Small Intestine Tumor Number						
	Region			Size			Total
	Proximal	Middle	Distal	< 1 mm	1-2 mm	> 2 mm	
Control (9)	9.56±5.0	11.11±2.4	17.89±4.9	13.22±2.4	19.22±3.4	6.11±3.6[a]	38.56±5.1
Control + 0.16% EGCG (7)	9.29±3.8	9.14±5.5	16.71±8.4	15.43±8.5	17.29±9.9	2.43±1.9[ab]	35.14±12.7
Fish oil (9)	5.78±3.8	12.00±8.7	18.67±10.9	14.78±11.2	18.56±14.2	3.11±3.0[ab]	36.44±21.3
Fish oil + 0.16% EGCG (10)	7.22±4.6	18.33±9.8	19.67±12.6	23.44±11.1	19.44±8.5	2.33±2.2[b]	45.22±18.8

*Aged 12 weeks at beginning of treatment. Data are presented as mean ± S.D. Different superscripted letters indicate statistical significance ($p<0.05$, one-way ANOVA)

Table 3.4. Effect of 0.16% EGCG and 12% fish oil (9-week treatment) on cell proliferation, apoptosis, PGE_2 levels and nuclear staining positivity of β-catenin and phospho-Akt, in small intestinal tumors*

Treatment	Adenomas analyzed	Proliferation index (%)	Apoptotic index (%)	PGE_2 levels pg/μg protein	Nuclear positivity (%)	
					β-catenin	phospho-Akt
Control	20	65.1 ± 3.5^{a}	2.2 ± 0.4^{a}	28.29 ± 3.97^{a}	53.4 ± 5.1^{a}	78.7 ± 1.9^{a}
Control + 0.16% EGCG	16	24.2 ± 3.1^{c}	8.3 ± 2.8^{b}	20.98 ± 3.81^{ab}	29.5 ± 5.0^{b}	35.5 ± 5.9^{b}
Fish oil	15	52.3 ± 5.5^{ab}	8.3 ± 1.1^{b}	2.90 ± 0.49^{b}	20.0 ± 3.1^{b}	32.6 ± 6.4^{b}
Fish oil + 0.16% EGCG	15	39.1 ± 6.0^{bc}	9.7 ± 1.8^{b}	5.53 ± 2.03^{b}	19.9 ± 4.4^{b}	41.9 ± 4.8^{b}

*Proliferation activity was evaluated by immunohistochemical analysis using antibody against Ki-67 and apoptotic activity was determined by immunohistochemistry using antibody against cleaved caspase-3. Proliferation or apoptotic index or positivity of β-catenin and phospho-Akt is the number of positively stained cells expressed as a percentage in the total number of tumor cells counted. Different superscripted letters indicate statistical significance ($p<0.05$, one-way ANOVA).

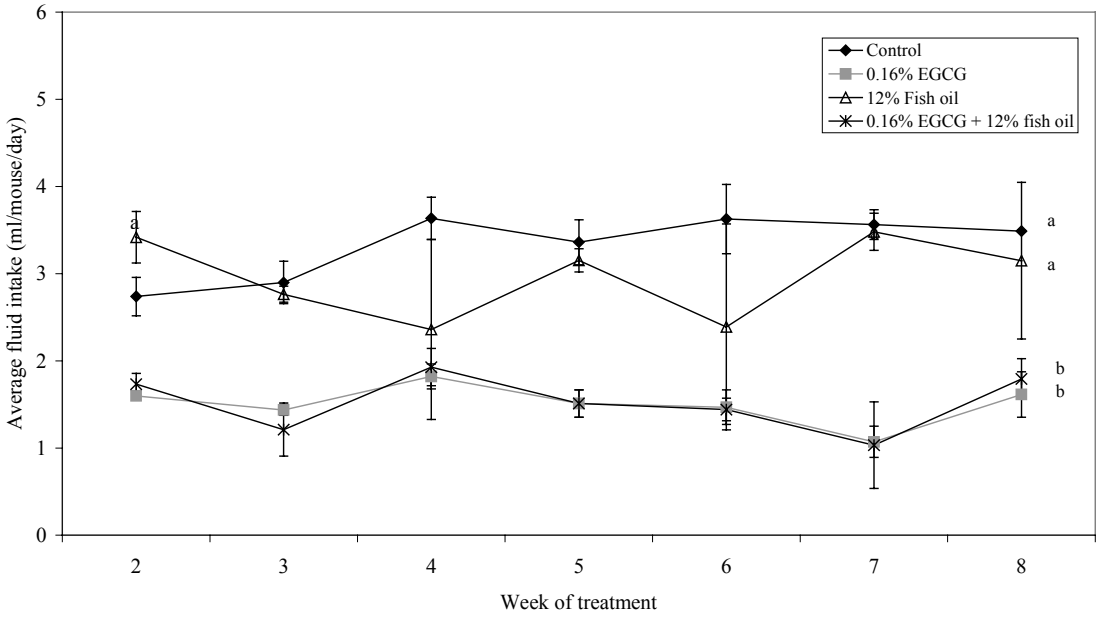
3.6. Figure legends and figures

Figure 3.1. Average body weight (A), food consumption (B), and fluid intake (C) in *Apc*$^{Min/+}$ mice treated with 0.16% EGCG, 12% fish oil, or a combination of agents. Body weight was measured weekly. Mice were sacrificed at 14 weeks of age. Data are presented as mean ± S.E. Different letters indicate statistical signficance at $p<0.05$ (Repeated measures ANOVA).

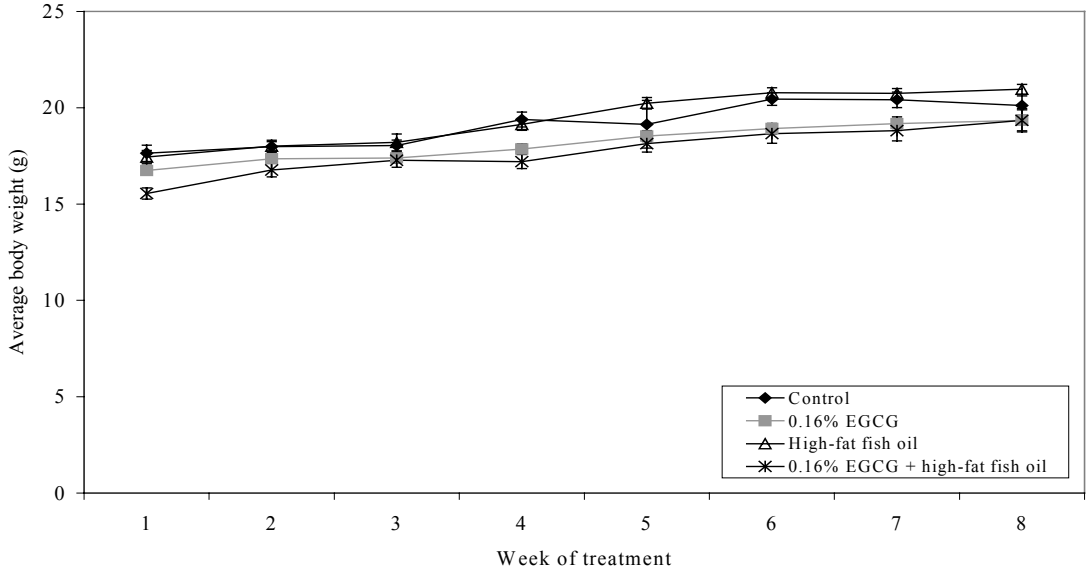
Figure 3.2. Effect of 0.16% EGCG and 12% fish oil on the proliferation (A), apoptosis (B), β-catenin (C) and phospho-Akt levels (D) in *Apc*$^{Min/+}$ mice. Strong Ki-67 nuclear staining is seen at the base of the normal crypts from control (A1) and treated mice (A2 and A3) and reduced Ki-67 staining level in adenomas from treated mice with EGCG (A2), fish oil (A3), and their combination (A4), in comparison to the control. Apoptosis is visualized by detecting cleaved caspase-3. Treatment with EGCG (B2), fish oil (B3), and the combination (B4) increased the number of apoptotic cells in the adenomas in comparison to the control (B1). Strong β-catenin membrane staining is observed in normal mucosa from both control (C1) and treated mice (C2, C3 and C4). Adenomas from untreated mice showed strong nuclear and cytoplasmic β-catenin staining (C1). Treatment with EGCG (C2), fish oil (C3), and the combination (C3) reduced nuclear staining and increased membrane staining. Treatment with EGCG (D2), fish oil (D3), and the combination (D4) reduced phospho-Akt staining in adenomas in comparison to the control (D1).

Figure 3.1

(A)


Control
0.16% EGCG
12% Fish oil
0.16% EGCG + 12% fish oil
Average fluid intake (ml/mouse/day)
Week of treatment
a
a
b
b

(B)


Control
0.16% EGCG
High-fat fish oil
0.16% EGCG + high-fat fish oil
Average body weight (g)
Week of treatment

(C)

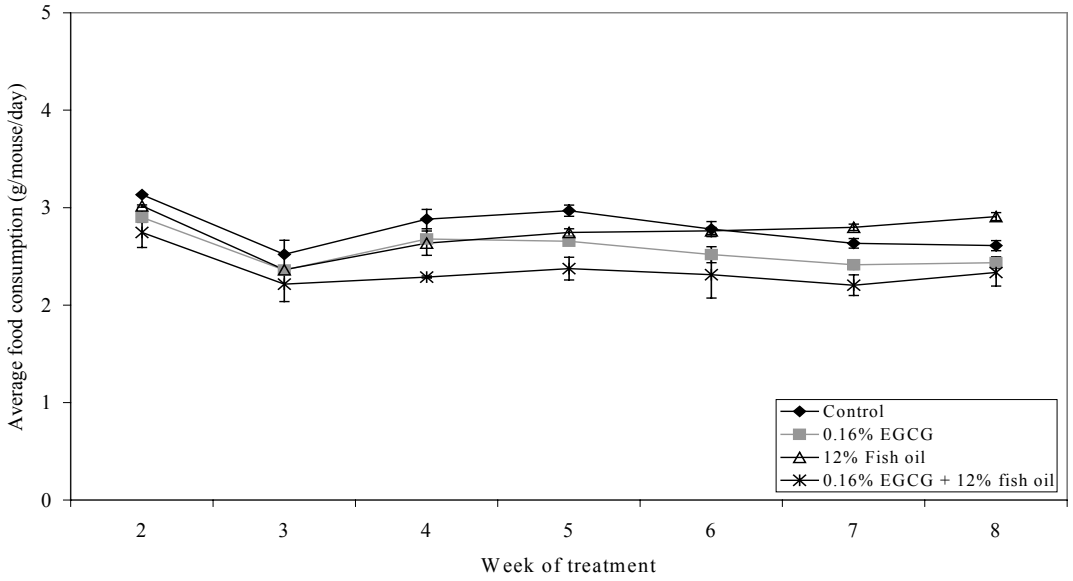
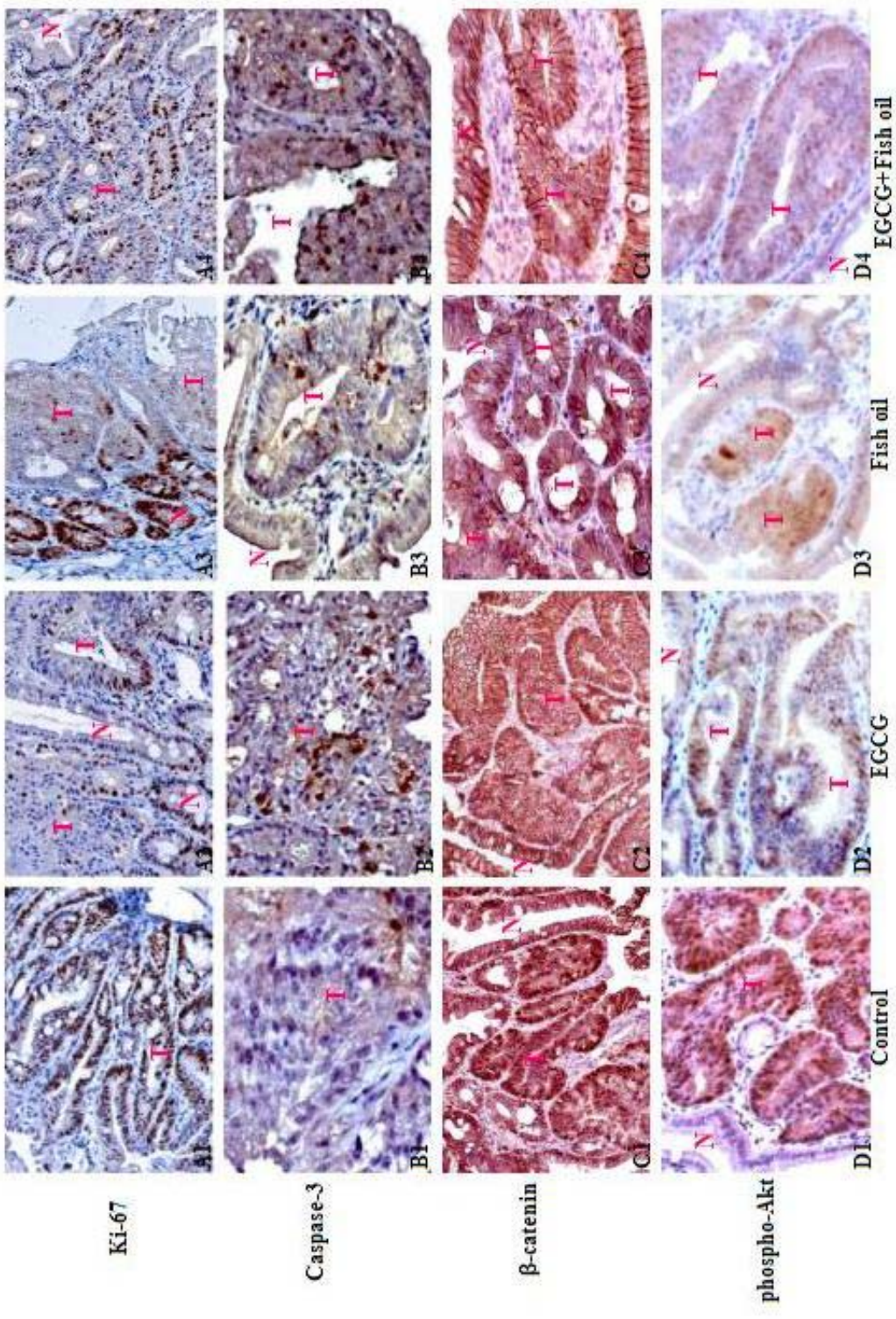

Average food consumption (g/mouse/day)
0
1
2
3
4
5
2
3
4
5
6
7
8
Week of treatment
Control
0.16% EGCG
12% Fish oil
0.16% EGCG + 12% fish oil

Figure 3.2


Ki-67
Caspase-3
β-catenin
phospho-Akt
Control
EGCG
Fish oil
EGCG+Fish oil
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
T
N

CHAPTER 4

GENERAL DISCUSSION

The results presented in Chapters 1 and 2 show that EGCG reduces high-fat diet-induced obesity and metabolic syndrome by decreasing weight gain, visceral fat accumulation, insulin resistance, hypercholesterolemia, and hepatic steatosis. These effects were observed with a dose of EGCG that is comparable to about 10-12 cups of green tea per day for humans. The results from Chapter 3 show that EGCG, in combination with fish oil, inhibits intestinal tumorigenesis in a transgenic mouse model. These effects were observed with a dose of EGCG that is comparable to about 4-5 cups of green tea per day for humans. Taken together, these studies suggest that dietary EGCG may be effective at reducing risk for chronic diseases that are associated with inflammation.

As described in Chapter 1, obesity and metabolic syndrome are characterized by increased inflammation and this inflammation may be the cause of the metabolic effects of obesity, including insulin resistance, increased risk for atherosclerosis, and hepatic steatosis. In Chapter 2, I also found that EGCG reduces plasma monocyte chemoattractant protein (MCP-1) levels, a marker of systemic inflammation. Inflammation also increases the risk for colon cancer. Several studies have reported a positive correlation between colon cancer risk and plasma C-reactive protein (CRP) levels in humans [239, 240]. A recent study found that white blood cell count was positively correlated with mortality due to colon cancer in a Korean cohort [241]. It is also widely accepted that inflammatory conditions such as ulcerative colitis, Crohn's disease and inflammatory bowel disease are positively associated with risk for colon

cancer [242]. There are several studies in rodent models of colitis that showed tea treatment was effective at reducing colitis-related pathologies and inflammatory markers in the colon [243-245]. Furthermore, studies reported a positive association between obesity and colon cancer in several cohorts [8, 10-12], similar to the association of obesity with increased risk for metabolic syndrome. However, I did not find that EGCG treatment affected the levels of the inflammatory prostaglandin E_2 (PGE_2) in adenomas of *Apc*$^{Min/+}$ mice (Chapter 3). Future studies are needed to determine if EGCG affects other markers of inflammation in intestinal tumorigenesis. It is possible that obesity-induced chronic inflammation increases the risk for colon cancer, and that EGCG reduces this risk by attenuating inflammation.

In contrast to EGCG alone, treatment with fish oil decreased PGE_2 levels in adenomas of *Apc*$^{Min/+}$ mice (Chapter 3), suggesting decreased inflammation caused by modulation of arachidonic acid metabolism. Fish oil and ω-3 polyunsaturated fatty acids (ω-3 PUFA) have shown to be anti-inflammatory. Several studies have shown that dietary fish and fish oil treatment resulted in weight loss, increased insulin sensitivity, and decreased risk factors for the metabolic syndrome in both humans and animal models [246-250]. Although we did not test the effects of fish oil in our high-fat mouse model for obesity, it would interesting to determine the effects of fish oil alone and in combination with EGCG on weight gain, fat gain, inflammation, and the metabolic syndrome in this model.

Although these studies suggest that EGCG may reduce risk for both obesity and symptoms associated with the metabolic syndrome, epidemiological studies are conflicting as to whether tea consumption affects body mass index (BMI) or

characteristics of the metabolic syndrome [28, 83, 251]. This variability in data may be due to a number of factors, including type of tea used, duration of the study diet of the population studied, and the genetic differences between the populations studied. Another factor that may play a role is the inter-individual variability in the bioavailability of tea constituents among study populations.

A study in our laboratory indiacted that when human subjects consumed a one-time dose of EGCG or tea polyphenols (equivalent to the catechin content of 2 cups of green tea), peak plasma levels of EGCG were < 1μM [252]. Similar results were found in a study by Chow *et al* [253]. This may be due to the metabolism and efflux of EGCG in the intestine and liver. One study in rats showed that EGCG undergoes first pass hepatic metabolism [254], although another study found that intestinal absorption and metabolism played a more important role in its bioavailability [255]. A study in intestinal epithelial cells showed that EGCG and ECG (50 μM) were rapidly absorbed into the cell; however, the accumulation of ECG was blunted by active efflux and poor transport to the basolateral membrane of the cell, suggesting low systemic bioavailability of catechins [256]. Studies conducted n our laboratory have also shown active efflux of EGCG in human colon cancer cells [257]. Such active transport mechanisms must be validated *in vivo.* The above-mentioned studies may be important in determining the relevance of various *in vitro* experiments investigating the mechanisms by which EGCG confers its health effects.

An individual's diet may affect the levels of EGCG in target tissues, and therefore determine whether or not consumption of EGCG will confer any health benefit. In our laboratory, we showed that the dietary constituent piperine enhance the bioavailability of

EGCG [182]. In addition, different constituents may also modulate the actions of EGCG. Westerterp-Plantenga *et al*. showed that green tea is effective in weight maintenance after body weight loss only in consumers who had low baseline levels of caffeine [36]. Lorenz *et al.* reported that addition of milk to tea prevents its cardiovascular benefits [258]. Future studies are necessary to confirm these effects of other dietary constituents on the health benefits of tea and EGCG.

One alternative to drinking several cups of green tea daily to achieve potential health benefits is consumption of green tea supplements. There are several green tea capsules, patches and extracts currently available on the market. These supplements can have catechin content equivalent to 30 cups of green tea. Although these supplements increase the dose of EGCG and other catechins delivered to the body compared to drinking tea, it is important to note that there are increasing reports that suggest supplements derived from dietary constituents may have toxic side effects if consumed in excessive quantities. Two studies published in 2005 showed that long-term consumption of Exolise, a green tea extract, caused hepatitis and liver toxicity in individuals that were taking the supplement for weight loss purposes [259, 260]. One of these case studies reported that the supplement was associated with liver failure, which required transplantation after taking the supplement [260]. Preliminary studies in our laboratory found that a large dose of EGCG (1500 mg/kg, *i.g.*) significantly increased plasma alanine aminotransferase (ALT) and MCP-1 levels in mice on a standard diet (in contrast to the studies in Chapter 2 with chronic dietary EGCG). In addition, this dose of EGCG (1500 mg/kg, *i.g.*) decreased survival in mice on a standard diet. These findings suggest that consumption of EGCG may have potential health benefits, but in large quantities,

green tea extracts may produced toxicity. More studies are required to determine the effective and safe doses of EGCG for various beneficial health effects.

In conclusion, dietary administration of EGCG is effective at preventing obesity and the metabolic syndrome, and in combination with fish oil, it inhibits intestinal tumorigenesis in mouse models. Well-designed clinical trials will be needed to determine whether these findings have application in the prevention of diseases in humans.

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Abstract of unpublished results

Bose, M., Chin, K-V., Liu, Y-Y., Park, S., Husain, A., Liao, J., Lambert, J.D., Ju, J., Vittal, R., Kopelovich, L., Huang, M-T., and C.S. Yang. Inhibition of colon carcinogenesis and modulation of gene expression by (-)-epigallocatechin-3-gallate and sulindac in azoxymethane-treated mice.

The purpose of this study was to investigate the inhibitory actions of (-)-epigallocatechin-3-gallate (EGCG) and sulindac against colon carcinogenesis in azoxymethane (AOM)-treated mice. EGCG and sulindac, administered after AOM treatment as 0.1% solution in the drinking fluid and in the diet at 200 ppm, respectively, for 32 weeks, decreased tumor incidence by 55% and 61%, respectively. Both agents also decreased tumor multiplicity in tumor bearing mice. Gene expression analysis using Affymetrix U74Av2 Genechip revealed several interesting alterations by the treatments. Heat shock protein (Hsp) 86, a chaperone for several oncogenic proteins, was elevated in both tumorous and non-tumorous colonic tissues in AOM-treated mice, but was suppressed in these tissues by treatment with EGCG and sulindac. A group of calcium-regulated genes (such as endothelial calcium/calmodulin-activated myosin light chain kinase and actin interacting protein) were induced by EGCG and sulindac in non-tumorous tissues. Distinct transcription profiles by both agents in tumor tissue samples were also observed. The gene encoding RhoC, a protein involved in cell motility and whose expression has been associated with the progression of several types of cancer, was down-regulated by 0.1% EGCG treatment in tumor samples. Some of these gene expression changes may be directly involved in the chemopreventive mechanisms of EGCG and sulindac.

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PUBLICATIONS

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