EFFECT OF DIETARY PRETREATMENT AND OBESITY ON \((-\)-EPIGALLOCATECHIN-3- GALLATE (EGCG) MEDIATED HEPATOTOXICITY AND THE UNDERLYING MECHANISM

A Dissertation in
Food Science
by
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Submitted in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

May 2016
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ABSTRACT

A growing body of evidence suggests the hepatotoxic potential of high doses of EGCG. *In vitro* several mechanisms of EGCG cytotoxicity have been examined; however potential mechanisms of in vivo toxicity remain understudied. Additionally factors that can potentiate EGCG induced hepatotoxicity, such as pre-existing physiological conditions that attenuate liver function have not been investigated. With the lack of data to support an association between dietary supplements and liver toxicity, I hypothesized that dietary pretreatment with EGCG markedly decreases EGCG bioavailability and can potentially mitigate the toxic potential of EGCG, and that ORFLD increases susceptibility to high dose EGCG-mediated hepatotoxicity. In addition to the effect of these factors on high dose EGCG hepatotoxicity, I further hypothesized that the underlying mechanism by which EGCG mediates hepatotoxicity is through induction of mitochondrial oxidative stress and increased mitochondrial dysfunction.

This study investigated the effect of dietary pretreatment with EGCG in male CF-1 mice. EGCG pretreated (EP) mice were pretreated with 3.2mg/kg EGCG in their diet for 2 weeks, a dietary dose that is equivalent to 500 mg/kg body weight. Non-pretreated (NP) and negative control (NC) mice were given a basal diet. NP mice showed increased levels of ALT and phosphorylated histone 2Ax compared to control mice. Pretreatment with EGCG reduced these elevated levels. NP mice showed reduced levels of glutathione, which was partially ameliorated in EP mice. In addition to blunting the markers of oxidative stress dietary pretreatment enhanced hepatic level of antioxidant genes and reduced plasma and tissue concentrations of unconjugated EGCG indicating that EGCG impacted its own bioavailability. In summary, these results show that dietary pretreatment with EGCG reduced the bioavailability and hepatotoxic potential of subsequent acute high dose oral bolus EGCG.
In another study we examined the effect of pre-existing obesity and ORFLD on EGCG-mediated hepatotoxicity in mice. EGCG treatment decreased survival rates of both lean and obese mice, but the effects were more pronounced in obese mice. EGCG caused increased liver injury as seen in elevated levels of liver ALT in all treatment groups. EGCG treatment increased DNA oxidative damage in both lean and obese mice. Markers of liver tissue damage assessed using histopathology showed that EGCG caused a dose dependent increase in tissue apoptosis, necrosis, hemorrhage, inflammation and hepatic lipidosis in both lean and obese mice. Lipid peroxidation increased following EGCG treatment, and depleted glutathione levels were observed. Obese mice exhibited significantly lower levels of glutathione compared to lean mice indicating elevated levels of oxidative stress caused by EGCG treatment. This study showed that EGCG treatment caused hepatotoxicity in lean and obese mice in a dose dependent manner, with the effects being more pronounced in obese mice.

Our third study examined the effects of EGCG on hepatic markers of antioxidant response and mitochondrial biogenesis/function in lean and obese mice. EGCG significantly reduced hepatic antioxidant capacity. Lean and obese mice showed reduced gene and protein expression, and activity of antioxidant enzymes. EGCG treatment significantly reduced mitochondrial superoxide dismutase (Sod2) mRNA levels in lean mice; as well as catalase mRNA levels in both lean and obese mice. Obese mice showed lower catalase activity and protein expression following EGCG treatment. A significant decrease in glutathione peroxidase 1 (Gpx1) protein expression was also seen. mRNA expression of glutathione transferase zeta 1 (Gstz1) and peroxiredoxin were significantly decreased by EGCG. In response to oxidative stress induced by EGCG, increases in gene expression of metallothionein transcription factor 1 (MTF-1) and metallothionein I and II were observed. Reduced antioxidant activity was a result of the impairment of antioxidant regulators by EGCG treatment. EGCG reduce mRNA levels of Sirtuin 3 (Sirt3), forkhead box O3 (Foxo3a) and nuclear factor (erythroid-derived 2)-like 2 (Nrf 2).
EGCG treatment reduced mitochondrial biogenesis by decreasing mRNA levels of transcription factor nuclear respiratory factor 1 (Nrf1), co-activator peroxisome proliferator-activator receptor coactivator-1a (Pgc-1α) and mtDNA copy number in both lean and obese mice. EGCG impaired mitochondrial function by reducing gene expression of mitochondrial transcription factors A, (Tfam), B1 (Tfb1m) and B2 (Tfb2m), as well as subunits of mitochondrial Complex I and mitochondrial complex III.

Overall, based on our results and observations, EGCG treatment induced hepatotoxicity in lean and obese mice in a dose dependent manner and it induced oxidative stress by inhibiting antioxidant response. Mitochondrial function was impaired based on reduced biogenesis and inhibition of complexes following EGCG treatment. However, EGCG was shown to mitigate its toxic effects by reducing its own bioavailability following chronic dietary treatment.
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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Dr. Lambert. I appreciate the guidance and support he gave me during my research tenure, particularly when I got stuck or was pessimistic about my results. He always pointed out the value of my work and helped me see the brighter side of what I was doing. Dr. Lambert maintained a positive attitude, was very approachable, and made himself readily available to discuss any issues that arose no matter how busy he was. I was fortunate to have an advisor with his background that was genuinely interested in my project’s findings, which bolstered my own interest in this project and provided ideas and direction for my future endeavors. I enjoyed his candor and views on research, science and life as an academic. My research would not have been possible without the funding I received, so I would also like to thank Dr. Lambert and the Department of Food Science for my assistantship and tuition.

A special thank you to my other committee members, Dr. Elias for his input on the chemistry aspects of my work and encouraging me to think about the chemical processes taking place and their significance to my project; Dr. Perdew for his thought provoking questions that ensured that I considered many perspectives and covered all my bases, and Dr. Vanamala for encouraging me to always look at the bigger picture and broader implications of my work. I am very grateful for their insight and approaches to problem solving; they helped me produce my best work.

I am thankful to my lab mates Shannon Glisan, Weslie Khoo, Mingyao Sun, Kuier Zhao, Ben Chrsifield, and Qiaoqiao Dai for their support, guidance, input, critiques, and discussions throughout my project and during my defense and seminar. Special thanks to Ben Chrsifield for his help with my experiments when my workload became overwhelming. Everyone played an important role in my journey.
I want to thank my friends in State College for being there for me, supporting me, helping me grow, recognize my strengths, being my family and making this one of the most enriching experiences of life. Last but not least my heart goes out to my family for their continued love and support, especially my dad to whom I dedicate all of this work and because of whom all of this was possible. Without him I would not be the woman I am today. His love and support was a source of motivation, strength and sustenance.
Chapter 1

Literature Review and Research Objectives

Green Tea Processing and Chemistry

Tea is derived from the processing and brewing of leaves from the *Camellia sinensis* plant. Drinking of the brew is a practice that has been around for centuries and valued for its medicinal properties. Tea consumption, which originated in China and Southeast Asia, is very high, and represents the second most popular beverage in the world [1][2]. Green tea is one of three different types of tea produced based on processing, and it is the most popular and widely studied for its health benefits. A typical cup of green tea (1.5 g in 150 mL water contains approximately 450 – 675 mg of water extractable solids. Many of these solids are polyphenolic compounds such as quercetin and myricitin [3]. Characteristic of green tea are catechins (Figure 1-1), which are polyphenols that make up 30 – 40 % of the water extractable solids [2]. (-)-Epigallocatechin-3-gallate (EGCG) is the most abundant and potent catechin found in green tea, making up approximately 50 % of catechins by weight [4].
Processing of tea leaves produces three types of tea based on the method; green tea, black tea and oolong tea. Green tea is processed in such a way as to minimize oxidation and retain the major components that are found in the fresh tea leaf. Quickly after plucking, green tea leaves are either steamed, pan fried, rolled or air dried at high temperature to inactivate polyphenol oxidase, the oxidizing enzyme [5]. As a result, green has been shown to have the highest amount of catechins (Graham 1992, Bhagwat et al. 2011). Black tea which is more commonly consumed and manufactured compared to green tea is processed in order facilitate enzymatic oxidation of the catechins in a process known as fermentation. Oxidation results in a decreased level of catechins, and yields theaflavins, which are catechin dimers, and polymers called thearubigins [5], [7]. Oolong tea processing allows for partial oxidation of tea leaves to occur and as a result it contains varying levels of catechins. Oolong tea is considered an intermediate between green and black tea and as such has components of both types of teas, but in different proportions. The
partial oxidation process also yields components that are not found in either green or black tea, for example, theasinensins (bisflavanols) [5].

Catechins have been widely studied for their antioxidant activities. The hydroxyl groups on the A and B rings of the catechins are at the center of the antioxidant activity. This antioxidant capacity is further increased by the presence of the gallate ester group (D-ring) as found in ECG and EGCG. Based on this EGCG has been shown to exhibit the highest antioxidant capability of the tea catechins [8].

The antioxidant capabilities of EGCG in biological systems have been extensively explored. EGCG functions as a chain-breaking antioxidant that can trap peroxyl radicals and interrupt detrimental reactions such as lipid peroxidation *in vitro* (Figure 1-2) (reviewed in [4]). EGCG has also been shown to be effective in scavenging reactive oxygen, nitrogen and chlorine species *in vitro* [4], [9]. EGCG antioxidant capacity extends to chelating transition metals, which participate in the Fenton reaction and can initiate lipid peroxidation [4], [8], [10]. The relevance of these direct antioxidant effects of EGCG *in vivo* remain unclear.

![Figure 1-2. Redox Activity of EGCG adapted from Yang et al [11]](image)
Recent studies have demonstrated that EGCG has the propensity to exhibit pro-oxidant activities as well, and this has been implicated in its anti-cancer capabilities (reviewed in [9]). In a review by Lambert and Elias polyphenols such as EGCG were shown to undergo oxidation and produce ROS in a process known as auto-oxidation, summarized in Figure 1-2.

Figure 1-2 shows EGCG reacting with molecular oxygen to produce the superoxide anion and an EGCG semiquinone radical. The superoxide anion produced can further react with EGCG to produce more EGCG semiquinone radicals and hydrogen peroxide (H$_2$O$_2$). The semiquinone radical is very reactive and it can either react with EGCG to form reactive dimers or produce a more stable quinone. Transition metals such as copper and iron are also able to initiate catechin oxidation and can catalyze EGCG auto-oxidation. This contributes to the in vitro and in vivo pro-oxidant effects of EGCG [4].

**Putative Health Benefits of Green Tea and EGCG**

Green tea and EGCG have been extensively studied for their numerous health benefits. Numerous studies have demonstrated the effects of green tea catechins in treatment and prevention of a host of diseases. They have been shown to have significant chemopreventative effects, play a role in the treatment of cardiovascular disease, diabetes, arthritis, and neurodegenerative disorders; and exhibit anti-inflammatory and anti-obesity effects [3], [12]. Many of those health benefits have been attributed to the antioxidant capabilities of EGCG. Recent studies have shown that the pro-oxidant activities are very important as well, particularly in the treatment of cancer [4], [9].
Antioxidant Activity of EGCG

The antioxidant capabilities of EGCG have been explained by its ability to scavenge ROS, chelate transition metal ions, inhibit redox-sensitive transcription factors, inhibit “pro-oxidant” enzymes and induce phase II detoxification enzymes [12]. Several studies and reviews have shown the antioxidant effects of EGCG in vitro and to a lesser extent in vivo [4], [9], [12].

For example, Nakagawa et al showed the direct scavenging of nitric oxide and superoxide by green tea catechins in in vitro systems generating these reactive oxygen species [13]. One study using 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) to generate radicals in renal epithelial cells showed that green tea catechins protected the cells from oxidative damage with EGCG showing the highest antioxidant activity [14]. Kumamoto et al showed the metal chelating abilities of EGCG when its antioxidant capacity was measured in the presence of 13 metals. EGCG was shown to have the highest antioxidant activity against copper (II) ions [15]. A study by Khan et al showed mice fed 0.2% (w/v) GTP for 30 days had significantly increased activities of glutathione peroxidase, catalase, and quinone reductase in small bowel, liver, and lungs, and glutathione S-transferase in small bowel and liver. Additionally there was considerable enhancement of glutathione reductase activity in the liver [16].

Pro-oxidant Activity of EGCG

Depending on experimental conditions, green tea polyphenols undergo auto-oxidation and generate ROS. For example, the addition of 50 μM EGCG to cell culture media caused the production of hydrogen peroxide in time dependent manner to reach a maximum of 25 μM after 120 minutes [17]. The pro-oxidant activity of EGCG has been widely studied, particularly its role in cancer prevention. Lambert and Elias, and Forester and Lambert have conducted reviews
detailing the pro-oxidant activities of green tea polyphenols as it pertains to cancer prevention [4], [9]. In vitro, EGCG was shown to induce apoptosis and inhibit cell proliferation in various cell lines, as well as affect key cell signaling pathways that induce EGCG-mediated oxidative stress [4]. Tao et al showed that preceding apoptosis, EGCG induced mitochondrial ROS and mitochondrial dysfunction in oral cancer cells. SCC-25 cancer cells treated with 0 – 200 µM EGCG for 0 – 6 hours resulted in formation of extracellular reactive oxygen species (ROS) and increased the production of mitochondrial hydrogen peroxide in SCC-25 cells (0–6 h) before the induction of apoptosis. Subsequent opening of the mitochondrial transition pore and a decrease in mitochondrial membrane potential were observed. Additionally, EGCG down regulated expression of genes related to antioxidant defense including superoxide dismutase 2/3 and thioredoxin reductase 2 [18].

The pro-oxidant activity of EGCG in vivo has not been widely studied. One study by Li et al showed that oral administration of EGCG to H1299 human lung cancer xenograft-bearing nu/nu mice dose-dependently inhibited tumor growth and induced tumor cell apoptosis. EGCG treatment resulted in tumor cell-specific increases in 8-hydroxy-2-deoxyguanosine (8-OHdG) and phosphorylated histone 2A variant X (γ-H2AX) [19]. Lambert et al found that salivary H₂O₂ levels increased when healthy subjects either held tea solution (0.1–0.6%) in the oral cavity or chewed green tea leaves. This study provides some support for the pro-oxidant activity of green tea polyphenols [20]. These data suggest that pro-oxidant activity of polyphenols is impacted by concentration and experimental conditions. Further studies are warranted to identify mechanisms of pro-oxidant activity in vivo.
Anti-Obesity Properties

The anti-obesity properties of green tea and EGCG have also been extensively studied (reviewed in [21]. EGCG was shown to stimulate BAT thermogenesis, reduce adipocyte differentiation and proliferation; lipogenesis, fat absorption, plasma levels of triglycerides, free fatty acids, and increase beta-oxidation in in both cell culture and animal models of obesity [21].

High-fat fed C57BL/6J mice supplemented with 0.2-0.5% tea catechin in the diet showed reduced body fat (epididymal, retroperitoneal, perirenal fat) as well as a significant reduction of high-fat diet-induced body weight gain, visceral and liver fat accumulation [22], [23]. Green tea reduced body fat accumulation in male Sprague–Dawley rats a high-fat diet for 14 days and increased in brown adipose tissue thermogenesis through adrenoceptor activation [24]. High-fat fed C57BL/6J mice treated with 0.32% dietary EGCG for 16 weeks saw a reduction in body weight gain, percent body fat, and visceral tissue weight compared to high fat-fed controls [25]. Additionally, EGCG was shown to decrease liver weight, liver triglycerides, and plasma alanine aminotransferase concentrations in high-fat fed mice. C57BL/6J mice on a high fat diet supplemented with 1% w/w TEAVIGO (90% EGCG) for 5 months showed lower body weight gain and reduced levels subcutaneous and epididymal adipose tissue [26]. In the same study, obese Sprague-Dawley rats treated with 1% w/w TEAVIGO (90% EGCG) for 1 month showed a reversal of obesity. In other study, the beneficial effects of EGCG on high-fat/Western-style diet-induced obesity in C57BL/6J mice were examined. Treatment with 0.32% EGCG for 17 weeks significantly lowered body weight gain weight of brown adipose tissue, total visceral adipose tissue, levels of plasma cholesterol and plasma alanine aminotransferase compared to high-fat/Western-style-fed mice [27].

EGCG has been shown to affect fat metabolizing enzymes and effect changes in expression of genes related to fat metabolism and oxidation. Changes in these factors represent
the mechanism by which EGCG causes the changes seen in body weight. A recent publication using animal models showed that EGCG reduces body weight gain in high fat-fed mice by inhibiting pancreatic lipase which is essential for the breakdown and absorption of fats in the intestines [28]. Another study using high fat-fed C57bl/6J mice treated with 0.32% dietary EGCG for 16 weeks observed that EGCG can increase the expression of genes related to lipid oxidation, such as nuclear respiratory factor (nrf)1, medium chain acyl coA decarboxylase (mcad), uncoupling protein (ucp)3, and peroxisome proliferator responsive element (ppar)α. The authors also found that EGCG treated mice had reduced body weight gain and final body weight compared to high fat-fed controls. EGCG-treatment decreased fasting blood glucose, plasma insulin, and insulin resistance by 18.5%, 25.3%, and 33.9%, respectively [29]. New Zealand black mice fed a high-fat diet supplemented with (0.5 and 1%) EGCG purified from green tea (TEAVIGO) showed a dose-dependent decrease of body fat accumulation, reduction of gene expression of stearoyl-CoA desaturase-1 (SCD1), malic enzyme (ME), and glucokinase GK and an increase in UCP2. These data support a decrease in lipogenesis and an increase in fat oxidation by EGCG treatment [30]. Changes in expression of genes related to fat metabolism and fatty acid oxidation were measured in Male C57BL/6J mice fed a high fat diet for 8 weeks then supplemented with 0, 0.2 or 0.5% EGCG for another 8 weeks. EGCG treatment reduced body weight, and plasma triglyceride and liver lipid levels. Additionally, EGCG significantly reduced mRNA levels of as peroxisome proliferator-activated receptor-γ (PPAR-γ), CCAAT enhancer-binding protein-α (C/EBP-α), regulatory element-binding protein-1c (SREBP-1c), adipocyte fatty acid-binding protein (aP2), lipoprotein lipase (LPL) and fatty acid synthase (FAS), and increased mRNA levels of carnitine palmitoyl transferase-1 (CPT-1) and uncoupling protein 2 (UCP2) [31].

In many cases of obesity the progression of hepatic steatosis or obesity related fatty liver disease (ORFLD) is seen. A number of studies have reported the effect of green tea polyphenols
on ORFLD in animal models. Sae-Tan *et al.* showed that EGCG reduced liver injury marker, alanine aminotransferase (ALT) in high fat fed mice [32]. Murase *et al.* showed that 0.5% green tea extract in the diet for 11 months significantly decreased liver lipid accumulation caused by a high-fat diet in C57BL/6J mice [23]. Bose *et al.* have also shown that in C57BL/6J mice fed high fat diets supplement with 0.32% EGCG for 16 weeks, EGCG was able to ameliorate high fat-diет induced ORFLD [25].

**Anti-Inflammatory Properties**

Inflammation is an important immune response to trauma and infection and aids in the host’s recovery process. Chronic inflammation is cause for concern as it can result in DNA damage, cell death, and cancer promotion [33]. Similar to studies demonstrating the anti-carcinogenic effects of green tea polyphenols, laboratory studies have generally supported anti-inflammatory effects of green tea polyphenols. EGCG has been shown to exert its inflammatory effects by inhibiting inducible nitric oxide synthase (iNOS) gene expression and enzyme activity; protecting against neutrophil-mediated inflammation induced by topical application of 12-O-tetra-decanoylphorbol-13-acetate, in the ears of SENCAR mice; inhibiting infiltration of leukocytes and markers of oxidative stress (H_{2}O_{2} and NO) in the skin of UVB-treated C3H/HeN mice following topical application; and preventing infiltration of macrophages and neutrophils on UVB-irradiated human skin, also following topical application [34]–[37]. Kawai *et al.* in a recent study showed that EGCG was able to induce apoptosis in monocytes. Monocytes were isolated from blood incubated without or with catechin; Epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and EGCG. Apoptosis was evaluated by annexin V and propidium iodide double-staining or terminal deoxynucleotidyl assay. Of the four catechins evaluated, EGCG was the only one to induce apoptosis [38].
In another study, EGCG was shown to have an inhibitory effect on T-cell–mediated inflammation. CD8+ T cells incubated without or with catechin; epicatechin gallate (ECG), and EGCG, were assessed for changes in the surface expression of integrin molecules and the direct binding of catechin to CD11b molecule. Also, the effect of catechin on the ability of CD8+ T cells to bind intracellular adhesion molecule 1 and to migrate in response to chemokines was evaluated. Both catechins bound directly to CD11b expressed on CD8+ T cells, and caused a decrease of CD11b expression, but EGCG was more prominent. This resulted in decreased ability of CD8+ T cells to adhere intercellular adhesion molecule 1, and consequently decreased migration in response to chemokines [38].

In addition to its anti-inflammatory effects on monocytes and T-cells, EGCG has been shown to inhibit neutrophil elastase and apoptosis of neutrophils. Additionally EGCG inhibited chemokine-induced neutrophil chemotaxis in vitro, blocked neutrophil-mediated angiogenesis in vivo, and enhanced resolution in a pulmonary inflammation model, significantly reducing consequent fibrosis [39].

**Anti-Diabetes Properties**

Insulin resistance, like obesity is a symptom of metabolic syndrome, which has been shown to increase the risk of diseases such as cardiovascular disease, diabetes and cancer [40]. The International Diabetes Federation defines metabolic syndrome as having central adiposity based on waist circumference (> 102 cm in men and > 88 cm in women) and two or more of the following factors: elevated serum of triglycerides, dysglycemia, elevated blood pressure, and reduced concentration of high-density lipoprotein (HDL) associated cholesterol [41]. A comorbidity of metabolic syndrome is ORFLD. Data from clinical, experimental and epidemiological studies indicate that ORFLD may be the hepatic manifestation of metabolic
syndrome [42]. Insulin resistance is a key pathogenic factor in the development of type II diabetes and has been shown to be associated with obesity [29].

Many laboratory, epidemiological and intervention studies have shown the role of green tea polyphenols in the treatment and prevention of symptoms of metabolic syndrome (reviewed in [43]). In the previous section we discussed the anti-obesity effects of green tea polyphenols. Here we look at the role of green tea polyphenols in prevention and treatment of insulin resistance and type II diabetes. Several studies have examined the effects of green tea polyphenols in type I and type II diabetes in animal model. Sae-Tan et al conducted an extensive review of these findings, and their data was summarized as seen in Table 1-1.

**Table 1-1. Effects of green tea polyphenols on markers of diabetes**¹. [29]

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Type</th>
<th>Dose</th>
<th>Duration</th>
<th>Models</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood/plasma glucose</td>
<td>GTE</td>
<td>0.5% in fluid</td>
<td>4 wk</td>
<td>STZ-treated rats</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>GTE</td>
<td>2.0% in fluid</td>
<td>4 wk</td>
<td>STZ-treated rats</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>GTE</td>
<td>1.25% in diet</td>
<td>3 mo</td>
<td>STZ-treated rats</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>GTE</td>
<td>0.5% in fluid</td>
<td>4.6 wk</td>
<td>SDR (non-diabetic models)</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>GTC</td>
<td>200 mg/kg</td>
<td>6 wk</td>
<td>Wistar rats</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>EC</td>
<td>30 mg/kg (bid)</td>
<td>0 d</td>
<td>STZ-treated rats</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>EGC</td>
<td>0.25 – 1% in diet</td>
<td>7 wk</td>
<td>ZDF rats</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>EGC</td>
<td>0.5% in diet</td>
<td>11 mo</td>
<td>C57BL/6J mice</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>EGC</td>
<td>25 mg/kg (bid)</td>
<td>8 wk</td>
<td>STZ-treated Wistar</td>
<td>↓</td>
</tr>
<tr>
<td>Plasma insulin</td>
<td>GTE</td>
<td>0.5% in fluid</td>
<td>4.6 wk</td>
<td>SDR (non-diabetic models)</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>GTC</td>
<td>200 mg/kg</td>
<td>6 wk</td>
<td>Wistar rats</td>
<td>↓</td>
</tr>
<tr>
<td>Insulin sensitivity &amp;Glucose tolerance</td>
<td>GTE</td>
<td>80 mg kg</td>
<td>12 wk</td>
<td>Beagle dogs</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>GTE</td>
<td>0.5% in fluid</td>
<td>4–12 wk</td>
<td>SDR (non-diabetic models)</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>GTE</td>
<td>0.5 – 2.0% in fluid</td>
<td>4 wk</td>
<td>STZ-treated rats</td>
<td>improved</td>
</tr>
<tr>
<td></td>
<td>EGC</td>
<td>0.2 – 1% in diet</td>
<td>7 wk</td>
<td>db/db mice</td>
<td>improved</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>GTE</td>
<td>80 mg kg</td>
<td>12 wk</td>
<td>Beagle dogs</td>
<td>↓</td>
</tr>
</tbody>
</table>
These data suggest that green tea polyphenols are capable of improving symptoms of insulin resistance and diabetes.

**Dietary supplements**

With all these seemingly beneficial effects of EGCG in cancer prevention and obesity prevention shown mostly in animal models and cell lines, as well as its consumption as beverage with no adverse effect, green tea and EGCG have been considered safe. Studies show that the oral bioavailability of EGCG in humans is low compared to *in vitro* systems, and as such it has no serious adverse effects when consumed in low doses, for example in green tea beverage [44]. The relative safety of EGCG and its anti-obesity properties have in recent years lead to the translation of green tea and EGCG into dietary supplements, for a variety of indications, particularly for their potential to manage body weight [45].

US Food and Drug administration data shows that dietary supplements, including vitamins, were consumed by 158 million Americans in the year 2000 compared with a previous 1997 survey that showed that alternative medical therapies, principally herbals, were used by 83 million people. The sales of dietary supplements in the United States doubled after passage of the Dietary Supplement Health and Education Act (DSHEA) in 1994, to $17.1 billion in 2000, and are anticipated to continue increasing by 10% per year [46]. According to the Nutrition Business Journal, dietary supplement sales have reached $36 billion as of 2014 and projected to continue to increase [47]. Regulation of dietary supplements is governed by the U.S. Food and Drug Administration (FDA). According to DSHEA, a dietary supplement is a product taken by mouth that contains a "dietary ingredient" intended to supplement the diet. The "dietary ingredients" in
these products may include: vitamins, minerals, herbs or other botanicals, amino acids, and substances such as enzymes, organ tissues, glandulars, and metabolites. Dietary supplements are not as strictly regulated as drugs in that Under DSHEA, a firm is responsible for determining that the dietary supplements it manufactures or distributes are safe and that any representations or claims made about them are substantiated by adequate evidence to show that they are not false or misleading. This means that dietary supplements do not need approval from FDA before they are marketed [48]. This aspect can give rise to many inconsistencies in the products found on the market, including variations in the concentrations of the different products and raises issues regarding the safety of these products. By law, manufacturers may make three types of claims for their dietary supplement products: health claims, structure/function claims, and nutrient content claims. If a dietary supplement label includes a claim other than those mentioned, it must state in a "disclaimer" that FDA has not evaluated this claim. The disclaimer must also state that this product is not intended to "diagnose, treat, cure or prevent any disease," because only a drug can legally make such a claim [48]. This often leads to manufacturers making claims the do not necessarily have sufficient scientific evidence to support them.

Green tea based dietary supplements are especially popular for weight loss. For example, Sales of green tea-based dietary supplements in the US totaled (USD) 5.6 million in 2005, an increase of 94% from 2004 [49]. Green tea supplements can contain 0.4–8 mmol EGCG with a recommend dosing of 1–2 capsules up to 3 times/d. giving rise to a total recommended dose that may be up to 5.2 mmol/d. Green tea is commonly consumed as a beverage, and a typical cup of green tea contains about 0.4 mmol EGCG [50]. Whereas no reported adverse effects associated with normal green tea beverage consumption, green tea-based dietary supplements represent a different dosage form and have the potential to deliver a much higher dose of catechins than green tea beverages. For example Lambert et al showed that treatment of mice with a single oral bolus dose of 1.1 mg/kg EGCG result in peak plasma concentrations of 2.0 mM, whereas the
same total daily dose given via the diet resulted in plasma concentrations of 0.5 mM [50]. This shows the stark difference in dosage forms and their potential implications in the safety of dietary supplements, which I discuss further in the next section.

**Impact of dosage form on bioavailability**

 Whereas historical exposure to EGCG has been in the form of a beverage, these dietary supplements are generally in the form of capsules or pills. The safety of these alternative formulations is generally based on the historical safety of green tea beverage. However, when compared to green tea beverages, green tea based dietary supplements can deliver between 700 and 2100 mg of green tea polyphenols based on recommended dosing. These concentrations are several times higher than what is found in a cup of tea, 240-320 mg of catechins, thus supplements can deliver a higher dose of green tea catechins [4], [50]. Equally as important to consider is the dosage form. Previous studies have shown that oral bolus dosing, such as capsules results in greatly increased peak plasma concentrations of EGCG compared with dietary administration of the same total daily dose [51]. This data suggests that the dosing form impacts the bioavailability of the polyphenols. In this section I will discuss the metabolism and bioavailability of EGCG

The metabolic pathways of green tea catechins have been shown to be methylation, glucuronidation, sulfation and ring fission metabolism [8]. EGCG is methylated by catechol-O-methyltransferase (COMT) to form 4″-O-methyl-(−)-EGCG and 4′,4″-O-dimethyl-(−)-EGCG and was found to be time- and concentration-dependently sulfated in human, mouse and rat liver. But, EGCG-4″-O-glucuronide is the major metabolite formed in human, mouse and rat microsomes [8], [52]. Following i.p. or i.g. administration of EGCG mouse urine have shown that methylated EGCG (or glucuronidated or sulfated EGCG) can be further glucuronidated and/or sulfated (or
methylated) to form related mixed EGCG metabolites [8]. In addition to these conjugation reactions, the tea catechins undergo metabolism in the gut to form the ring fission products 5-(3’,4’,5’-trihydroxyphenyl)-γ-valerolactone (M4) and 5-(3’,4’-dihydroxyphenyl)-γ-valerolactone (M6). These products were present in human urine and plasma approximately 13 hours following oral ingestion of 20 mg/kg decaffeinated green tea [8]. The extensive biotransformation of tea catechins results in low systemic bioavailability. Figure 1-3 summarizes the biotransformation of tea catechins.

Figure 1-3. Biotransformation of the green tea catechins. [53]

4’-MeEGC, 4’-O-methyl-(-)-epigallocatechin; 4’,4’’-di-O-methyl EGCG, 4’,4’’-di-O-methyl-(-)-epigallocatechin-3-gallate; COMT, catechol-O-methyltransferase; EGC, (-)-epigallocatechin; EGCG, (-)-epigallocatechin-3-gallate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase
Studies have found that the oral bioavailability of EGCG and other green tea catechins in humans is low [44]. For example, a study conducted by Nakagawa et al in which human volunteers were given 3, 5 or 7 capsules of green tea corresponding to 225, 375 and 525 mg of EGCG showed that 90 minutes after ingestion plasma levels of free EGCG concentrations increased to 300, 1970 and 2020 ng/ml in the subjects who received 3, 5 and 7 capsules respectively [54]. Chow et al showed that the plasma levels seen in the previous study by Nakagawa et al were higher than levels obtain in their studies, although not high enough to exert any potential toxicity. In their study healthy subjects were randomly assigned doses of 200, 400, 600, and 800 mg based on EGCG content. Analysis of blood and urine samples 24 hours after oral administration showed plasma levels of EGCG observed were 73.7, 111.8, 169.1 and 438.5 ng/mL after 200, 400, 600, and 800 mg dose of EGCG respectively [44], [55]. Further research by Chow et al investigated the effect of dosing conditions on green tea catechin availability, and found that oral bioavailability of EGCG in humans increased under fasting conditions, when compared to fed conditions. Fasting subjects given 400, 800 and 1200 mg of EGCG showed 450, 420, and 270 %, respectively increase in plasma concentrations of free catechins when compared to fed subjects. [44]

Some studies show that mice and humans have a greater ability to glucuronidate EGCG compared to rats and are thus may represent a better animal model for EGCG biotransformation in humans compared to rats. Lambert et al examined EGCG bioavailability in mice following intravenous and intragastric administration. Plasma and urine levels of EGCG were analyzed. It was found that bioavailability of EGCG after intragastric administration was 12.4-25.6%. Plasma levels of EGCG after intravenous and intragastric administration were 2.7 and 0.28 µmol/L respectively. Results showed that EGCG was present in the plasma mainly in the conjugated form after intragastric administration. This high level of conjugated EGCG and not free EGCG was
attributed to the fact that intragastric administration results in the EGCG entering the small intestines and liver prior to reaching the plasma. In these organs enzyme activity results in conjugation of EGCG. In intravenous administration EGCG reached all the tissues at the same time. As a result EGCG levels in various organs such as the lung, liver, small intestines, colon and spleen was dependent on method of EGCG administration. It was concluded from this study that mice and humans have similar EGCG bioavailability compared to rats and that EGCG bioavailability is impacted most by glucuronidation of EGCG. This is consistent with research that has shown that EGCG-4''-O-glucuronide is the major formed in human, mouse and rat microsomes and also takes into highlight species differences in EGCG biotransformation [56]. Based on these studies, and a review by Yashin et al it appears that EGCG bioavailability is greatly impacted by the method of EGCG administration, and that bioavailability is increased in this order, oral < intragastric < intravenous [57]. Figure 1-4 illustrates peak levels of EGCG following route of administration.
Figure 1-4. Tissue concentration of EGCG following oral and intragastric administration
The red peak represents gavage administration and the blue peak represents dietary administration.

Figure 1-4 shows that following intragastric administration tissue concentrations are significantly higher when compared to oral administration. This suggests that intragastric administration has the potential to cause concentrations to increase pass a safe level thus becoming unsafe or toxic. Whereas oral administration, especially dietary administration does not increase concentrations nearly as high mainly because dietary administration occurs in increments, allowing the concentration to fluctuate. In a study by Henning et al thirty healthy subjects were randomly assigned to 3 different sequences of green tea, black tea, or a green tea extract supplement in a 3 x 3 crossover design with a 1-wk washout period. They showed that catechin absorption was enhanced when tea polyphenols were administered as a green tea supplement in capsule form and led to a small but significant increase in plasma antioxidant activity compared with when tea polyphenols were consumed as black tea or green tea [58].
Understanding the mechanisms behind EGCG bioavailability will prove useful in understanding the potential for EGCG toxicity.

Potential Toxic Effects of EGCG and Green Tea Extracts

A growing number of human cases have been reported citing adverse effects, mainly liver toxicity associated with consumption of dietary supplements containing high levels of EGCG and green tea extract. As previously stated, EGCG has the highest antioxidant potential among green tea catechins because of the presence of the gallate ester, which increases the number of hydroxyl groups in the compound; a moiety that is at the center of the antioxidant activity of catechins [8]. These functional groups in addition to increasing antioxidant capacity also increase the pro-oxidant capabilities of EGCG and thus its overall redox activity. Due to this high redox activity of EGCG, it has the greatest potential to cause toxicity when compared to other green tea catechins. In addition to published cases of liver toxicity in humans, laboratory studies have reported toxicity associated with administration of high doses EGCG in animal models. In the following sections I will discuss data supporting EGCG mediate hepatotoxicity with a focus on laboratory studies as well as human studies.

Human studies/Case Reports

Human studies for evaluating EGCG toxicity are very limited. Hepatotoxicity has not been widely observed in controlled human intervention studies [59]. Many of the studies done do not provide consistent findings. This indicates that there is a need to better understand the factors which determine sensitivity to EGCG-induced hepatotoxicity, and the mechanism by which it
occurs. With a large number of green tea based dietary supplements available on the market, and more being produced, many of which contain high levels of EGCG (as high as 90%), the risk for EGCG-mediated hepatotoxicity is a major cause for concern. Case reports are the common source of data, but they do not provide conclusive evidence for hepatotoxicity from green tea. In a review completed by Mazzanti et al 34 reported cases of liver toxicity believed to be associated with green tea were reviewed Table 1-2 [modified from [45]].

**Table 1-2.** Case reports of hepatotoxicity with green tea-based dietary supplements. [45]

<table>
<thead>
<tr>
<th>Sex, age (years)</th>
<th>Herbal product composition (Brand name)</th>
<th>Time (duration of treatment in weeks)</th>
<th>Evidence of Toxicity</th>
<th>Dechallenge/rechallenge</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>f, 81</td>
<td>Green tea dry aqueous extract (90% EGCG)</td>
<td>4</td>
<td>ALT-1996/2368 HEP</td>
<td>+/-No</td>
<td>[45]</td>
</tr>
<tr>
<td>f, 72</td>
<td>Green tea extract; (Hydroxycut)</td>
<td>12</td>
<td>ALT&gt;700 HEP Cholestatic hepatitis</td>
<td>+/-No</td>
<td></td>
</tr>
<tr>
<td>m, 28</td>
<td>Green tea extract; (Hydroxycut)</td>
<td>12</td>
<td>ALT-1049/2272 HEP</td>
<td>+/-NR</td>
<td>Shim et al. 2008</td>
</tr>
<tr>
<td>Sex, age (years)</td>
<td>Herbal product composition (Brand name)</td>
<td>Time (duration of treatment in weeks)</td>
<td>Evidence of Toxicity</td>
<td>Dechallenge/rechallenge</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------------------------------</td>
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<td>----------------------</td>
<td>------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>f, 60</td>
<td></td>
<td>8</td>
<td>62N/73N MIX</td>
<td>+/NR</td>
<td></td>
</tr>
<tr>
<td>f, 56</td>
<td></td>
<td>12</td>
<td>8N/41N MIX</td>
<td>+/NR</td>
<td></td>
</tr>
<tr>
<td>f, 64</td>
<td></td>
<td>5</td>
<td>77N/89N HEP</td>
<td>+/NR</td>
<td>Bjornsson et al 2007</td>
</tr>
<tr>
<td>f, 35</td>
<td></td>
<td>11</td>
<td>55N/95N HEP</td>
<td>+/NR</td>
<td></td>
</tr>
<tr>
<td>m, 40</td>
<td>Green tea</td>
<td>20</td>
<td>33N/25N MIX</td>
<td>+/NR</td>
<td>Federico et al 2007</td>
</tr>
</tbody>
</table>

- **62N/73N MIX**: Mixed normal and toxic responses.
- **NR**: Not reported.
- **+NR**: Positive dechallenge/rechallenge.
- **MIX**: Mixed response.
- **HEP**: Hepatocellular injury.
- **Centrilobular hepatocytes drop out with bridging necrosis, heavy inflammatory reaction**: Describes the type of liver damage observed.
- **Federico et al 2007**: Cite the reference for more detailed information.
- **Bjornsson et al 2007**: Cite the reference for more detailed information.
<table>
<thead>
<tr>
<th>Sex, age (years)</th>
<th>Herbal product composition (Brand name)</th>
<th>Time (duration of treatment in weeks)</th>
<th>Evidence of Toxicity</th>
<th>Dechallenge/rechallenge</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>f, 51</td>
<td>Green tea infusion (NR)</td>
<td>260</td>
<td>4-5N COL Mild cholestasis</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>f, 20</td>
<td></td>
<td>NR</td>
<td>~ 60 COL NR</td>
<td>+/-/No</td>
<td></td>
</tr>
<tr>
<td>f, 44</td>
<td>Green tea extract, Vitamin E, wheat germ oil, soy oil, beeswax, glycerol esters of fatty acids (NR)</td>
<td>26</td>
<td>2393/3583 HEP Hepatocellular necrosis, mixed inflammatory infiltrates. The patient underwent orthotopic liver transplant</td>
<td>NR/No</td>
<td>Molinari et al 2006</td>
</tr>
<tr>
<td>f, 46</td>
<td>Green tea infusion (NR)</td>
<td>31</td>
<td>1188/1100 HEP NR</td>
<td>NR/NR</td>
<td>Javaid et al 2006</td>
</tr>
<tr>
<td>f, 26</td>
<td>Green tea (75%), <em>Menta piperita</em> (25%) infusion (Té verde Hacendado, Mercadona, Valencia, Spain)</td>
<td>18</td>
<td>1813/3314 HEP Toxic liver disease</td>
<td>+/-</td>
<td>Martinez-Sierra et al 2006</td>
</tr>
<tr>
<td>Sex, age (years)</td>
<td>Herbal product composition (Brand name)</td>
<td>Time (duration of treatment in weeks)</td>
<td>Evidence of Toxicity</td>
<td>Dechallenge/rechallenge</td>
<td>Reference</td>
</tr>
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<td>------------</td>
</tr>
<tr>
<td>m, 45</td>
<td>Green tea infusion (NR)</td>
<td>18</td>
<td>1037/1613 HEP</td>
<td>NP</td>
<td>Jimenez-Saenz et al 2006</td>
</tr>
<tr>
<td>f, 37</td>
<td>Green tea extract (Tegreen 97: polyphenols 97%, cathechins 64%), Magnolia officinalis, Epimedium koreanum and Lagerstroemia speciosa extract, calcium, chromium, L-Theanine, β-sitosterol, vanadium (USA)</td>
<td>18</td>
<td>1783/1788 HEP</td>
<td>Marked interface necrosis, mild lobular inflammation</td>
<td>Bonkovsky et al 2006</td>
</tr>
<tr>
<td>f, 52</td>
<td>Green tea, Citrus aurantium, Citrus paradisi, Cynara scolymus, Petroselinum sativum extracts (X-elles)</td>
<td>1.2</td>
<td>2.5N/6.5N COL</td>
<td>Portal inflammation, mild cholestasis, mixed centrolobular inflammatory infiltrates, necrosis</td>
<td>Mathieu et al 2005</td>
</tr>
<tr>
<td>Sex, age (years)</td>
<td>Herbal product composition (Brand name)</td>
<td>Time (duration of treatment in weeks)</td>
<td>Evidence of Toxicity</td>
<td>Dec challenge/rec challenge</td>
<td>Reference</td>
</tr>
<tr>
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</tr>
<tr>
<td>f, 48</td>
<td>Green tea extract (AR25: EGCG 25%), caffeine (19%) (Exolise, Arkopharma, Carros, France)</td>
<td>8</td>
<td>140N/102N HEP</td>
<td>NR/No</td>
<td>Gloro et al 2005</td>
</tr>
<tr>
<td>f, 35</td>
<td>See Gloro, 2005 [20] (Exolise, Arkopharma, Carros, France)</td>
<td>1.2</td>
<td>1191/2885 HEP NP</td>
<td>+/-No</td>
<td>Abu el Wafa et al 2005</td>
</tr>
<tr>
<td>m, 27</td>
<td>Green tea extract (catechins 70%, EGCG 45%); Garcinia cambogia, Gymnema silvestre, Salix spp. and Paullinia cupana extract, calcium, chromium, potassium, glucomannan, α-lipoic acid, L-carnitine, caffeine, gelatin, silica, cellulose (HydroxyCut, Iovate Health Sciences Research, Mississauga, Canada)</td>
<td>5</td>
<td>1808/3131 HEP NR</td>
<td>+/-No</td>
<td>Stevens et al 2005</td>
</tr>
<tr>
<td>Sex, age (years)</td>
<td>Herbal product composition (Brand name)</td>
<td>Time (duration of treatment in weeks)</td>
<td>Evidence of Toxicity</td>
<td>Dechallenge/rechallenge</td>
<td>Reference</td>
</tr>
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</tr>
<tr>
<td>m, 30</td>
<td>Green tea leaves, <em>Ananas sativus</em> powder, citric acid, maltodextrine, magnesium stearate, silicium dioxide (Fitofruits grasas acumuladas)</td>
<td>0.6</td>
<td>59/45</td>
<td>COL</td>
<td>Porcel et al 2005</td>
</tr>
<tr>
<td>f, 53</td>
<td>Green tea leaves, <em>Ananas sativus</em> powder, citric acid, maltodextrine, magnesium stearate, silicium dioxide (Fitofruits grasas acumuladas)</td>
<td>2</td>
<td>927/1259</td>
<td>HEP</td>
<td>+/No</td>
</tr>
<tr>
<td>f, 25</td>
<td>Green tea leaves micronized powder (caffeine &gt;2%) (Camilina Arkocapsulas Arkopharma, Carros, France)</td>
<td>8</td>
<td>1943/2398</td>
<td>HEP</td>
<td>+/No</td>
</tr>
<tr>
<td>f, 42</td>
<td>Green tea leaves, <em>Gynostemma pentaphyllum</em>, Aloe sp. juice, <em>Raphanus sativus</em> semen, <em>Crataegus</em> sp fruit (Slim 10)</td>
<td>18</td>
<td>NR</td>
<td>–</td>
<td>Lau et al 2004</td>
</tr>
<tr>
<td>f, 35</td>
<td>See Gloro, 2005 [20] (Exolise, Arkopharma, Carros, France)</td>
<td>5</td>
<td>1108/1558</td>
<td>MIX</td>
<td>+/No</td>
</tr>
</tbody>
</table>

Reference:
- Porcel et al 2005
- Garcia-Moran et al 2004
- Lau et al 2004
- Dueñas Sadornil et al 2004
<table>
<thead>
<tr>
<th>Sex, age (years)</th>
<th>Herbal product composition (Brand name)</th>
<th>Time (duration of treatment in weeks)</th>
<th>Evidence of Toxicity</th>
<th>Dechallenge/rechallenge</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>f, 56</td>
<td>Green tea hydroalcoholic extract, <em>Cassia</em> sp. (Mincifit, Arkopharma, Carros, France)</td>
<td>2</td>
<td>33N/54N HEP NP – Rech: cholestasis, mild necrosis, steatosis of 20% hepatocytes</td>
<td>+/-^e^</td>
<td>Peyrin-Biroulet et al 2004</td>
</tr>
<tr>
<td>f, 46</td>
<td>See Gloro, 2005 [20] (Exolise, Arkopharma, Carros, France)</td>
<td>13</td>
<td>61N/75N HEP NP</td>
<td>+/-</td>
<td>Vial et al 2003</td>
</tr>
<tr>
<td>f, 35</td>
<td></td>
<td>5</td>
<td>976/1558 MIX NP</td>
<td>+/-No</td>
<td></td>
</tr>
<tr>
<td>f, 34</td>
<td></td>
<td>Several</td>
<td>NR – NR</td>
<td>NR/No</td>
<td></td>
</tr>
<tr>
<td>f, 69</td>
<td></td>
<td>5</td>
<td>3N/4N COL NP</td>
<td>+/-No</td>
<td>Pedros et al 2003</td>
</tr>
<tr>
<td>f, 29</td>
<td>See Gloro, 2005 [20] (Exolise, Arkopharma, Carros, France)</td>
<td>6.5</td>
<td>1023/1674 HEP NR</td>
<td>+/-No</td>
<td></td>
</tr>
<tr>
<td>Sex, age (years)</td>
<td>Herbal product composition (Brand name)</td>
<td>Time (duration of treatment in weeks)</td>
<td>Evidence of Toxicity</td>
<td>Dechallenge/rechallenge</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------------------------------</td>
<td>--------------------------------------</td>
<td>----------------------</td>
<td>------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>m, 44</td>
<td>As in Stevens, 2005 [22] + <em>Ephedra sinica</em> extract (Hydroxycut Iovate Health Sciences Research, Mississauga, Canada)</td>
<td>16</td>
<td>2046/3600 HEP Mild periportal fibrosis, periportal and parenchymatous inflammatory infiltrates</td>
<td>+/-No</td>
<td>Bajaj et al 2003</td>
</tr>
<tr>
<td>f, 52</td>
<td>Green tea, <em>Gynostemma pentaphyllum</em>, <em>Nelumbo</em> sp., <em>Chrysanthemum</em> sp., <em>Lycium barbarum</em>, <em>Crataegus monogyna</em>, <em>Citrus aurantium</em>, <em>Cassia mimosoides</em>, <em>Raphanus sativus</em>, beer yeast, Blc Golden tang, raifukushi®(Be-petite)</td>
<td>9</td>
<td>1539/1920 MIX Dramatic portal inflammation with characteristic fibrosis</td>
<td>+/-No</td>
<td>Kanda et al 2003a</td>
</tr>
<tr>
<td>f, 31</td>
<td>Green tea leaves, <em>Gynostemma pentaphyllum</em>, barbaloin, total saponin, polyphenol®(Onshidou-genbi-kounou)</td>
<td>4</td>
<td>9310/8820 HEP NR</td>
<td>+/-No</td>
<td>Kanda et al 2003b</td>
</tr>
<tr>
<td>f, 39</td>
<td>Green tea (7%), Oolong tea, <em>Cassia angustifolia</em> leaves, <em>Momordica grosvenori</em>, <em>Malva verticillata</em> (Ooloon tea fine tonic)</td>
<td>2</td>
<td>1.9N/3.9N COL NP</td>
<td>+/-No</td>
<td>Thiolet et al 2002</td>
</tr>
<tr>
<td>Sex, age (years)</td>
<td>Herbal product composition (Brand name)</td>
<td>Time (duration of treatment in weeks)</td>
<td>Evidence of Toxicity</td>
<td>Dechallenge/rechallenge</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------------------------------</td>
<td>--------------------------------------</td>
<td>----------------------</td>
<td>-------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>f, 50</td>
<td>See Gloro, 2005 [20] (Exolise, Arkopharma, Carros, France)</td>
<td>4</td>
<td>31N/40N HEP Marked periportal hepatocytes necrosis, lobular inflammatory infiltrates, marked centrolobular cholestasis</td>
<td>+/-No</td>
<td>Seddik et al 2001</td>
</tr>
<tr>
<td>f, 19</td>
<td>Green tea powdered leaves (Arkocapsulas, Arkopharma, Carros, France)</td>
<td>8</td>
<td>1130/1110 HEP Necrosis of hepatocytes, mixed inflammatory infiltrates</td>
<td>+/-</td>
<td>Gavilan et al 1999</td>
</tr>
</tbody>
</table>

f, Female; m, male; AST, alanine aminotransferase; NR, not reported; No, not done; NP, not performed; HEP, hepatocellular; COL, cholestatic; MIX, mixed; -, not evaluable; ECGC, (-)-epigallocatechin gallate a x N, x times the upper normal value; b Recovery time (weeks);
cCapsules were adulterated with N-nitrosafenfluramine; dAdulteration with N-nitrofenfluramine is hypothesized; eRechallenge was performed with Dynasvelte (*Camellia sinensis* + * Coffea arabica* + chromium)

Elevated serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin and alkaline phosphatase were reported. In most of the cases reviewed in which histological analysis was conducted, hepatic tissue necrosis was very common. In several of the reported cases symptoms abated or ceased all together when consumption of the dietary supplement was discontinued. Re-injury was reported when consumption of supplement was resumed (reviewed in [45]). In another report, a 37-year-old woman suffered from abdominal
pain, nausea and jaundice following consumption of a green tea extract weight loss supplement for 4 months [60]. Her serum AST, serum alanine ALT, serum bilirubin and serum alkaline phosphatase levels were all much higher than the respective reference ranges.

In a case report, a 44 year old woman was hospitalized for progressive illness, the onset of abdominal pain and jaundice [1]. Blood tests revealed elevated serum bilirubin, serum AST, serum ALT and gamma glutamyl transferase levels. Other bodily functions and vital signs were normal. Investigation into the possible cause for her conditions revealed that the woman had been engaged in a weight loss program that required the intake of green tea extract containing dietary supplement with a dosage of 720 mg/day. Subsequent toxicology and hepatitis tests were all negative. Treatment with intravenous infusion acetylcysteine resulted in worsening of some of her conditions. A liver biopsy was performed and it revealed severe hepatocellular necrosis among other finding. Continued deterioration of her medical conditions resulted in a liver transplant. Further investigation prompted chemical analysis of the green tea extract supplements. The results were not conclusive, but the medical personnel attributed the acute liver failure to the green tea supplement the patient consumed as part of her weight loss regimen.

Federico et al presented the case of a 51 year old woman with a history of abnormal liver function tests; increased serum aminotransferases (four- to fivefold), $\gamma$-glutamyl-transpeptidase ($\sim 200$ U/L; normal range 0–50 U/L), and alkaline phosphatase (over 200 U/L; normal range 53–128 U/L) spanning a period of 5 years. Clinical tests were not able to identify the cause of the liver issues, but an investigation into her history revealed that she consumed green tea every day for the past 5 years. Cessation of tea consumption for two months improved liver function test. When she resumed consumption, liver function test reverted to being abnormal. Cessation again returned liver function tests to normal [61].

A 45-year-old man diagnosed with jaundice was reported to consume six cups/day of a marketed green tea infusion over the previous 4 months with no other medication. Laboratory
tests showed elevated serum AST 1033 U/l, ALT 1613 U/l (<40), lactate dehydrogenase 602 U/l (<425), alkaline phosphatase 310 U/l (<110), GGT 394 U/l (<30), total bilirubin 119 μg/dl (<17) and direct bilirubin 102 μg/dl. Discontinuation of the product led to normal liver function tests. Resumption of the product resulted in a significant increase of liver enzymes levels. Withdrawal again normalized liver function tests [62].

These data though not conclusive indicate the potential of dietary supplements containing high levels of EGCG to cause liver toxicity. One factor to consider is the bioavailability of EGCG, which can vary from one individual to the next. In the next section I discuss EGCG bioavailability and its impact on EGCG mediated liver toxicity.

**Laboratory Studies**

Animal models and cell lines have been used in laboratory studies to investigate the potential toxicity of EGCG. Hepatotoxicity of high oral dose EGCG in mice was studied. Lambert *et al* showed that high orally administered doses of EGCG in male CF-1 mice resulted in hepatotoxicity [63] Mice were fasted and treated with either single dose of 50-2000 mg/kg EGCG through oral administration or once-daily dose of 500 or 750 mg/kg EGCG through oral administration for 2-7 days. Blood and liver samples were collected and analyzed by both histological and biochemical methods, with plasma ALT used as the biochemical marker for hepatotoxicity. Results showed that EGCG toxicity was time and dose-dependent. In mice given once daily doses of 1500 mg/kg EGCG, and mice given two once-daily 750 mg/kg EGCG through oral administration resulted in high mortality (85%) and toxic potency of EGCG increased with this dose. Hepatic necrosis and apoptotic cells were observed in liver samples from mice given two once-daily 750 mg/kg EGCG through oral administration. Plasma ALT levels, showed an 8 and 108 fold increase in plasma ATL levels after 24 and 48 hours after
administration of that dose. Increased oxidative stress was also observed after treatment with 1500 mg/kg EGCG through oral administration and two once-daily treatments with 750 mg/kg EGCG through oral administration. A similar study in Beagle dogs observed a dose-dependent toxicity of EGCG (in Polyphenon E) when administered at 200, 500, and 1000 mg/kg/day to fasted dogs [64].

These results from Lambert et al suggest that at some threshold, or at a certain concentration present in the cells, EGCG exhibits pro-oxidant activities, which induces oxidative stress and results in hepatotoxicity in the mouse. The toxicity levels in mice (500-1500 mg/kg) translates to (30-90 mg/kg) in humans, which is equivalent to 10.5-32 cups of green tea [63]. Although these levels may not pose a significant risk for hepatotoxicity from tea consumption alone, risk levels increase with the introduction of green tea dietary supplements. Some supplements may contain sufficient levels of EGCG to reach the toxic threshold, or the persons taking more than the recommended dosage could potentially reach the toxic threshold.

Galati et al studied the cytotoxicity of green tea catechins and phenolic acids [65]. Hepatocytes from male rats were obtained and treated with various stock solutions of the phenolic compounds. Mitochondrial membrane potentials, hepatocyte GSH and GSSG, and reactive oxygen species (ROS) formation were all measured. They found that the most cytotoxic tea phenolic was EGCG. EGCG collapsed the hepatocyte mitochondrial membrane potential proving to be the most effective mitochondrial toxin. They also found that EGCG was the most effective at inducing ROS formation. The H$_2$O$_2$ generated caused oxidative damage to isolated and cellular DNA in the rat hepatocytes. The same group studied hepatotoxicity in vivo in male mice and found that EGCG increased plasma ALT levels in a dose-dependent manner, which indicates liver injury, and EGCG treatment of 150 mg/kg i. p. resulted in mice death less than 24 hrs after treatment. No actual references to hepatotoxicity in healthy cells were made in this study, making it difficult to form conclusions on the actual hepatotoxicity of EGCG. The in vivo study reports
that the toxic level of EGCG in male mice is 150 mg/kg when administered intraperitoneally. This data though essential, is not relevant when assessing the potential toxicity of EGCG as it pertains to green tea extract containing dietary supplements, which are administered orally.

In this study, Schmidt et al investigated the possible hepatotoxicity from hydro-alcoholic green tea extracts as well as the components involved [66]. Hepatocytes isolated from adult rats were treated with hydro-alcoholic green tea extracts of varying concentrations of catechins and polyphenols and cytotoxicity was assessed. Results based on the two methods of analysis used to determine cytotoxicity; EGCG was found to be mostly responsible compared to other green tea components tested, for causing cytotoxicity in rat hepatocytes. This study is relevant to EGCG toxicity as it pertains to green tea extract containing dietary supplements. Many supplements are prepared using hydro-alcoholic extracts from green tea leaves. Exolise® is one such example, and it is responsible for many of the reported cases of liver toxicity, resulting in its removal from French and Spanish markets [45]. In their study Schmidt et al showed that the cytotoxicity of EGCG occurred at levels ≥ 1000 µg/ml of green tea extract, which according to the authors is a very high concentration, when compared to other hepatotoxins such as acetaminophen, which cause toxicity at concentrations of 1-50 µM [66]. Based on their findings, the authors conclude that hydro-alcoholic green tea extracts display low cytotoxicity in rat hepatocytes in vitro and most of this toxicity is due to EGCG. The authors also concluded that due to EGCG’s low bioavailability in humans, it does not appear to be the cause of liver toxicity associated with supplements containing hydro-alcoholic green tea extracts. However, several of the studies that I have looked at directly contradict this conclusion.

Kapetanovic et al examined the exposure and toxicity of green tea in fasted and non-fasted dogs over the course of 13 week. Beagle dogs were treated with 0, 200, 500, and 1000 mg/kg/day of unformulated Polyphenon E, a highly purified and standardized green tea extract containing 85-95% total catechins (56-72% EGCG) in gelatin capsules. The dogs were fasted
prior to treatment with Polyphenon E. Blood and urine samples were collected from the fasted dogs, and analyzed. The high dose of 1000 mg/kg/day had to be discontinued due to observed levels of toxicity and subsequent death of one dog. During the 9 month study, the 1000 mg/kg/day administration resulted in the highest mortality (100 %), with the majority of deaths occurring in the first 13 weeks. Overall there were high levels of mortality in the fasted dogs. Some of the toxicities observed after analysis of samples include decreased red blood cell, and increase in white blood cells, lesions in GI tract, lymph nodes, liver, kidney, lung, heart and tonsils. Necrosis was also observed in several organs. The authors concluded that fasting increased bioavailability of EGCG resulting in enhanced toxicity [64]

The underlying mechanism of EGCG-induced hepatotoxicity in vivo remains to be studied. Lambert et al in a study showed that high doses of EGCG caused oxidative stress in liver tissue resulting in hepatotoxicity [63]. The pro-oxidant activities of EGCG have been studied and have been implicated in its chemopreventative effects. Studies by Galati et al and Tao et al show that EGCG induces ROS in cancer cells resulting in cytotoxicity. Tao et al also showed that at the doses used normal cells were unaffected by EGCG. The ROS including activities of EGCG in vitro could have implications in vivo and could provide insight into high does EGCG induced liver toxicity, which warrants further study.

Dietary and Lifestyle Factors Affecting Risk of Green Tea-Related Liver Toxicity

The benefits of EGCG particularly its ability to modulate body weight, has made it popular as a dietary or weight loss supplements. In a study by Bailey et al, which examined the use of supplements in adults and the reason for taking them, it was found that 45% of the adults surveyed took supplements to improve and 33% to maintain overall health [67]. The study
highlighted that many adult took multiple supplements for a variety of reason, and many were not recommended by a physician or health care provider. As was discussed, many of these supplements are capable of delivering a higher dose of green tea catechins compared to the traditional beverage. When consumed with limited supervision, especially when not recommended by a physician or health care provider the risk of improper use of supplements increases. Many supplement takers do not consider the risks involved as some are taking multiple supplements at once and many are of the belief that supplements are natural and are therefore safe. As such, lifestyle factors that are potentially harmful are not considered when taking supplements. Users have not evaluated potential health risks if supplements are taken with pre-existing physiological conditions, or how taking multiple supplements or other aspects of the diet may affect the safety of the supplements.

Previous studies have shown that chronic administration of EGCG to mice subsequently decreased EGCG bioavailability [68]. Rats and mice treated with a 0.6% green tea polyphenol preparation as the drinking fluid for 28 days showed an initial increase in plasma catechin levels from Day 1 and peaked on day 14. The plasma levels of tea catechins the decreased, to Day 1 values on Day 28. The results suggest that consumption of tea by rodents could induce adaptive responses affecting blood and tissue levels of tea catechins with time. Studies using other compounds, for example alcohol, which is liver toxin, have shown that chronic consumption of alcohol elicit an adaptive response over time, essentially allowing test subjects to develop a tolerance [69]. With supplements capable of delivering high doses of EGCG and inducing hepatotoxicity, the potential of chronic dietary administration of EGCG in modulating these effects warrants further investigation.

Green tea supplements are marketed primarily for weight loss because of a pre-existing obese condition. As I will discuss, obesity has the potential to increase drug induced liver injury and the potential role it play in EGCG induced liver toxicity warrants further investigation.
Numerous studies show that obesity is a major issue not only in the United States, but worldwide and is considered a major public health problem. Obesity has been shown to be a significant risk factor for many diseases such as type 2 diabetes, cardiovascular disease, obesity-related fatty liver disease (ORFLD), and certain cancers [70]. Obesity is highly associated with non-alcoholic fatty liver disease (NAFLD), which is the most common type of liver disease. NAFLD can develop into non-alcoholic steatohepatitis (NASH), and in some cases hepatocellular carcinoma (HCC), making obesity a risk factor for HCC [71], [72]. Studies have shown that ORFLD effects changes in liver physiology and function. This impacts drug metabolism and it has been shown to increase instances of drug induced liver injury (DILI) [73], [74]. Obesity has been shown to increase blood flow to the liver and the activity of cytochrome P450 (CYPs) metabolizing enzymes which can subsequently increase the generation of toxic metabolites. This increases the risk for drug induced liver injury by inducing oxidative stress, mitochondrial dysfunction and tissue necrosis. This is illustrated in Figure 1-5 [73]–[75].

Figure 1-5. Illustration of drug induced liver injury in obesity. [74]
In addition to altering CYP activity, obesity has been shown to increase oxidative stress by depleting glutathione (GSH) levels, which can inhibit the mitochondrial respiratory chain (MRC) and lead to mitochondrial dysfunction. In addition depleted GSH levels in the mitochondrial can prevent the removal of CYP-generated toxic metabolites, further impairing the MRC [76].

Many studies support the role of obesity as a risk factor for DILI. In studies conducted by Corcoran et al. obesity was shown to increase the risk of acetaminophen and furosemide induced liver injury. Acetaminophen and furosemide, both of which generate toxic metabolites when metabolized by CYPs, caused dose dependent increases in liver tissue necrosis when fed to obese mice [77].

A review by Browning et al, (summarized in Figure 1-6) showed that in ORFLD there is an increase in the release of free fatty acids (FFA) which can lead to insulin resistance, increase oxidative stress and induce proinflammatory cytokines and eventually promote liver injury [78].

![Figure 1-6. Mechanisms of lipid-induced cellular injury in ORFLD. [78]](image-url)
As stated earlier, EGCG has been well-regarded for its anti-obesity properties, so much so that dietary supplements containing high levels of EGCG have been marketed particularly for weight loss [21], [50]. With the growing body of evidence of high dose EGCG mediated liver toxicity, and studies showing increased DILI in obesity, obesity is likely to increase susceptibility to high dose EGCG mediated hepatotoxicity.

With a large number of green tea-based dietary supplements available on the market, and more being produced, many of which contain high levels of EGCG (as high as 90%), EGCG toxicity and the risk of hepatotoxicity are a major cause for concern. Understanding the mechanism by which EGCG mediates liver toxicity and the potential risk factors associated with EGCG induced hepatotoxicity are significant, particularly from a public health perspective. Consumers are exposed to doses of EGCG that are potentially toxic. With many of the green tea supplements listing other ingredients such as acai berry, known for its antioxidant properties, consumers are more inclined to purchase these supplements. Many are under the misconceptions that more is better and naturally derived products are 100% safe. Also, with the growing trend of eating and being healthy, especially in the face of an obesity epidemic many consumers believe that taking dietary supplements will help them lead a healthy life. As such, studies investigating the potential mechanism of EGCG mediated liver toxicity and the effect of dietary and physiological conditions on toxicity are warranted to identify factors which increase susceptibility to green tea supplement-associated hepatotoxicity.
Research Hypothesis and Specific Aims

A growing body of evidence suggests the hepatotoxic potential of high doses of EGCG. In vitro several mechanisms of EGCG cytotoxicity have been examined; however potential mechanisms of in vivo toxicity remain understudied. Additionally factors that can potentiate EGCG induced hepatotoxicity, such as pre-existing physiological conditions that attenuate liver function have not been investigated. With the lack of data to support an association between dietary supplements and liver toxicity, I hypothesized that dietary pretreatment with EGCG markedly decreases EGCG bioavailability and can potentially mitigate the toxic potential of EGCG, and that ORFLD increases susceptibility to high dose EGCG-mediated hepatotoxicity. In addition to the effect of these factors on high dose EGCG hepatotoxicity, I further hypothesized that the underlying mechanism by which EGCG mediates hepatotoxicity is through induction of mitochondrial oxidative stress and increased mitochondrial dysfunction.

To test this hypothesis the following specific aims were proposed:

1. To determine the effect of dietary pretreatment with EGCG on the bioavailability and hepatotoxicity of subsequent oral bolus dosing with EGCG in mice.

2. To determine the effect of pre-existing obesity and ORFLD on EGCG-mediated hepatotoxicity in mice.

3. To examine the effect of EGCG on hepatic markers of antioxidant response and mitochondrial biogenesis/function in lean and obese mice.
Abstract

The green tea catechin, (-)-Epigallocatechin-3-gallate (EGCG), may have obesity preventive effects. Recent case-studies, however, have reported an association between green tea-based dietary supplement use and hepatotoxicity in human subjects. Additionally, laboratory studies have demonstrated the hepatotoxicity of an acute high-dose oral bolus EGCG in mice and dogs. Here, we examined the effect of pretreatment with dietary EGCG on the bioavailability and hepatotoxicity of acute oral bolus EGCG in male CF-1 mice. EGCG (750 mg/kg, i.g., once daily for 3 days) increased plasma levels of alanine aminotransferase (80-fold increase), decreased both reduced (59% decrease) and total (33% decrease) hepatic glutathione, and increased hepatic levels of γ-histone 2AX compared to control mice. Pretreatment with dietary EGCG (3.2 mg/g diet) for 2 wk significantly blunted these markers of hepatotoxicity. Acute oral bolus dosing with EGCG also decreased mRNA expression of glutathione reductase and glutathione peroxidase (Gpx) 2 compared to control mice: dietary pretreatment prevented these decreases and significantly increased expression of Gpx3, Gpx5, and Gpx7. We hypothesized that these effects were related to modulation of EGCG bioavailability. We found that pre-treatment with dietary EGCG significantly reduced the peak plasma (38% reduction) and hepatic (57% reduction) EGCG concentrations following oral bolus dosing compared to mice that were not pretreated. In conclusion, it appears that EGCG can modulate its own bioavailability and that
chronic exposure to dietary EGCG may reduce the toxic potential of acute high oral bolus doses of EGCG. These data may help explain the variation in hepatotoxic response to green tea-containing dietary supplements.
Introduction

(-)-Epigallocatechin-3-gallate (EGCG), is the most abundant polyphenol in green tea (*Camellia sinensis*), and has been widely-studied for its potential health benefits including modulation of body weight gain [26], [28], [79]. One result of these reports of positive health effects has been the proliferation of green tea-based dietary supplements for a variety of indications. Whereas historical exposure to EGCG has been as a beverage, green tea-based dietary supplements are generally capsules or pills. The result is the ability to deliver an equivalent dose of green tea components in a much smaller volume. The safety of these alternative formulations is generally based on the historical safety of green tea beverage, however, there is increasing data to suggest that this assumption may not be valid [45].

Laboratory studies in mice and dogs have demonstrated the potential hepatotoxicity of acute oral bolus dosing with EGCG or green tea extracts. We have previously reported that EGCG induces dose-dependent hepatotoxicity in CF-1 mice [63]. Once daily dosing with EGCG (750 mg/kg, *i.g.*) induced oxidative liver damage and hepatic necrosis. Similar results were observed in fasted Beagle dogs treated with oral bolus doses of Polyphenon E, a defined tea polyphenol mixture containing 60% EGCG [64]. Although the underlying mechanisms of EGCG-induced hepatotoxicity remains understudied, it has been proposed that the acute oral doses of EGCG, depending on dose, can result in oxidative stress leading to liver injury [63], [80].

More than 34 case-reports have associated human hepatotoxicity with the use of green tea-containing dietary supplements since 1999 [reviewed in [45]]. Although the causative role of these supplement in the hepatotoxicity has not been clearly established, several case-reports have indicated that cessation of supplement use led to resolution of symptoms and re-challenge led to renewed liver injury [45]. It is interesting to note that hepatotoxicity has not been widely
observed in controlled human intervention studies [55], [59]. These inconsistent findings indicate that genetic or life-style factors may play a role in susceptibility to EGCG-mediated hepatotoxicity.

Previous studies have shown that chronic, dietary administration of green tea can impact the oral bioavailability of the tea polyphenols. Kim et al., have previously reported that long term treatment of rats and mice with dietary green tea resulted in an initial increase in plasma EGCG levels over the first 14 d of treatment [68]. Over the subsequent 14 d, plasma levels of EGCG decreased with continued treatment and eventually reached baseline levels again. In addition, EGCG has been shown to induce the expression of genes related to antioxidant response and Phase II biotransformation in vivo [81]. These changes have been shown to alter the metabolic profile of toxicants and carcinogens [82]. Given that EGCG, itself, is subject to Phase II metabolism, it is plausible that chronic administration of EGCG or green tea may influence the biotransformation and bioavailability of EGCG.

In the present study we determined the effect of dietary pretreatment EGCG on the bioavailability and hepatotoxicity of subsequent oral bolus dosing with EGCG in mice. This study was meant to mimic the potential hepatotoxic effects of high oral bolus dosing with EGCG in chronic tea consumers to those in non-consumers, and should aid in identifying factors which predispose subjects to green tea supplement-associated hepatotoxicity.

**Materials and methods**

**Chemicals and reagents**

EGCG (93% pure) was purchased from Taiyo Green Power (Wuxi, Jiangsu, China). Diets (Table 2-1) were prepared by Research Diets, Inc. (New Brunswick, NJ).
Table 2-1. Composition of Experimental Diets.

<table>
<thead>
<tr>
<th>Macronutrient Composition</th>
<th>NC</th>
<th>NP</th>
<th>EP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, % kcal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrate, % kcal</td>
<td>20.8</td>
<td>20.8</td>
<td>20.8</td>
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<tr>
<td>Fat, % kcal</td>
<td>67.7</td>
<td>67.7</td>
<td>67.7</td>
</tr>
<tr>
<td>Ingredient, g/kg</td>
<td>11.5</td>
<td>11.5</td>
<td>11.5</td>
</tr>
<tr>
<td>Casein, 30 Mesh</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Cellulose, BW 200</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>^aMineral Mix S10001</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>^bVitamin Mix V10001</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>EGCG</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

*aMineral mix adds the following components (per g mineral mix): sodium chloride, 259 mg; magnesium oxide, 41.9 mg; magnesium sulfate, 257.6 mg; chromium K sulfate, 1.925 mg; cupric carbonate, 1.05 mg; sodium fluoride, 0.2 mg; potassium iodate, 0.035 mg; ferric citrate, 21 mg; manganous carbonate, 12.25 mg; ammonium molybdate, 0.3 mg; sodium selenite, 0.035 mg; zinc carbonate, 5.6 mg. *bVitamin mix adds the following components (per g vitamin mix): vitamin A palmitate, 400 IU; vitamin D3, 100 IU; vitamin E acetate, 5 IU; menadione sodium bisulfite, 0.05 mg; biotin, 0.02 mg; cyanocobalamin, 1 µg; folic acid, 0.2 mg; nicotinic acid 3 mg; calcium pantothenate, 1.6 mg; pyridoxine-HCl, 0.7 mg; riboflavin, 0.6 mg; thiamin HCl, 0.6 mg
Primers for quantitative reverse transcriptase (q)PCR (Table 2-2) were synthesized by the Genomics Core Facility at The Pennsylvania State University (University Park, PA). Phosphorylated histone 2AX (γH2AX) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were purchased from Cell Signaling Technologies (Danvers, MA). All other chemicals were of the highest grade commercially-available.

Table 2-2. Primer sequences for qPCR analysis of hepatic antioxidant response in male CF-1 mice.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gsr</td>
<td>GACACCTCTTCCCTCGACTACC</td>
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<td>Gpx7</td>
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<td>Sod2</td>
<td>CAGACCTGCCTACGACTATGG</td>
<td>CTCGGTGCGCTTGGATAGTT</td>
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</table>

Animal studies

All animal studies were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University (University Park, PA, IACUC 37115). Mice were maintained on a 12 h light-dark cycle at 21°C and 38% relative humidity. Mice were acclimatized for 1 wk
prior to the start of experiments and had *ad libitum* access to food and water. The dietary dose of EGCG (3.2 mg/g diet) is equivalent to a total daily dose of 500 mg/kg, body weight. The dietary EGCG dose and the oral bolus EGCG dose (750 mg/kg, body weight) are equivalent to 10 and 16 cups of green tea prepared using a standard method (2.5 g tea leaves in 250 mL) [56].

**Hepatotoxicity Studies**

Male CF-1 mice (6 – 8 wks old, Charles River Laboratory, Wilmington, MA) were randomized into three treatment groups; EGCG pretreatment (EP), no pretreatment (NP), and negative control (NC). EP mice were fed AIN76A diet supplemented with EGCG (3.2 mg/g) for 14 d, whereas NP and NC mice received AIN76A diet. Following the pretreatment period, mice were fasted for 7 h (0700 – 1400 h) and then given a single dose of EGCG (750 mg/kg, i.g.) once daily for 3 d. On d 3, 1 h post-gavage, mice were anesthetized and blood was collected by cardiac puncture. Plasma was prepared by centrifugation at 3200 g for 15 min and frozen at -80°C. The livers were harvested, washed with ice-cold PBS and frozen at -80°C for later analysis.

**Bioavailability Studies**

Male CF-1 mice (n = 24 per group) were randomized into EP, NP and NC pre-treatment groups. After a pretreatment period of 14 days, NP and EP mice were fasted for 7 h and then given a single dose of EGCG (750 mg/kg, i.g.). At 0.5, 1.5, 4 and 6 h post-gavage, mice were anesthetized and blood was collected via cardiac puncture. Plasma was prepared as above, combined with 0.1 vol of ascorbate preservative (20% ascorbic acid: 0.1% EDTA) and frozen at -80°C. Livers were harvested and frozen as above.
Determination of hepatotoxicity and oxidative stress

Plasma alanine aminotransferase (ALT) levels were determined spectrophotometrically ($\lambda_{\text{max}} = 340 \text{ nm}$) using a commercially-available assay (CATACHEM Bridgeport, CT) according to the manufacturer’s instructions. Expression of γ-H2AX was determined by western blot. Protein was extracted from liver samples using Tissue Protein Extraction Reagent (TPER) containing 1% (v/v) phosphatase and protease inhibitor cocktails (Thermo-Scientific, Rockford, IL). Protein samples (30 μg protein) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. Membranes were probed with γH2AX primary antibody and a fluorescent-conjugated secondary antibody (Li-Cor Co., Lincoln, NE). Bands were visualized using a Licor Odyssey Imaging System (LI-COR Corporate). GAPDH was used as a protein loading control. Total and reduced glutathione levels were determined using the GSH-Glo™ Glutathione assay kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

Gene expression studies

The mRNA expression of genes related to antioxidant response (Table 2) was examined by qPCR. RNA was isolated from liver tissue using Tri-reagent (Sigma Chemical Co. St. Louis, MO) according to the manufacturer’s instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer. cDNA was reverse transcribed and amplified using SYBR Green PCR Master Mix and an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Standard curves were made by using serial dilutions from pooled cDNA samples. mRNA levels were normalized to the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (Gapdh).
HPLC analysis of EGCG

EGCG levels in plasma and liver samples were determined as described previously [56]. In brief, plasma samples were combined with 0.4 M sodium phosphate monobasic (pH 6.8) and 20% ascorbic acid and then extracted with dichloromethane and ethyl acetate. The ethyl acetate fraction was dried under vacuum and resuspended in 10% aqueous acetonitrile for HPLC analysis. The resultant samples represented unconjugated EGCG. To determine the total EGCG, samples were treated with β-glucuronidase (250 U) and sulfatase (1 U) prior to extraction. Liver samples were homogenized in a 1:1 mixture of hydrosulfite buffer (0.4 M sodium phosphate monobasic containing 0.3 M sodium hydrosulfite and 0.1% disodium EDTA) and methanol:ethyl acetate (2:1, v:v). The supernatant was collected after centrifugation for 4 min at 16 000 g. After removing the methanol and ethyl acetate under vacuum, samples were treated in a manner analogous to plasma. All samples were analyzed using a Shimadzu HPLC system equipped with two LC-20AD pumps (Shimadzu Co, Columbia, MD), an ESA 5600A Coularray detector (Chelmsford, MA), and a Supelcosil LC18 column (4.6 x 150 mm, 5 μm particle size, Supelco, Bellefonte, PA).

Statistical Analysis

All experiments were repeated twice with at least two replicates for each treatment and time point. Maximum plasma concentrations (C_{max}) and exposure (AUC_{0→6h}) were determined using GraphPad Prism 5.0 (San Diego, CA). One-way ANOVA with Dunnett’s post-test was used to compare differences in plasma ALT, hepatic glutathione, and gene expression data. Pharmacokinetic parameters were compared using the Student’s t test. Statistical analysis was
performed using GraphPad Prism 5.0. Data are expressed as mean ± SEM and significance was reached at $P<0.05$. 
Results

Effect of EGCG pretreatment on EGCG-induced hepatotoxicity and oxidative stress

Figure 2-1. Hepatotoxic effects of acute oral bolus EGCG in naive or dietary EGCG-pretreated mice.

(A) Plasma ALT levels were determined following administration of 3 daily doses of 750 mg/kg, i.g. EGCG to male CF-1 mice pretreated with dietary EGCG [3.2 mg/g diet] for 14 days [EP] or not [NP] using spectrophotometric methods. (B) Hepatic levels of reduced glutathione [GSH] and (C) total glutathione in EP and NP mice following 3 daily doses of 750 mg/kg, i.g. EGCG using a luminescence-based assay. N = 8–16. (D) Hepatic levels of γH2AX were determined by western blot analysis in EP and NP mice following 3 daily doses of 750 mg/kg, i.g. EGCG. Expression was determined by densitometry and normalized to GAPDH. The blot shows representative expression patterns. The histogram shows quantification of all samples analyzed (N = 10 per treatment group). Error bars represent SEM. Data were analyzed by one-way ANOVA with Dunnett’s post-test (* = P < 0.05; ** = P < 0.01). All biomarkers were compared to vehicletreated control mice [NC].
Treatment of naïve male CF-1 mice (NP) with oral bolus EGCG (750 mg/kg, i.g., once daily) for 3 days resulted in an 80-fold increase in plasma ALT levels compared to vehicle-treated control mice (NC) (Figure 2-1A). By contrast, pretreatment with EGCG (3.2 mg/g diet [equivalent to 500 mg/kg, body weight total daily dose], EP) for 2 weeks reduced this elevation in plasma ALT by 75%. Treatment with oral bolus EGCG (750 mg/kg, i.g. once daily for 3 d) decreased both reduced (GSH) and total glutathione in NP mice by 57% and 33%, respectively, compared to vehicle-treated controls (Figure 2-1B and 2-1C). These reductions were ameliorated in EP mice (Figure 2-1B and 2-1C). Western blot analysis showed that the hepatic levels of the oxidative stress marker, phosphorylated histone 2AX (γ-H2AX), were increased in NP mice compared to NC mice (Figure 2-1D). By contrast, hepatic γ-H2AX expression was mitigated in EP mice.

**Hepatic expression of antioxidant responsive genes**

A panel of genes related to antioxidant response was examined in the hepatic tissue of mice treated with daily oral bolus EGCG (Figure 2-2). The expression of glutathione reductase (Gsr) and glutathione peroxidase (Gpx)2 were significantly suppressed in NP compared to NC mice (Figure 2-2). These decreases in expression were ameliorated in EP mice. In addition, hepatic expression of Gpx3, Gpx5, and Gpx7 was increased in EP mice compared to NP mice (Figure 2-2).
Figure 2-2. Effect of dietary pretreatment with EGCG on the expression of antioxidant response genes in mice following subsequent acute oral bolus dosing with EGCG.

Hepatic gene expression of the genes of interest were determined following administration of 3 daily doses of 750 mg/kg, i.g. EGCG to male CF-1 mice pretreated with 3.2 mg/g dietary EGCG for 14 days [EP] or not [NP]. Levels were determined 0.5 and 1.5 hours after the last EGCG treatment and normalized to Gapdh expression. Each bar represents the mean of N = 4–8. Error bars represent SEM. Data were analyzed by one-way ANOVA with Dunnett’s post-test (*P < 0.05, ** = P < 0.01).
Effect of EGCG pretreatment on EGCG bioavailability

Figure 2-1. Effect of dietary pretreatment with EGCG on the oral bioavailability of a single oral bolus EGCG in mice.

(A) Plasma and (B) liver levels of unconjugated EGCG were determined following a single oral bolus dose of 750 mg/kg, i.e. EGCG to male CF-1 mice pretreated with 3.2 mg/g dietary EGCG for 14 days [EP] or not [NP]. EGCG levels were determined by HPLC with electrochemical detection. Data represent the mean of N = 5–6 mice. Error bars represent SEM. Data were analyzed by one-way ANOVA with Dunnett’s post-test (*P < 0.05, ** = P < 0.01).

Table 2-3. Comparative maximum concentration and exposure to EGCG in pretreated and non-pretreated CF-1 mice.a

<table>
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<th>Parameter</th>
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<tr>
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<tr>
<td>$C_{\text{max}}$</td>
<td>µM</td>
<td>14.5 ± 7.4</td>
<td>33.7 ± 7.6</td>
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<tr>
<td>$\text{AUC}_{0\to6h}$</td>
<td>µM · h</td>
<td>36.2 ± 8.4*</td>
<td>83.3 ± 12.5</td>
</tr>
<tr>
<td>Liver</td>
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</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>µg/g</td>
<td>0.34 ± 0.11</td>
<td>0.79 ± 0.35</td>
</tr>
<tr>
<td>$\text{AUC}_{0\to6h}$</td>
<td>µg/g · h</td>
<td>0.9 ± 0.2†</td>
<td>3.1 ± 0.7</td>
</tr>
</tbody>
</table>

a Data represent the mean ± SEM.
* Indicates P < 0.05.
† Indicates P = 0.0637.
Plasma and liver concentrations of unconjugated EGCG were determined by HPLC in both EP and NP mice (Figure 2-3). We found that following a single oral bolus dose of 750 mg/kg, \( i.g. \) EGCG, the maximum plasma concentration (\( C_{max} \)) of and exposure (\( \text{AUC}_{0\rightarrow6h} \)) to unconjugated EGCG was 1.3-fold higher in NP mice compared to EP mice (Table 2-3). Similar to the plasma, EGCG \( C_{max} \) and \( \text{AUC}_{0\rightarrow6h} \) were higher in the livers of NP mice than EP mice (Table 2-3).
Discussion

In the present study we investigated the effect of dietary pretreatment with EGCG on hepatotoxicity induced by high oral bolus dose EGCG mediated in male CF-1 mice. These experiments were meant to evaluate dietary conditions which mitigate the hepatotoxic effects of high oral bolus doses of EGCG. They were also meant to model a comparison of the potential hepatotoxic effects of a high oral bolus dose of EGCG, as might be expected through use of green tea-based dietary supplements, in regular tea consumers (EP mice in this study) to naïve subjects (NP mice in this study). Such circumstances of exposure to green tea-based dietary supplements are likely to occur in free-living human subjects.

The doses of EGCG selected for the present study, 500 mg/kg total daily dose (3.2 mg/g in the diet) or 750 mg/kg, i.g. bolus dose are equivalent to a human dose of 20 or 30 mg/kg body weight, respectively, based on allometric scaling [63]. These doses are somewhat higher than the typical recommended dose of EGCG derived from commercially-available supplements (2 – 12 mg/kg/d), but are well within the range achievable in humans subjects who exceed the recommended dose either inadvertently or due to a belief that higher doses of EGCG will provide additional benefit.

Previous studies by our laboratory and others have reported that high oral bolus doses of EGCG induced liver toxicity in the livers of mice and Beagle dogs, whereas dietary dosing with up to 1000 mg/kg body weight/d was not toxic (Lambert et al. 2010; Isbrucker et al. 2006). These differences likely result from the fact that the $C_{\text{max}}$ of EGCG achieved following oral bolus dosing with EGCG is significantly higher than that achieved by the same dose given in the diet (Lambert et al. 2006; Klaus et al. 2005).

Here, we observed that oral bolus dosing with EGCG (750 mg/kg once daily with 3 d) elevated plasma ALT levels in non-pretreated (NP) mice compared to vehicle-treated controls.
These effects are consistent with our previous studies[63]. By contrast, dietary pretreatment with EGCG for 2 weeks (EP) reduced this elevation in plasma ALT by 75%, indicating a reduction in hepatotoxicity induced by oral bolus EGCG.

EGCG has been shown to exhibit pro-oxidant activity both \textit{in vitro} and \textit{in vivo} (reviewed in ([9]), and previous studies have shown that EGCG-mediated liver toxicity was associated with increased hepatic oxidative stress (Lambert et al. 2010; Galati et al. 2006). Here, we found that both reduced (57 % decrease) and total glutathione (33 % decrease) was significantly diminished in NP mice compared to vehicle-treated NC mice, which is consistent with previous studies demonstrating EGCG-mediated increases in hepatic oxidative stress. By contrast, the reductions in both GSH and total glutathione were blunted in EP mice.

Increased phosphorylation of histone 2AX (\(\gamma\)H2AX) is a marker of oxidative stress and double strand DNA breaks. Previously we found that EGCG treatment could increase hepatic levels of \(\gamma\)H2AX [63]. Consistent with this previous work, we found that NP mice exhibited increased hepatic levels of \(\gamma\)-H2AX compared to NC mice. By contrast, pretreatment with dietary EGCG mitigated this increase in hepatic \(\gamma\)-H2AX. Taken together, these results suggest that pretreatment with dietary EGCG can ameliorate oxidative stress induced by high oral bolus doses of EGCG.

In addition to blunting the expression of biomarkers of oxidative stress, dietary pretreatment also preserved or enhanced the hepatic expression of several genes related to antioxidant response in mice following treatment with a single high dose oral bolus of EGCG. Following oral bolus dosing with EGCG, hepatic expression of glutathione reductase (\(Gsr\)), glutathione-S-transferase zeta 1 (\(Gstz1\)), and glutathione peroxidase (\(Gpx\))2, \(Gpx3\), \(Gpx5\), and \(Gpx7\) was higher in mice that had been pretreated with dietary EGCG compared to those that did not receive EGCG pretreatment. Increased expression of these genes would be expected to translate into reduced hepatic oxidative stress and are similar to the results of previous studies.
Previous studies have shown that oral dosing with EGCG can modulate hepatic antioxidant gene expression and function. For example, oral bolus administration of non-toxic doses of EGCG (200 mg/kg once daily) has been shown to enhance the expression of a number of antioxidant-related genes in mice in a Nuclear factor (erythroid-derived 2)-like 2-dependent manner [81]. Another study found that dietary green tea extract could enhance glutathione-mediated detoxification of aflatoxin in piglets [86]. Taken together, the present results and these previous studies provide a putative mechanism by which dietary EGCG can mitigate the pro-oxidant effects of toxic oral bolus doses of EGCG.

Previous studies in mice and rats have indicated that longer term treatment with dietary green tea can reduce the overall plasma and tissue bioavailability of EGCG and the other green tea polyphenols [68]. These authors reported that in A/J mice given 0.6% green tea solids as the sole source drinking fluid, plasma and liver levels of EGCG increased from day 0 to day 4 and then declined over the subsequent 10 days of treatment. Consistent with these results, we found that following a single oral bolus dose of 750 mg/kg, i.g. EGCG, the plasma Cmax and AUC0→6h of unconjugated EGCG was 1.3-fold higher in NP mice compared to EP mice. Similarly, the hepatic EGCG Cmax and AUC0→6h were higher in NP mice than EP mice. These results indicate that EGCG can impact its own bioavailability and that chronic exposure can result in lower plasma and tissue levels of unconjugated EGCG.

Unconjugated EGCG has been reported to have greater biological activity than glucuronidated and methylated conjugates of EGCG [52], [87], therefore this observed decrease in bioavailability, coupled with increased expression of hepatic antioxidant genes, provides a mechanism by which dietary pretreatment with EGCG can reduce acute oral bolus-related hepatotoxicity. Further studies are needed to determine the molecular mechanism by which EGCG can modulate its own bioavailability; however, it is reasonable to speculate that EGCG may induce Phase II metabolic enzymes as well as active efflux transporters. We and others have
previously shown that the bioavailability of EGCG is modulated by uridine diphosphate glucuronyltransferases, catechol-O-methyltransferase, and the multidrug resistance-associated proteins in the liver or small intestine [17], [52], [56], [88], [89].

Previous studies have shown that these systems are inducible by other compounds [90]. Further studies are needed to determine if chronic oral EGCG can induce such changes and the extent to which such induction may influence the oral bioavailability of acute oral bolus doses of EGCG. Of note, there have been a limited number of studies that have examined the impact of repeating dosing on the bioavailability of EGCG in human subjects. Chow et al. have reported that daily dosing with 800mg EGCG for 4 weeks increased both the plasma Cmax and AUC [55]. No such increase was observed at a lower dose (400 mg per day). The underlying mechanism for these results remains unclear, and no further studies have reported similar findings. It is possible that the differences between this study in humans and our present study in mice are related to species differences in the response of inducible Phase II and active transport systems. Alternatively, the differences could result from the dosage form for the chronically administered EGCG. In our study, it was incorporated into the diet, and mice are to be exposed continuously to lower concentrations throughout the feeding period. In the human study, EGCG was administered in pill form once or twice daily. The result would be higher peak plasma concentrations and more frequent periods of very low levels. To date there have been no studies to determine the impact of such differences on bioavailability of EGCG over long periods of time.
Chapter 3

Effect of Obesity on High Dose Oral EGCG-mediated Liver toxicity

Abstract

Fatty liver disease is a co-morbidity of obesity which is characterized by increased hepatic oxidative stress and increased sensitivity to drug induced liver injury (DILI). Green tea-based dietary supplements containing high levels of the polyphenol (-)-epigallocatechin-3-gallate (EGCG) have become popular for weight loss. Case-studies have reported hepatotoxicity associated with consumption of some of these supplements and laboratory studies have shown the potential hepatotoxicity of EGCG in animals. With the growing body of evidence demonstrating the hepatotoxic potential of high oral doses of EGCG, and studies showing increased sensitivity to DILI in obese subjects, it is reasonable to hypothesize that obesity may increase susceptibility to EGCG-mediated hepatotoxicity. We tested this hypothesis in high fat-fed obese C57BL/6J mice. Obese and age-matched lean controls were treated with 0, 250, 500, or 750 mg/kg EGCG by oral gavage, once daily for 2 days. Lean and obese mice treated with 750 and 500 mg/kg EGCG suffered many casualties over the course of the treatment period. Obese mice treated with 750 mg/kg and 500 mg/kg EGCG had survival rates of 25% and 68% respectively, while lean mice had 91% and 86% survival rates. At 500 mg/kg, EGCG treatment increased plasma alanine aminotransferase (ALT) and hepatic malondialdehyde (MDA) levels to a greater extent in obese mice compared to lean, age-matched controls. By contrast, plasma ALT and hepatic MDA levels were greater in lean mice treated with 750 mg/kg EGCG compared to obese mice. EGCG treatment reduced hepatic levels of reduced glutathione more significantly in obese mice compared to lean mice. Obese mice also had significantly higher liver weights. EGCG treatment
dose dependently increased liver toxicity, makers of liver tissue and DNA damage and in both lean and obese mice.
Introduction

The incidence of obesity has increased in recent years. Also on the rise are obesity associated disorders such obesity related fatty liver disease (ORFLD) which is a potential risk factor for diabetes, cardiovascular disease and cancer [71]. As a result, many obese patients take more drugs compared to non-obese patients to manage some of the associated disorders [74]. Many drugs have been shown to induce liver injury (reviewed in [91]). Additionally there is a growing body of evidence showing that obesity and ORFLD can increase the risk of drug induced liver injury (DILI), which could make obese subjects more susceptible to DILI [74].

Obesity related DILI can occur in a few ways. Some drugs such as tamoxifen can aggravate ORFLD in subjects with a pre-existing condition, while others such as acetaminophen can induce acute hepatitis in obese subjects [92]. Obesity has been shown to increase oxidative stress in the liver due to an increase in fatty acid oxidation [93], [94]. This results in a decrease in antioxidant capacity, such as glutathione levels. When combined with drug induced oxidative stress the already reduced antioxidant capacity accelerates the progression of DILI [74].

(-)-Epigallocatechin-3-gallate (EGCG) is the most abundant and studied polyphenol in green tea [5], [95]. Many health benefits have been attributed to EGCG such as the prevention of cancer, obesity, diabetes and neurodegenerative diseases [96]. In many cases, these biological effects of green tea have been associated with the antioxidant and a pro-oxidant effects of EGCG [4], [97], [98]. EGCG has been shown to scavenge reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals as well as reactive nitrogen species such as nitric oxide and peroxynitrite in vitro [4], [9]. By contrast EGCG can also generate ROS by undergoing auto-oxidation [4].

Many studies have focused on the ability of EGCG to modulate obesity. In a review by Sae-Tan et al on the effects of green tea polyphenols on body weight and metabolic syndrome,
EGCG was shown to significantly reduce the gaining of body or adipose tissue weight in mice [29]. Many studies used mice on high-fat diets to demonstrate the anti-obesity effects of EGCG. In one study for example, mice fed a high-fat (60% of calories) diet, supplemented with 3.2 g/kg EGCG for 16 weeks showed significantly reduced body weight gain, percent body fat, and visceral fat weight compared to mice without EGCG treatment [25]. EGCG treatment also attenuated insulin resistance, and plasma cholesterol, and ameliorated high-fat-diet-induced hepatic steatosis in mice on the high-fat diet.

The effects of EGCG on body weight in human subjects have been studied in a number of small, randomized controlled trials (RCTs). Reviews and meta-analyses have been conducted by Hursel et al in (covering 11 RCTs) and by Phung et al (covering 15 RCTs). The effects of EGCG (in green tea extract containing caffeine) were studied for 8 to 12 weeks in subjects with normal weight or who were overweight. As compared with caffeine-free controls, most studies observed a lowering of body weight [99], [100]. A review by Rains et al on intervention studies showed that consumption of green tea catechins has beneficial effects on body composition [101]. A randomized placebo-controlled trial with moderately overweight Chinese subjects showed that daily consumption of 458 to 886 mg of green tea catechins (with less than 200 mg caffeine) for 90 days reduced total body fat and percent body fat [102]. Although caffeine has been shown to be a confounding variable in many of these studies, the effects of EGCG on modulating body weight are observed.

The suggested beneficial effects of EGCG against obesity have encouraged the consumption of EGCG as a dietary and weight loss supplement. Laboratory and human case reports have since made an association been these supplements and liver injury. A review by Mazzanti et al reported several instances where persons showed liver injury as result of taking EGCG supplements. Symptoms abated when supplementation ceased [45]. Laboratory studies have demonstrated the hepatotoxic effects of EGCG in mice. For example, Lambert et al showed
a dose dependent increase in toxicity in mice treated with once daily dosing of 750 mg/kg \textit{i.g.} EGCG [63]. As discussed, many people consume EGCG supplements for weight loss purposes and this can cause liver injury. Obesity has also been shown to increase instances of drug induced liver injury. For example, obesity has been shown to increase toxicity of acetaminophen, a known hepatotoxin in rats [77]. To date the potential of obesity to increase susceptibility to EGCG induced hepatotoxicity has not been studied. In the present study, I investigate the effect of obesity on EGCG mediated hepatotoxicity.
Materials and methods

Chemicals and reagents

EGCG (93% pure) was purchased from Taiyo Green Power (Wuxi, Jiangsu, China).

Diets (Table 3-1) were prepared by Research Diets, Inc. (New Brunswick, NJ).

Table 3-1. Composition of Experimental Diets.

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Table 3-1 continued

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</tbody>
</table>

*a*Mineral mix adds the following components (per g mineral mix): sodium chloride, 259 mg; magnesium oxide, 41.9 mg; magnesium sulfate, 257.6 mg; chromium K sulfate, 1.925 mg; cupric carbonate, 1.05 mg; sodium fluoride, 0.2 mg; potassium iodate, 0.035 mg; ferric citrate, 21 mg; manganous carbonate, 12.25 mg; ammonium molybdate, 0.3 mg; sodium selenite, 0.035 mg; zinc carbonate, 5.6 mg. *b*Vitamin mix adds the following components (per g vitamin mix): vitamin A palmitate, 400 IU; vitamin D3, 100 IU; vitamin E acetate, 5 IU; menadione sodium bisulfite, 0.05 mg; biotin, 0.02 mg; cyanocobalamin, 1 µg; folic acid, 0.2 mg; nicotinic acid 3 mg; calcium pantothenate, 1.6 mg; pyridoxine-HCl, 0.7 mg; riboflavin, 0.6 mg; thiamin HCl, 0.6 mg

**Animal care and treatment**

All animal studies were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University (University Park, PA, IACUC Protocol 37115). Male C57BL/6J mice (4 – 5 wks old) were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained on a 12 h light-dark cycle at 21°C and 38% relative humidity. After a 2 week acclimation period, mice were randomized into two groups and given *ad libitum* access to either a high fat (HF- 60% kcal from fat) diet or a low fat (LF – 10 % kcal from fat) diet. After 9 weeks, mice were fasted for 7 h (0700 – 1400 h) and then given a single oral bolus dose of saline (0 mg/kg) or EGCG (250, 500 or 750 mg/kg, *i.g.*) once daily for 2 days. On day 2, mice were anesthetized and blood was collected by cardiac puncture 1 hour post-gavage. Plasma was
prepared by centrifugation at 3200 g for 15 min and frozen at -80°C. The livers were harvested, washed with ice-cold PBS, a section was fixed in formalin for histopathology analysis, a 1mm³ section was fixed in modified Karnovsky’s fixative (2.5 % formaldehyde, 1.5 % gluteraldehyde, 0.1 M sodium Cacodylate buffer, 4 % sucrose) for transmission electron microscopy (TEM) analysis and the remainder was frozen at -80°C for later analysis. Formalin-fixed liver tissues were embedded, sectioned 1 micron thick and stained with hematoxylin and eosin by the Animal Diagnostics Laboratory, Pennsylvania State University (University Park, PA). The oral bolus EGCG doses (250, 500 and 750 mg/kg, body weight) are equivalent to 5, 10 and 16 cups of green tea prepared using a standard method (2.5 g tea leaves in 250 mL) [56].

**Western blot analysis**

Expression of proteins of interest was determined by western blot. Protein was extracted from liver samples using Tissue Protein Extraction Reagent (TPER) containing 1% (v/v) phosphatase and protease inhibitor cocktails (Thermo-Scientific, Rockford, IL). After homogenization and centrifugation the protein concentration in supernatant was determined by the Bradford assay (Sigma-Aldrich, St.Louis, MO). Lysates were then combined with an equal volume of laemmlie sample buffer (Bio-Rad, Hercules, CA) and denatured at 90°C for 5 min. Protein samples (75 μg protein) were resolved by SDS-polyacrylamide (15 %) gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were probed with primary antibody for protein of interest (1:1000 dilution) (Cell Signaling Technology, Danvers, MA) overnight at 4 °C. After incubation with a fluorescent-conjugated secondary antibody (1:10, 000 dilution) (Li-Cor Co., Lincoln, NE), bands were visualized using a Licor Odyssey Imaging System (LI-COR Corporate). Band density was
quantified using Odyssey Application Software Version 3.0. Beta actin was used as a protein loading control.

**Determination of hepatotoxicity**

Plasma alanine aminotransferase (ALT) levels were determined spectrophotometrically \( (\lambda_{\text{max}} = 340 \text{ nm}) \) using a commercially-available assay (CATACHEM Bridgeport, CT) according to the manufacturer’s instructions.

Images of hematoxylin and eosin stained liver tissue taken using under microscope were visually analyzed for instances of inflammation, necrosis, apoptosis, hepatic lipidosis and hemorrhage. Each image, 5 each representing 9 – 12 livers per treatment group was scored on a scale from 0 – 4 based on increase in severity for each pathology. Scores were averaged according to diet and EGCG treatment to determine overall severity of the different pathologies.

**Examination of Mitochondrial Structure**

Formaldehyde/glutaraldehyde-fixed samples were washed in 0.1 M Cacodylate buffer, 10 mins each. Liver samples were incubated for 1 hour in 1 – 2 % Osmium tetroxide in 0.1 M Cacodylate buffer to fix lipids in the sample. This was followed by 2 washes with 0.1 M Cacodylate buffer and 1 wash in milliQ water. En Bloc staining for 1 hour in 2 % Uranyl acetate was performed followed by dehydration for 5 – 15 min each in 50, 70, 85, 90, 95, and 100% ethanol. Three washes for 5 – 15 mins each in EM grade ethanol came next, followed by three washes with acetone, also for 5 – 15 mins each. Samples were infiltrated with Eponate resin
overnight (50% acetone, 50% Eponate). Resin was removed and samples were infiltrated with 100% Eponate resin three times each for 2 hours. Samples were placed in molds and covered with fresh 100% resin and allowed to polymerize overnight at 60 °C. Blocks containing samples were first trimmed and cut into 50–100 nm thick sections with a Leica UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany). Slices were collected on mesh grids, stained with 3.4 mg/mL lead citrate for 12 mins, rinsed and allowed to dry prior to imaging with Transmission electron microscope.

TEM images were analyzed using NIH Image J software. Area of mitochondria was determined by tracing each whole mitochondrion in the field, and then averaged per field and number of samples. Density was determined as the number of mitochondria per field and averaged based on number of fields per sample. Lipid droplets per field were counted and averaged per sample. Disruption of cristae was determined based on visual analysis of fields and scored a scale of 0–4, with 0 representing absolutely no disruption, 1 = 0–25% disruption, 2 = 25–50% disruption, 3 = 50–75% disruption and 4 = 75–100% disruption. These values were averaged according to diet and EGCG treatment.

**Measurement of hepatic oxidative stress**

Malondialdehyde (MDA) levels were determined using a standard Thiobarbituric acid reactive substances test modified from McDonald and Hultin 1987. Briefly, liver homogenate prepared for western blot analysis (see above) were also used for MDA analysis. Homogenate was combined with 6 volume of 100:3 ratio of A (15% TCA and 0.375% (w/v) in 0.25M HCl) and B (2% BHT in ethanol (w/v)). Mixture was heated at 95 °C for 15 mins and cooled on ice for 10 mins. Sample was centrifuged at 14 000 g for 15 mins and then incubated for 10 mins at room
temperature. Absorbance was measured at 532 nm using a plate reader. Quantification was made based on a MDA standard curve.

Reduced glutathione levels were determined using a modified HPLC method from [103]. Briefly, liver tissue (10 – 30 mg) was perfused with PBS containing heparin (0.15 mg/mL). Tissue was homogenized in 1 – 2 mL PBS containing 2 mM EDTA, centrifuged at 13, 000 g for 10 mins at 4 °C, and the supernatant was collected. The supernatant was combined with 2 vol of PBS and 3 vol of 1 % metaphosphoric acid, and centrifuged at 13, 000 g for 10 mins at 4 °C. The resultant supernatant was analyzed by HPLC. Samples were resolved using a SUPELCOSIL LC-18 column (15 cm x 4.6 mm x 5 µm) and an isocratic mobile phase of 50 mM NaH₂PO₄ (pH 3.0). The column was maintained at 30 °C, the flow rate was 0.3 mL/min and the injection volume was 10 µL. GSH detection was carried out on a four channel ESA 5600 CoulArray detector (ESA Inc. Chelmsford MA) with increasing potentials of 250, 400, 600, and 800 mV applied. Quantified was based on comparison to a standard curve of GSH in metaphosphoric acid at 600 mV.

Statistical Analysis

All experiments were repeated twice with at least three replicates for each treatment. GraphPad Prism 5.0 (San Diego, CA) was used for statistical analysis. In this experiment two outcomes were focused on; the effect of varying doses of EGCG and the difference between diets at each dose of EGCG. To determine differences between control (0 mg/kg) and varying doses of EGCG (250, 500, and 750 mg/kg), one-way ANOVA with Dunnett’s post-test was used to compare differences in plasma ALT, hepatic glutathione, MDA levels, liver weight and severity of tissue damage induced by different doses of EGCG, (* = P < 0.05, ** = P < 0.01, *** = P < 0.001). The Student’s t-test was used to compare differences between diets (HF and LF) at each
dose of EGCG, (# = P < 0.05, ## = P < 0.01, ### = P < 0.001). Data are expressed as mean ± SEM and significance was reached at P<0.05.
Results

Effect of EGCG on general markers of toxicity

**Figure 3-1.** Survival rate and changes in body weight of male C57BL/6J mice following treatment with EGCG.

Mice were fed either a high fat or low fat diet for mice 9 weeks and then treated with 0, 250, 500, or 750 mg/kg b.w. EGCG once daily for 2 days.

At the end of the first day of treatment lean and obese mice given 0 and 250 mg/kg EGCG, and lean mice given 500 mg/kg EGCG suffered no casualties (100% survival) (Figure 3-1). By contrast, obese mice given 500 and 750 mg/kg EGCG, and lean mice given 750 mg/kg EGCG had 77%, 67%, and 91% survival rates, respectively. After the second day of treatment survival rates for lean and obese mice given 0 and 250 mg/kg EGCG stayed the same at 100%, but lean mice given 500 mg/kg had an 86% survival rate. Lean mice given 750 mg/kg EGCG had a 91% survival rate. By contrast, obese mice given 500 and 750 mg/kg EGCG had rates of 68% and 25%. Body weight for male C57BL/6J mice treated with 0, 250, 500, or 750 mg/kg b.w. EGCG were higher in obese mice compared to lean mice. Obese mice treated with 750 mg/kg EGCG showed the largest decrease in body weight over the 2 day treatment period, but it was not
significant. Obese mice treated with 250 and 500 mg/kg also showed decreasing trends in body weight, but these too were not significant. Body weight of obese control mice remained constant. An insignificant decreasing trend in body weight can be seen in lean mice treated with 750 and 500 mg/kg EGCG. Lean mice treated with 0 and 250 mg/kg EGCG remained fairly constant over the 2 day period.

Effect of EGCG on general markers of liver toxicity

**Figure 3-2.** Plasma alanine aminotransferase levels and liver weight in male C57BL/6J mice treated with oral EGCG.

ALT levels and liver weights were determined following administration of 2 once daily doses of EGCG by oral gavage. Each bar represents the mean of N= 8-18 and error bars represent SEM. ALT levels represent mice treated with 0, 250, 500, or 750 mg/kg b.w. EGCG. Liver weights represent mice treated with 0, 250 or 500 mg/kg b.w. EGCG. Mice were fed either a low fat (LF) or a high fat (HF) diet. Liver tissue was weighed immediately after it was excised for the mouse. For liver weight each bar represents the mean of N= 6 – 10. Error bars represent SD. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test and compared to controls (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) and t-test for diets (# = P < 0.05, ## = P < 0.01, ### = P < 0.001).
Alanine aminotransferase (ALT), a marker of liver injury was measured in plasma samples of lean and obese mice treated with 0, 250, 500, 750 mg/kg EGCG (Figure 3-2). A dose dependent increase was observed in lean mice. ALT levels increased 50 and 150 fold at the 500 and 750 mg/kg dose respectively, in lean mice. In mice fed a HF diet a significant increase in ALT can only be seen in mice given a 500 mg/kg dose, (80 fold) when compared to controls. A 55 fold increase occurred for mice that received 750 mg/kg EGCG, but it was not significant. The decrease seen at the 750 mg/kg dose in obese mice could be as a result of the mice that succumbed to EGCG treatment during the study, so plasma from these subjects were not available for analysis at that dose (refer to survival data, Figure 3-1). These mice potentially had higher levels of ALT compared to the ones that survived, hence the reason they did not survive, and could account for why the remaining mice had lower ALT levels. There were no significant differences between diet groups at the different doses of EGCG. Treatment with EGCG had no significant effect on liver weights of obese or lean mice. In general obese mice had higher liver weights than lean mice with significant differences seen in mice treated with 250 and 500 mg/kg EGCG. At these two doses obese mice had significantly higher liver weights than lean mice.
Histological evaluation of the hepatotoxic effect of EGCG

Figure 3-1. Representative images of histopathological analysis of stained liver tissue from male C57BL/6J mice.

Mice were fed either a low fat (LF) or a high fat (HF) diet and treated with 0, 500, or 750 mg/kg b.w. EGCG. Tissue was fixed in formalin and slices were stained with hematoxylin and eosin for imaging and analysis. Images are shown at 200 x magnification. Arrows indicate blood vessels (central veins) and circles indicate area necrotic tissue.
Images of liver tissue stained with hematoxylin and eosin were analyzed to examine the effect of treatment with EGCG. Representative images (Figure 3-3) show normal liver tissue for control obese and lean mice (HF-0 and LF-0). Clear and defined cellular structures are present as well as blood vessels such as portal and central veins (denoted by arrows). Effects of EGCG can be seen in the HF-500 image, where the circled area denotes the presence of necrosis and hemorrhage. Compared to HF-500, the image for LF-500 does not show any effects of EGCG. Effects of EGCG can be seen in the images for both LF-750 and HF-750, indicated by circled regions. Based on these images a dose dependent effect of EGCG can be seen, but is not sufficient to demonstrate the extent of EGCG toxicity. Quantification of the markers of liver tissue damage will provide a more comprehensive analysis of the hepatotoxic effects of EGCG (Figure 3-4).

**Figure 3-4.** Quantification of markers of liver tissue damage based on histopathological analysis.

Inflammation, necrosis, apoptosis, hemorrhage, hepatic Lipidosis were assessed on a scale of 0-4 for images of each section, where 0 was no presence and 4 was highest presence. Severity was determined as the average presence within a treatment group. Each bar represents the mean of N=
Quantification of stained liver tissue severity of inflammation, necrosis, apoptosis, hepatic lipidoses and hemorrhage, showed an overall dose dependent increase in severity for all pathologies. Both obese and lean mice showed significant increases in inflammation (Figure 3-4) at the 750 mg/kg dose compared to the controls (0 mg/kg). The increase in severity of tissue necrosis was significant for both the 500 and 750 mg/kg doses in lean and obese mice compared to controls. EGCG caused significant increases in apoptosis at both the 500 and 750 mg/kg doses compared to controls for mice fed a HF diet. Mice fed a LF diet showed a significant increase in apoptosis only when given a 750 mg/kg dose of EGCG. There was a dose dependent increase in hemorrhaging for mice fed both a HF and LF diet. The increase was only significant for mice given a 750 mg/kg dose of EGCG. Hepatic lipidoses increased significantly for both diet groups. There was a significant increase for the obese mice only at the 750 mg/kg dose, but for the lean mice the increase was significant at both the 500 and 750 mg/kg doses. No significant differences were observed between HF treated mice and LF treated mice at the different doses of EGCG.
Effect of EGCG on Mitochondrial Structure

Figure 3-5. Representative images from transmission electron microscopy (TEM) analysis of liver tissue of male C57BL/6J mice.

Mice were fed either a low fat (LF) or a high fat (HF) diet and treated with 0, or 500 mg/kg b.w. EGCG. Liver tissue was fixed formaldehyde and glutaraldehyde, treated with Osmium tetroxide, negatively stained with Uranyl acetate, dehydrated with ethanol, infiltrated with resin, polymerized, sectioned, stained positively with lead citrate and imaged. Images are shown at 1000 x magnification. Arrows indicate fat droplets. Circles indicate mitochondria.

Transmission electron microscopy enabled us to obtain images of the mitochondria in obese and lean mice treated with 0, or 500 mg/kg EGCG at 1000 x magnification (Figure 3-5). These images were analyzed using Image J software and for lean and obese mice treated with 0 mg/kg EGCG the most significant difference observed is in the lipid droplet content. Lipid droplets, indicated with an arrow, are in a larger quantity in obese mice when compared to lean
mice. At that dose, the density of mitochondria; mitochondria are indicated with a circle, appears to be higher in lean mice. At 500 mg/kg EGCG, obese mice showed a higher number of lipid droplets compared to lean mice and a higher mitochondrial density. Changes in mitochondrial area and density, disruption of cristae, and number of lipid droplets are shown in Figure 3-6.

![Figure 3-6](image)

**Figure 3-6.** Quantification of mitochondrial parameters from TEM analysis.

Images were analyzed for mitochondrial area, density, number of lipid droplets, and cristae disruption. Quantification was done using Image J software. Each bar represents the mean of N=5-10 fields. Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test and compared to controls (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) and t-test for diets (# = P < 0.05, ## = P < 0.01, ### = P < 0.001).

The areas of the mitochondria were measured for mice fed both diets that received the doses of EGCG (Figure 3-6). Although not significant, the data shows a decreasing trend in areas of mitochondria of obese mice and an increasing trend in areas of mitochondria of lean mice. There was a significant difference in areas between lean and obese not treated with EGCG.
Obese mice had significantly larger mitochondria when compared to lean control mice. Treatment with 500 mg/kg EGCG yielded no difference between mice.

Another parameter measured was the density of mitochondria in the images obtained (Figure 3-6). Mitochondrial density defined as the number of mitochondria per image revealed no difference for obese mice at the various doses of EGCG. Lean mice, however, had significantly less mitochondria per image at 500 mg/kg EGCG, when compared to controls (P < 0.05). Also at this dose, obese mice had significantly more mitochondria than lean mice.

The cristae of the mitochondria, which are formed by the inner mitochondrial membrane, were analyzed to measure how intact they were. Disruption of cristae is a marker of mitochondrial dysfunction. There was no significant difference in obese mice from 0 to 500 mg/kg EGCG. Lean mice showed a decreasing trend, but it was not significant. When compared, control lean mice appear to have higher levels of cristae disruption compared to their obese counterparts. This difference however, was not significant. No significant difference was seen between lean and obese mice for the 500 mg/kg dose of EGCG.

The number of lipid droplets in each image was measure for lean and obese mice. Figure 3-6 shows that obese mice had significantly lower number of lipid droplets at 500 mg/kg when compared to controls. There was no significant difference for lean mice in the number of lipid droplets at the different doses of EGCG, but an increasing trend from 0 to 500 mg/kg EGCG can be seen. Additionally, HF control mice had a significantly higher number of lipid droplets when compared to lean control mice.
Effect of EGCG on Markers of Oxidative Stress

Hepatic levels of reduced glutathione (GSH) were lower in obese mice compared to mice fed a LF diet regardless of the EGCG treatment (Figure 3-7). This difference between diets was significant only at the 0 and 500 mg/kg doses (P < 0.001 and P < 0.05 respectively). EGCG treatment caused an insignificant dose dependent decrease in levels of GSH in lean mice. Similar results were observed in obese mice from 250 to 750 mg/kg EGCG, but that too was not significant.

![Graph showing GSH levels](image)

**Figure 3-7.** Hepatic levels of reduced glutathione (GSH) were measured using HPLC and normalized to tissue weight.

Each bar represents the mean of N= 8-18. Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test and compared to controls, and t-test for diets (# = P < 0.05, ## = P < 0.01, ### = P < 0.001).

Phosphorylated histone 2Ax, a marker for DNA oxidative damage increased in a dose dependent manner for mice in both diet groups (Figure 3-8). No expression of γH2Ax was observed at the low dose, 250 mg/kg. A significant increase (P < 0.01) was seen for mice given a 750 mg/kg dose of EGCG in both diet groups when compared to controls.
Figure 3-8. Hepatic levels of γ-H2AX determined by western blot analysis.

γ-H2AX was determined in mice following 2 daily doses of 0, 250, 500, or 750 mg/kg, i.g. EGCG. Expression was determined by densitometry and normalized to β-actin. The blot shows representative expression patterns. The histogram shows quantification of all samples analyzed (N = 8-18 per treatment group). Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test and compared to controls (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) and t-test for diets.

Levels of MDA in liver tissue which correlated to the extent of lipid peroxidation in cells increased significantly for mice in both diet groups (Figure 3-9). The increase was dose dependent for mice on a LF diet with those given a 750 mg/kg dose having the most significant increase compared to controls. Mice on a HF diet had a significant increase in MDA levels when given a 500 mg/kg dose of EGCG. This increase was also significantly higher than mice on the LF on that dose. An increase was observed for HF mice given a 750 mg/kg dose of EGCG, but it was not significant.
MDA was measured using the standard thiobarbituric acid-reactivity test as a marker of lipid peroxidation and peroxidative tissue injury. Measurements were normalized to tissue protein concentrations. Each bar represents the mean of N= 8-18. Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test and compared to controls (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) and t-test for diets (# = P < 0.05, ## = P < 0.01, ### = P < 0.001).
Discussion

In the present study we examined the effect of obesity on EGCG mediated hepatotoxicity. Survival data shows that EGCG treatment induced toxicity most in obese mice treated with 750 mg/kg, evident by a 25% survival rate at the end of the two day treatment when compared to all other mice (Figure 3-1). Toxic effects were also seen in obese mice treated with 500 mg/kg EGCG as they had a survival rate of 68% at the end of the treatment period. When compared to the lean mice at the 500 and 750 mg/kg doses the survival data shows that obese mice had lower survival rates indicating that they were more susceptible to EGCG toxicity.

Using a decrease in body weight as a general marker of toxicity, it can also be seen in the data that obese mice treated with 750 mg/kg EGCG showed the most decrease in body weight (Figure 3-1). Decreases in body weight after the 2 day treatment period was evident for all obese mice except controls. When compared to lean mice EGCG treatment caused slight decreases only in lean mice treated with 500 and 750 mg/kg EGCG. This also indicates that obese mice were more affected by the EGCG treatment.

As previously stated, obesity has been shown to increase instances of drug induced liver injury. We examined the effects of EGCG treatment on general markers of liver injury. Plasma alanine aminotransferase (ALT) levels increased for both diet groups at the 500 and 750 mg/kg doses of EGCG (Figure 3-2). This data is consistent with findings from previous studies [63]. At the 500 mg/kg dose of EGCG, mice on a HF diet had higher levels of plasma ALT compared to lean. Though not significant, this difference indicates that HF mice were more susceptible to the toxic effects of EGCG at that dose. This is in keeping with studies that have shown that obesity can increase drug induced liver injury. In contrast, at the 750 mg/kg dose, EGCG appeared to have more toxic effects on mice fed a LF diet. Based on the survival data, obese mice treated with 750 mg/kg EGCG had a low survival rate (25%) at the end of the 2 treatment period. The loss of
mice during this time decreased the number of samples obtained for plasma based analyses, but not liver tissue based analyses. As a result this affected the changes seen at the 750 mg/kg dose for markers measured using plasma samples. The death of these mice suggests they succumbed to EGCG mediated toxicity at this dose, which is not reflected in the data due to loss of these mice. In the case of ALT levels it can be seen at the 750 mg/kg dose of EGCG, obese mice had lower levels compared to lean mice. This could be explained by the fact that obese mice given a 750 mg/kg dose of EGCG and having higher levels of plasma ALT, succumbed to the toxic effects prior to completion of the study. As a result these measurements could not be reflected in data, thus showing lower ALT levels at this dose, compared to the higher levels that were expected.

Liver weight data showed no differences between obese and lean control mice. However, when treated with 250 and 500 mg/kg EGCG obese mice had significantly higher liver weight, indicative of larger livers. This can be attributed to obese mice developing hepatomegaly as a result of EGCG treatment. Hepatomegaly has been shown to be increased in instances of DILI [104]. This data is consistent with that obtained so far, and supports the conclusion that EGCG has caused more liver injury in obese mice compared to lean mice.

Representative images of stained liver tissue of obese and lean mice treated with 0, 500, or 750 mg/kg EGCG showed markers of liver tissue damage evidenced by signs of hemorrhaging, the presence of apoptotic bodies, necrosis, inflammation and hepatic lipidosis. Based on images, the severity of tissue damage increased with dose of EGCG in both diet groups, Figure 3-3. These observations correlate well with data from previous studies done in our lab, where EGCG caused a dose dependent increase in liver tissue damage [63], [105]. However, no discernable differences were observed between diet groups based solely on images.

Data obtained from quantification of histological analysis supported the observations seen in tissue images. It can be seen from Figure 3-4 that EGCG caused significant increases in severity of tissue inflammation, necrosis, apoptosis, hemorrhage and hepatic lipidosis in mice fed
HF and LF diets. Closer examination of the data showed that there was no difference between obese and lean mice in severity of inflammation when mice received 0, 500, or 750 mg/kg dose of EGCG, indicating that EGCG affected mice on both diets in much the same way when triggering an inflammatory response. A similar effect was seen in the severity of tissue necrosis caused by EGCG, whereby there were no significant differences between mice on HF diet or LF diet.

The effect of EGCG on the severity of apoptosis, though not significant, can be seen to vary between lean and obese mice at the different doses of EGCG. Mice on HF diet recorded no incidence of apoptosis when they received 0 mg/kg EGCG. This observation is interesting to note as obesity has been shown to induce apoptosis in both liver tissue and skeletal muscle [106], [107], but this was not observed here. We expected to see more significant differences in apoptosis between obese and lean mice because of the ability of an obese diet to induce apoptosis, and when compounded with the toxic effects of EGCG, the severity would be higher in obese mice, but we did not see that. Obesity has been shown to increase blood flow to the liver as a result of increased lipid accumulation. Consequently, this increased blood flow and increased fat deposition compromises blood vessels and increases the risk of rupture [73]. This suggests that obese mice are at increased risk of tissue hemorrhage. However, we observed no significant differences between lean and obese mice.

In mice treated with acetaminophen, a known hepatotoxin, hepatic lipidosis was shown to increase along with hepatic necrosis [108]. Similar results were seen in this study, confirming the hepatotoxic effects of EGCG. As expected high fat fed control mice had higher levels of hepatic lipidosis. Interestingly, the severity of hepatic lipidosis in mice fed a LF diet was not significantly different from HF fed mice as one would expect. Several studies have shown that a high carbohydrate diet can also lead to lipid accumulation in the liver and contribute to nonalcoholic fatty liver disease (NAFLD) [109][110] [111]. Additionally, the type of carbohydrate in the diet
has also been shown to cause fat accumulation in the liver with rapidly absorbed carbohydrates (RAC) contributing more significantly to hepatic steatosis [112]. Based on the diet composition from Table 3-1 it can be seen that mice on LF diet had significantly more carbohydrates in their diet than mice on HF diet, (LF = 70 kcal from carbs, HF = 20 kcal from carbs). Also from Table 3-1 we see that the LF diet has 315 g/kg corn starch vs 0 g/kg for the HF diet. Corn starch is made up mostly of amylopectin, which is a RAC, and as studies have shown, these contribute significantly to fat accumulation in the liver. The high carbohydrate content may only be part of the reason we see no differences between lean and obese mice at the 500 and 750 mg/kg doses, because lean control mice had no hepatic lipidosis. It could be that EGCG treatment exacerbates hepatic lipidosis in mice on a high carbohydrate diet.

To get a better understanding of the hepatotoxic effects of EGCG, we examined the effect of EGCG on mitochondrial structure. Transmission electron micrographs of treated liver tissue were assessed for changes in mitochondria area, density, circularity, lipid droplet size and disruption of mitochondria cristae. EGCG has been shown to increase mitochondrial ROS and trigger mitochondrial dysfunction [18]. Changes in mitochondrial structure may be able to confirm the hepatotoxic capabilities of EGCG. Mitochondrial are constantly undergoing fusion and fission, which can impact their size and density at any given time, illustrated in Figure 3-10.
Fusion and fission work to maintain the mitochondrial function especially under environmental stress such as an increase in ROS. While fusion results in larger, more elongated mitochondria, fission results in fragmented or smaller mitochondrial, and the rates typically balance each other [114], [115]. Fusion of mitochondria requires intact membranes and stable membrane potential, interruption of fusion can lead to increased fission. This can be induced by ROS or apoptosis [116]. Fusion appears to play a role in protecting cells from cell death or in mitigating stress, in that mitochondria with damaged DNA or defects can fuse with other mitochondria to allow complementing of DNA for improved function [114]. Fission is generally triggered by apoptosis, and can result from loss of mitochondrial membrane potential. This can lead to swelling and opening of mitochondrial permeability transition pore, and is necessary for the removal of damaged mitochondrial in times of stress, and for the formation of new mitochondria [115]–[117]. Non-apoptotic cells treated with 3.3, 9.8 or 16.3 mM hydrogen
peroxide for 4, 24 and 48 hours showed a dose dependent increase in fragmentation after 4 hours of treatment. After 24 hours, there was intermediate fragmentation and some recovery of mitochondria, evident by the increase in tubular (normal) shaped mitochondria. 48 hours of treatment showed recovered mitochondria [118]. In the same study, treatment with hydrogen peroxide caused an increase in both fission and fusion, and an increase in cristae disruption. An increase in spherical mitochondrial was also seen and was associated with increase fragmentation.

Our examination of mitochondrial area in electron microscopy images showed no significant differences in obese mice treated with 0 or 500 mg/kg EGCG. Obese control mice showed significantly larger mitochondrial areas compared to lean control mice. Since there is no effect of EGCG treatment to consider, the difference seen could be an effect of diet. Larger mitochondrial areas generally indicate the mitochondria are undergoing fusion. Fusion has been shown to be an adaptive mechanism when some mitochondria are damaged, and before they are destroyed they can complement other mitochondria [114]. In this case, stress brought on by an obese diet could be causing some mitochondrial damage, but not sufficient to induce mitophagy, so the mitochondria are probably complementing each other in response to the stress and as result they have larger areas.

Mitochondrial density, indicative of the number of mitochondria on the image, showed no differences in obese mice with respect to EGCG treatment. Lean mice given 500 mg/kg showed a significant decrease in density compared to control, which can indicate an increase in mitochondrial fusion. As previously discussed, increased mitochondrial density is an indication of mitochondrial fusion. This can occur when there is a need for mtDNA complementation. In this case, EGCG treatment could have caused changes in mitochondrial oxidative environment resulting in mtDNA damage, and warrant mitochondrial fusion. The significant difference observed between lean and obese mice at 500 mg/kg EGCG could also be explained by a difference in the fusion and fission cycles at that point.
Cristae disruption is a marker of mitochondrial dysfunction. Here we see that there is no effect of EGCG on cristae disruption in obese mice, which suggests the EGCG had no effect on obese mice. This conflicts with the data we have obtained thus far, which clearly indicates the hepatotoxic effects of EGCG. In lean mice, although a slight decrease in cristae disruption is observed, it is not significant.

The number of lipid droplets was determined from the TEM images. Obese mice showed a significant decrease in the number of lipid droplets at 500 mg/kg EGCG, when compared to control. This could be as a result of the anti-obesity effects of EGCG, as seen in previous studies, where EGCG was shown to reduce lipid accumulation and increase fecal lipid content in high fat fed mice [28]. Also seen, is a significant difference in the number of lipid droplets between obese and lean controls. This data is supported by the TEM images (Figure 3-5) and shows that for the samples chosen obese mice had more lipid droplets. This is also consistent with hepatic lipidosis data (Figure 3-4).

Hepatic levels of reduced glutathione (GSH) were evaluated as a marker for oxidative stress. Previous studies have shown that EGCG-mediated liver toxicity was associated with increased hepatic oxidative stress [85][63]. Here, we found that EGCG dose dependently depleted GSH levels in both diet groups (Figure 3-7). This data is consistent with previous studies [105]. When comparing GSH levels between diets, GSH levels were significantly lower in mice fed a HF diet. Studies have shown that fatty acid oxidation is a source of ROS in fatty livers, which causes oxidative stress and can lead to depleted GSH levels in cells [74], [78], [119]. Our findings support what has been seen in the literature.

Phosphorylation of histone 2Ax (γ-H2Ax) occurs as a result of double strand breaks to DNA, and is used a biochemical marker for DNA damage. In this study we measured protein expression of γ-H2Ax to assess the effect of EGCG treatment on cellular DNA as a marker of liver toxicity. As can be seen in Figure 3-8, EGCG caused a dose dependent increase in
phosphorylated histone 2Ax. Quantification of the bands corroborates the data seen in the western blot images. These results suggest that EGCG caused double strand breaks in cellular DNA. DNA damage has been shown to induce apoptosis and necrosis [120], both of which were previously seen to increase dose dependently by EGCG treatment. These findings are not only consistent with previous studies [63], but support other data obtained in the study. No differences were observed between diet groups at the different doses of EGCG, suggesting that EGCG treatment affected mice on either diet in a similar manner.

Lipid peroxidation of fatty acids in cells has been shown to be initiated by ROS and exacerbated by oxidative stress. This results in the formation of aldehydes among other compounds which can be used as markers for oxidative stress in tissue [121]. Malondialdehyde, one of the by-products of lipid peroxidation was measured in this study to determine the magnitude of oxidative stress in liver tissue. We found that MDA levels increased significantly in both obese and lean mice (Figure 3-9). MDA levels have been shown to increase in liver tissue by known hepatotoxicant-acetaminophen, and this has been used as a marker for acetaminophen induced toxicity [122]. Lambert et al have also showed that EGCG increased MDA levels in mice treated with high doses of EGCG [63]. Our data confirms that EGCG caused significant oxidative stress in mice fed an HF or LF diet. As seen with the ALT data, mice on a HF diet that received a 500 mg/kg dose of EGCG had significantly higher levels of MDA than LF fed mice. Obesity related fatty livers have been shown to have increased levels of MDA because of the presence of excess fatty acids, which increase ROS and levels of oxidative stress [78]. These effects as we have seen are worsened by EGCG and can explain why HF mice have higher levels of MDA. Similar to ALT data, at the 750 mg/kg the increase in not as significant, and is lower that of the 500 mg/kg dose. These results can be explained in much the same way, in that the death of obese mice treated with 750 mg/kg EGCG did not make it to the end of the study, due to severe liver damage, could not be included in the analysis, and as such were not represented.
In summary EGCG induced hepatotoxicity in a dose dependent manner in both lean and obese mice. Toxicity in the mice was evident in the decrease in survival rates in both lean and obese mice. Hepatotoxicity was characterized by an increase in liver tissue injury seen in increased ALT levels and tissue damage. EGCG treatment also increased oxidative stress in liver tissue seen by depleted glutathione levels and increase phosphorylated histone 2Ax and malondialdehyde levels. Survival data, liver weight data, GSH data, as well ALT and MDA data all indicate that obese mice, particularly mice treated with 750 mg/kg EGCG were more susceptible to EGCG treatment when compared to lean mice.
Chapter 4

Effect of High Dose Oral (-)-Epigallocatechin-3-gallate (EGCG) on Hepatic Mitochondrial Antioxidants, Biogenesis and Function

Abstract

(-)-Epigallocatechin-3-gallate (EGCG) has been shown to induce hepatotoxicity in a dose dependent manner in vivo. The underlying mechanism of these effects has been poorly studied. In vitro, EGCG was shown to cause cytotoxicity by inducing mitochondrial reactive oxygen species ROS and mitochondrial dysfunction. In this study we examined the effects of EGCG on factors involved in mitochondrial biogenesis and function as well as mitochondrial antioxidant gene expression and activity in an effort to shed light on the underlying mechanism of high dose oral EGCG-mediated hepatotoxicity. Additionally, we explored the effects of obesity in potentially exacerbating mitochondrial dysfunction. We found that EGCG significantly decreased mRNA expression of mitochondrial superoxide dismutase in lean mice when treated with 750 mg/kg, but not obese mice. EGCG treatment significantly decreased mRNA levels of catalase in both lean and obese mice, and catalase activity in obese mice, while both lean and obese mice showed significant decreases in protein expression of glutathione peroxidase. EGCG treatment significantly decreased mRNA levels of glutathione-S-transferase zeta 1 and peroxiredoxin 3. Metallothionein gene and protein expression was significantly upregulated by EGCG in both lean and obese mice. EGCG treatment caused significant down regulation of antioxidant gene regulators, Siruin 3 and forkhead box O3 and mitochondrial biogenesis regulator peroxisome proliferator-activator receptor coactivator-1a. Mitochondrial transcription factors A, B1 and B2 were significantly reduced by EGCG, as well as complex I subunit Ndufs 8 and complex III
subunit Uqcr 1. Mitochondrial uncoupling protein 2 was significantly upregulated. These data suggests that EGCG depletes mitochondrial antioxidant capacity by decreasing expression and activity of antioxidant enzymes and their regulators, and inhibits mitochondrial biogenesis and function by down regulating the regulator and transcription factors at the gene expression level.
**Introduction**

Green tea polyphenol (-)-Epigallocatechin-3-gallate (EGCG) has been widely studied for its numerous health benefits. EGCG has been shown to be effective in reducing fat accumulation and modulating effects of metabolic syndrome in laboratory models; it has demonstrated profound anti-inflammatory effects and shown to be effective in treatment and prevention of cancer [9], [123].

Many of the cancer chemopreventative effects of EGCG have been attributed to its ability to function as both an antioxidant and a pro-oxidant \textit{in vitro} and \textit{in vivo} [4], [9]. As an antioxidant EGCG has been shown to scavenge reactive oxygen species (ROS) and chelate transition metals such as iron \textit{in vitro}, which are capable of triggering the Fenton reaction and initiating lipid peroxidation [4], [8]. In its pro-oxidant capacity, EGCG has been shown to undergo auto-oxidation and generate ROS, which can among other things induce apoptosis in cancer cells [4], [97], [124].

Another property of EGCG that has been well-regarded is its anti-obesity effects. Several studies have highlighted the role of EGCG in the modulation of obesity and its comorbidities such as obesity related fatty liver disease (ORFLD). Grove \textit{et al} showed that EGCG inhibited digestive enzyme pancreatic lipase, and reduced body weight gain in high fat fed mice. Sae-Tan \textit{et al} showed that EGCG increased expression of genes related to fatty acid oxidation in high fat fed mice. Bose \textit{et al} showed that in high fat fed mice supplemented with 0.32% EGCG for 16 weeks, EGCG was able to ameliorate high fat-diet induced ORFLD [28], [29][25].

These beneficial properties of EGCG have led to its increased use in forms such as dietary supplements, as it has generally been considered natural and safe. Case reports, however, have since made an association between instances of liver injury and high concentrations of green tea polyphenols found in these formulations [45], [125]. Cellular and \textit{in vivo} studies have
confirmed the hepatotoxic potential of EGCG. Galati et al showed the cytotoxic effects of EGCG in isolated rat hepatocytes, where it collapsed mitochondrial membrane potential and generated ROS [85]. Lambert et al showed the hepatotoxic effects of EGCG in mice treated with a single daily dose of 1500 mg/kg i.g. or 2 daily doses of 750 mg/kg i.g, which increased tissue necrosis, plasma alanine aminotransferase (ALT) and markers of oxidative stress [63].

In a recent study by Tao et al., the cytotoxic effects of EGCG in oral cancer cells were shown to be mediated by the induction of mitochondrial ROS and mitochondrial dysfunction [18]. Qanungo et al also showed that EGCG induced mitochondrial membrane depolarization and caspase-dependent apoptosis in pancreatic cancer cells [126]. The mitochondria plays an important role in the energy requirements of the cell, and through the electron transport chain (ETC) it has been shown to be an endogenous source of ROS in the cell [127], [128]. As such, damage to the mitochondrial has been implicated in a number of pathogeneses such as drug induced liver injury (DILI) and non-alcoholic fatty liver disease (NAFLD) [91], [129].

Hepatotoxic drugs can cause mitochondrial dysfunction if a variety of ways, including direct inhibition of mitochondrial respiration, damage to mitochondrial DNA and most commonly transformation of the parent compound into reactive metabolites the can generate ROS [91]. There are many antioxidant systems in the mitochondria to combat ROS, and inhibition of any of these systems can increase oxidative stress and lead to injury and dysfunction. These include the glutathione and thioredoxin redox systems and antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and peroxiredoxin [130]. Acetaminophen, for example, has been shown to cause liver injury by generating toxic metabolites at high doses, which covalently bind to DNA proteins and depleting GSH levels which increases ROS. This leads to increased mitochondrial oxidative stress, opening of the mitochondrial permeability transition pore and eventually mitochondrial dysfunction [131]–[134].
Mitochondrial dysfunction has been implicated in the progression of NAFLD. Mitochondrial dysfunction affects lipid homeostasis in the liver that can lead to an overproduction of reactive oxygen species (ROS) that trigger lipid peroxidation, cytokine overproduction and cell death [135], [136]. Rector et al. showed that hepatic mitochondrial dysfunction as determined by reduced hepatic carnitine palmitoyl-CoA transferase-1 activity, fatty acid oxidation, and cytochrome c protein in an obese mouse model preceded insulin resistance and hepatic steatosis [137]. Liver tissue from individuals with non-alcoholic steatohepatitis was analyzed using electron microscopy and it was found that 9 out of 10 subject exhibited mitochondrial dysfunction as evidence by loss of mitochondrial cristae [138]. Thyfault et al. showed that rats bred for low aerobic capacity had reduced hepatic mitochondrial oxidative capacity and showed increased susceptibility to hepatic steatosis[139].

In the previous chapter we demonstrated the hepatotoxic effects of EGCG. The underlying mechanism associated with EGCG mediated hepatotoxicity remains unclear. The mitochondria have been shown to be a target of EGCG induced ROS in inducing cytotoxicity in cancer cells. The effects of EGCG mediated liver toxicity on factors involved in mitochondrial antioxidant capacity, mitochondrial function and biogenesis in vivo, have not been studied. Additionally, the effect of obesity on these factors in EGCG mediated toxicity remains unclear.

In this study, I investigate the effects of high dose EGCG on mitochondrial antioxidant capacity, function and biogenesis, as well as the effect of obesity in potentially exacerbating the hepatotoxic potential of EGCG.
Materials and methods

Animal studies

The analyses reported in this Chapter used samples derived from C57BL6/J mice treated in Chapter 3. In brief, male C57BL/6J mice (4 – 5 wks old, Jackson Laboratory, Jackson, MS) were randomized into two groups and were on either a high fat (HF - 60% kcal from fat) diet or a low fat (LF – 10% kcal from fat) diet. Following a 9 week period on the respective diets, mice were fasted for 7 h (0700 – 1400 h) and then given a single dose of saline (0 mg/kg) or EGCG (250, 500 or 750 mg/kg, i.g.) once daily for 2 days. On day 2, 1 hour post-gavage, mice were anesthetized and blood was collected by cardiac puncture. Plasma was prepared by centrifugation at 3200 g for 15 min and frozen at -80°C.

Gene expression studies

Primers for quantitative reverse transcriptase PCR q RT-PCR (Table 4-1) were synthesized by the Genomics Core Facility at The Pennsylvania State University (University Park, PA). RNA was isolated from liver tissue using Tri-reagent (Sigma Chemical Co. St. Louis, MO) according to the manufacturer’s instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer. cDNA was reverse transcribed and amplified using SYBR Green PCR Master Mix and an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Relative gene expressions were normalized to the house-keeping genes GAPDH for HF samples and 18S for LF samples. Data was represented by fold change in Ct values.
Table 4-1. Primer sequences for qPCR analysis of gene expression in liver tissue.

<table>
<thead>
<tr>
<th>Gene Abbreviation</th>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
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<tr>
<td>Cat</td>
<td>Catalase</td>
<td>AGCGACCAGATGAAGCAGTG</td>
<td>TCCGCTCTCTGTCAAAAGTG</td>
</tr>
<tr>
<td>Gpx1</td>
<td>Glutathione Peroxidase 1</td>
<td>GATGGAGCCCATTCCGAA</td>
<td>CCCTGTACTTATCCAGGCAAG</td>
</tr>
<tr>
<td>Sod2</td>
<td>Superoxide dismutase 2</td>
<td>CCGAGGAGAAAGTACCAG</td>
<td>GCTTGATAGCCTCCAGCAAC</td>
</tr>
<tr>
<td>Nrf1</td>
<td>Nuclear Respiratory factor 1</td>
<td>TATGGCAGAATGAAAGACG</td>
<td>CAACGTAAGCTCTGCCTTG</td>
</tr>
<tr>
<td>Pgc-1a</td>
<td>Peroxisome proliferator-activator receptor coactivator-1a</td>
<td>ACAGCCGTAGGCCCAGGTAC</td>
<td>GCCTTTCTGCTCATAGGC</td>
</tr>
<tr>
<td>Sirt3</td>
<td>Sirtuin 3</td>
<td>ATCCCGGACTTCAGATCCCAG</td>
<td>CAACATGAAAAAGGGGTT</td>
</tr>
<tr>
<td>Foxo3a</td>
<td>forkhead box O3</td>
<td>ACAAACGGCTCAGTCCAG</td>
<td>AGCTTTGCTGCTCAGTCCCTCATTCTG</td>
</tr>
<tr>
<td>Nrf2</td>
<td>nuclear factor (erythroid-derived 2)-like 2</td>
<td>CAACTCGGCGAAGAAAGAACA</td>
<td>AGGATACTGGGATTCGTC</td>
</tr>
<tr>
<td>Tfam</td>
<td>Mitochondrial Transcription Factor A</td>
<td>CACCCAGATGCCAAAACCTTCAG</td>
<td>CTGCTCTITTATATCTGCTACAG</td>
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### Table 4-1 Continued

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<th>Gene</th>
<th>Description</th>
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<th>Sequence 2</th>
</tr>
</thead>
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<tr>
<td><strong>Tfb1m</strong></td>
<td>Mitochondrial Transcription Factor B1</td>
<td>ATAGAGCCCAAGATCAAGCAG</td>
<td>TGTAACAGCCTTCCAGTGC</td>
</tr>
<tr>
<td><strong>Tfb2m</strong></td>
<td>Mitochondrial Transcription Factor B2</td>
<td>ACCAAAACCATCCCGTC</td>
<td>TCTGTAAGGGCTCAAATG TG</td>
</tr>
<tr>
<td><strong>Complex I (Ndusf8)</strong></td>
<td>Complex I (Ndusf8)</td>
<td>GTTCATAGGGTCAGAGTC AAG</td>
<td>TCCATTAAGATGTCTCTGTG CG</td>
</tr>
<tr>
<td><strong>Complex III (Uqrc1)</strong></td>
<td>Complex III (Uqrc1)</td>
<td>ATCAAGGCACTGTCCAAAG G</td>
<td>TCATTTTCCTGCATCTCCCG</td>
</tr>
<tr>
<td><strong>Mt-I</strong></td>
<td>Metallothionein 1</td>
<td>GCTGTCTCTCAAGCTAC AC</td>
<td>AGGAGCAGGCTCTTTCT TT G</td>
</tr>
<tr>
<td><strong>Mt-II</strong></td>
<td>Metallothionein 2</td>
<td>CAAAACCGATCTCTCGTC GAT</td>
<td>AGGAGCAGGCTTTTTCT TT G</td>
</tr>
<tr>
<td><strong>Mtf-1</strong></td>
<td>Metallothionein transcription factor 1</td>
<td>CAGTCGGAATGTCCAGAA ACG</td>
<td>CTGCACGTCACACTCAAAT GG</td>
</tr>
<tr>
<td><strong>Gapdh</strong></td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>TGAAGCAGGCATCTGAGG G</td>
<td>CGAAGGTGGAAGAGTGGA AG</td>
</tr>
<tr>
<td><strong>18s</strong></td>
<td>18S ribosomal RNA</td>
<td>GTAACCGTTGAACC CCTT</td>
<td>CCATCCAAATCGGTAGTGAGC G</td>
</tr>
<tr>
<td><strong>CoxIII</strong></td>
<td>Cytochrome c oxidase subunit III</td>
<td>CGTGAAGGAACCTACCAA GG</td>
<td>ATTCCTGTTGGAGGTCA GC A</td>
</tr>
<tr>
<td><strong>Gstz1</strong></td>
<td>Glutathione S Transferase Zeta 1</td>
<td>TCAGGAGGTACGAGTGTC TC</td>
<td>TCAGGAGGTACGGGCTGTC</td>
</tr>
<tr>
<td><strong>Ucp2</strong></td>
<td>Uncoupling protein 2</td>
<td>GAGCGGACCACCTCAGCG TC</td>
<td>TCACCACATCCGTTGGGCTG G</td>
</tr>
</tbody>
</table>
Western blot analysis

The expression of proteins of interest was determined by western blot. Protein was extracted from liver samples using Tissue Protein Extraction Reagent (TPER) containing 1% (v/v) phosphatase and protease inhibitor cocktails (Thermo-Scientific, Rockford, IL). After homogenization and centrifugation the protein concentration in supernatant was determined by the Bradford assay (Sigma-Aldrich, St.Louis, MO). Lysates were then combined with an equal volume of laemmli sample buffer (Bio-Rad, Hercules, CA) and denatured at 90°C for 5 min. Protein samples (75 μg protein) were resolved by SDS-polyacrylamide (15 %) gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were probed with primary antibody for protein of interest (1:1000 dilution) (Cell Signaling Technology, Danvers, MA) overnight at 4 °C. After incubation with a fluorescent-conjugated secondary antibody (1:10,000 dilution) (Li-Cor Co., Lincoln, NE), bands were visualized using a Licor Odyssey Imaging System (LI-COR Corporate). Band density was quantified using Odyssey Application Software Version 3.0. Beta actin was used as a protein loading control.

Antioxidant enzyme activity

Glutathione peroxidase (GPx)

Glutathione peroxidase activity was determined using a commercially available assay kit from Cayman Chemicals (Ann Arbor, MI). In brief, 50 mg of liver tissue was homogenized in 300 μL homogenization buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT) and centrifuged at 10,000 g for 15 mins 4 °C. The supernatant was collected and assayed as per
manufacturer’s instructions. Absorbance was measured at 340 nm and quantification was based on a standard curve.

**Superoxide dismutase (SOD)**

Superoxide dismutase activity was determined using a previously reported method [140]. In brief, 100 mg of tissue was homogenized in 500 µL of homogenization buffer (20 mM Hepes, 1 mM EGTA, 210 mM, 70 mM sucrose; pH 7.2) and centrifuged at 1,500 g for 5 mins at 4 °C. The supernatant was removed diluted 1:50. A volume of 0.1 mL of diluted sample (or standards) was added to a reaction mixture (2.8 mL) containing: 0.05 M Na₂CO₃ (pH 10.2), 3mM EDTA, 1.5 mg/mL BSA, 0.75 mM NBT, and 3 mM Xanthine was added. The reaction was initiated with 0.1 mL xanthine oxidase (1:200 dilution) and incubated for 20 minutes at room temperature. The reaction was terminated by addition of 6 mM CuCl₂. The inhibition of NBT reduction was determined spectrophotometrically at 560 nm.

**Catalase Activity**

Catalase activity was determined as described by [141]. Briefly, 50 mg of liver tissue were minced in ice-cold 25 mM KH₂PO₄-NaOH buffer, pH 7.0. The homogenates were then diluted with buffer as appropriate. Purified catalase or homogenized tissues were incubated with 5.9 M methanol and 4.2 mM hydrogen peroxide in 250 mM KH₂PO₄-NaOH buffer, pH 7.0 in a 96 well plate. The enzymatic reaction was initiated by addition of 100 µL of a catalase-containing sample. The reaction mixture was incubated with continuous shaking for exactly 20 min at room temperature. The enzymatic reaction was terminated by addition of 50 µL 7.8 M potassium hydroxide solution to each tube. Immediately thereafter the tubes were treated with 100 µL each
of 34.2 mM Purpald in 480 mM hydrochloric acid, and a second incubation with continuous shaking was performed for 10 min at 20°C. To obtain a colored compound, the product of the reaction between formaldehyde and Purpald was oxidized by adding 50 µL 65.2 mM potassium periodate in 470 mM potassium hydroxide to each tube. Absorbance was measured at 540 nm and quantified using a catalase standard curve.

**Statistical Analysis**

All experiments were repeated twice with at least three replicates for each treatment. GraphPad Prism 5.0 (San Diego, CA) was used for statistical analysis. In this experiment two outcomes were focused on; the effect of varying doses of EGCG and the difference between diets at each dose of EGCG. To determine differences between control (0 mg/kg) and varying doses of EGCG (250, 500, and 750 mg/kg), one-way ANOVA with Dunnett’s post-test was used to compare differences in enzyme activity, protein expression and gene expression for different doses of EGCG, (* = P < 0.05, ** = P < 0.01, *** = P < 0.001). The Student’s t-test was used to compare differences between diets (HF and LF) at each dose of EGCG, (# = P < 0.05, ## = P < 0.01, ### = P < 0.001). Data are expressed as mean ± SEM and significance was reached at P<0.05.
Results

Effect of EGCG on Antioxidant Response

Figure 4-1. Effect of EGCG on hepatic gene expression, protein expression and activity of superoxide dismutase 2 (Sod2) in male C57BL/6J mice.

Mice were treated with 0, 250, 500 or 750 mg/kg b.w. EGCG once daily for 2 days. Gene expression was normalized to GAPDH for mice on HF diet and 18S for mice on LF diet. Protein expression was normalized to β-actin. Each bar represents the mean of N = 8-18. Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) and t-test for diets.

Examination of the key components of the hepatic antioxidant defense system showed that EGCG treatment significantly down regulated mitochondrial superoxide dismutase (Sod2) in lean mice treated with 750 mg/kg dose of EGCG (Figure 4-1), but no significant changes were observed in mice fed a HF diet. No changes in protein expression were seen for any of the treatment groups based on band expression. Quantification of band intensity showed a significant increase only for mice on a LF diet that received a 750 mg/kg dose of EGCG. EGCG treatment
caused decreases in *Sod* activity in both lean and obese mice given a 750 mg/kg dose of EGCG when compared to controls, but the decrease was not significant.

**Figure 4-2.** Effect of EGCG on hepatic gene expression, protein expression and activity of catalase in male C57BL/6J mice.

Mice were treated with 0, 250, 500 or 750 mg/kg b.w. EGCG once daily for 2 days. Gene expression was normalized to GAPDH for mice on HF diet and 18S for mice on LF diet. Protein expression was normalized to β-actin. Each bar represents the mean of N = 8-18. Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) and t-test for diets.

EGCG treatment caused an initial upregulation in catalase gene expression at the 250 mg/kg dose (Figure 4-2) followed by a significant dose dependent down regulation in catalase for mice on both LF and HF diets. Quantification of protein expression levels showed that catalase tended to decrease in all treatment groups when compared to controls, but the changes observed were insignificant. Catalase activity in obese mice was significantly decreased in mice treated with 250, 500, and 750 when compared to controls. No differences were seen in lean mice following EGCG treatment.
Figure 4-1. Effect of EGCG on hepatic gene expression, protein expression and activity of glutathione peroxidase in male C57BL/6J mice.

Mice were treated with 0, 250, 500 or 750 mg/kg b.w. EGCG once daily for 2 days. Gene expression was normalized to GAPDH for mice on HF diet and 18S for mice on LF diet. Protein expression was normalized to β-actin. Each bar represents the mean of N = 8-18. Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) and t-test for diets (# = P < 0.05, ## = P < 0.01, ### = P < 0.001).

EGCG treatment caused no observable changes in gene expression of glutathione peroxidase 1 (Gpx1) (Figure 3-10 A), however, significant decreases in the protein expression of Gpx1 can be seen for both obese and lean mice treated with a 750 mg/kg dose of EGCG. Obese mice treated with 500 mg/kg EGCG also showed a significant decrease in Gpx1 protein expression compared to controls (P < 0.05). At the 500 mg/kg dose obese mice had significantly lower protein expression levels compared to lean mice. A similar pattern is seen at the 750 mg/kg dose, but the difference was not significant. Total glutathione peroxidase activity showed an initial but insignificant increase in Gpx1 activity for 250 mg/kg EGCG, followed by a dose dependent decrease, in both lean and obese mice, but it was not insignificant.
Figure 4-4. Effect of EGCG on hepatic gene expression of glutathione-S-transferase zeta 1 and peroxiredoxin 3 in male C57BL/6J mice.

Mice were treated with 0, 250, 500 or 750 mg/kg b.w. EGCG once daily for 2 days. Gene expression was normalized to GAPDH for mice on HF diet and 18S for mice on LF diet. Each bar represents the mean of N = 8-18. Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) and t-test for diets.

Other components of the cell’s antioxidant defense system, glutathione transferase zeta 1 (Gstz1) and peroxiredoxin 3 (Prx3) were measured to assess the effect of varying doses of EGCG. An increase in Gstz1 gene expression was observed in mice given a 250 mg/kg dose of EGCG (Figure 4-4), similar to results seen for catalase expression. However, Gstz1 was down regulated at the 500 and 750 mg/kg doses in both lean and obese mice, and this was significant only for mice on a LF diet. EGCG treatment caused a dose dependent decrease in expression of Prx3 in mice on HF diet. This down regulation was significant for mice given a 750 mg/kg dose. A down regulation of Prx3 was also seen in lean mice for both the 250 and 500 mg/kg doses of EGCG, but these changes were not significant when compared to the controls. Interestingly, an increase in Prx3 expression was seen for mice on LF diet given a 750 mg/kg dose, but this change was not significant when compared to controls.
Figure 4-5. Changes in metallothionein expression in male C57BL/6J mice.

Gene expression of metallothionein transcription factor 1 (MTF-1), metallothionein I (MT-1), and metallothionein II (MT-2) and protein expression and quantification of MT-1 and MT-2 (MT I/II) were measured. Mice were treated with 0, 250, 500 or 750 mg/kg b.w. EGCG once daily for 2 days. Gene expression was normalized to GAPDH for mice on HF diet and 18S for mice on LF diet. Protein expression was normalized to β-actin. Each bar represents the mean of N = 8-18. Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) and t-test for diets (# = P < 0.05, ## = P < 0.01, ### = P < 0.001).

Metallothioneins are low molecular weight sulphur-rich proteins that play a role in heavy metal detoxification and metal homeostasis. EGCG treatment caused a significant upregulation in gene expression of metallothionein transcription factor MTF-1 for both lean and obese mice (Figure 4-5). For obese mice the upregulation appears to occur in a dose dependent manner, with mice given a 750 mg/kg dose having the most significant upregulation (P < 0.05) when compared to controls. Mice on a LF diet had the most significant upregulation of MTF-1 when they
received 500 mg/kg EGCG. In general, lean mice exhibited higher expression levels of MTF-1 when compared to obese mice, with this difference being significantly highest in mice given 500 mg/kg EGCG.

The expression of MT-I was upregulated significantly by EGCG treatment at the 250 and 500 mg/kg doses for obese mice when compared to controls. Similar results were seen for lean mice, but an increase in MT-I expression was most significant for mice on a 500 mg/kg EGCG. Although there was upregulation of MT-I at the 750 mg/kg dose for both diets, this change was not significant when compared to the controls. No significant differences were seen between the diets at the various doses of EGCG.

The effect of EGCG on MT-II was similar to that seen for MTF-1. EGCG caused a dose dependent upregulation of MT-II gene expression in both diet groups which was significant in mice given 500 and 750 mg/kg EGCG. Expression of MT-II in lean mice is higher than obese mice, but the difference between diets at all the doses was not significant. Protein expression of metallothioneins supported the trends seen in MTF-1 and MT-II gene expression, for mice on LF diet. EGCG treatment caused an increase in the expression of MTs which was significant in mice given a 750 mg/kg dose (P < 0.05). Obese mice had significantly lower expression of MTs when compared to controls at the 250 and 500 mg/kg dose. A significant difference in the protein expression of MTs can also be seen between diets. Lean mice expressed significantly higher protein levels of MTs when compared to HF fed mice when given 500 and 750 mg/kg EGCG (P < 0.01).
Effect of EGCG on regulators of antioxidant genes

Figure 4-6. Effect of EGCG on hepatic gene and protein expression of Sirtuin 3 (Sirt3) in male C57BL/6J mice.

Mice were treated with 0, 250, 500 or 750 mg/kg b.w. EGCG once daily for 2 days. Gene expression was normalized to GAPDH for mice on HF diet and 18S for mice on LF diet. Protein expression was normalized to β-actin. Each bar represents the mean of N = 8-18. Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) and t-test for diets.

Hepatic mRNA expression of the mitochondrial NAD-dependent deacetylase, sirtuin-3 (Sirt3), was significantly down-regulated in mice treated with 750 mg/kg dose of EGCG when compared to controls (Figure 4-6). Although a decrease was seen at the 500 mg/kg dose for both diets, this down regulation was not significant. Interestingly, an upregulation was observed at the 250 mg/kg dose of EGCG in both lean and obese mice. This increase in Sirt3 expression was higher in obese mice compared to lean mice, but the difference was not significant. Western blot analysis showed a dose dependent decrease in protein expression of Sirt3, which was significant in obese mice. Quantification of the bands corroborated what we saw in the images. In mice treated with 500 and 750 mg/kg EGCG, obese mice showed lower protein expression when compared to lean mice, but the differences were not significant.
Figure 4-7. Effect of EGCG on the hepatic gene expression of forkhead box O3 and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) in male C57BL/6J mice.

Mice were treated with 0, 250, 500 or 750 mg/kg b.w. EGCG once daily for 2 days. Gene expression was normalized to GAPDH for mice on HF diet and 18S for mice on LF diet. Each bar represents the mean of \( N = 8-18 \). Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test (\( * = P < 0.05, ** = P < 0.01, *** = P < 0.001 \)) and t-test for diets (\( # = P < 0.05, ## = P < 0.01, ### = P < 0.001 \)).

Other mitochondrial antioxidant regulators assessed were Foxo3a and Nrf2 (Figure 4-7). Foxo3a mRNA expression was significantly dose dependently reduced by EGCG in both lean and obese mice. No significant differences in Foxo3a expression was observed between HF and LF mice at the different doses of EGCG, except in mice given a 500 mg/kg dose. A decrease in Nrf2 expression can be seen in lean and obese treated with 750 mg/kg EGCG, but the differences were not significant when compared to controls. No differences were observed in mice treated with other doses of EGCG when compared to controls.
Effect of EGCG on mitochondrial biogenesis

Figure 4-8. Effect of EGCG on the hepatic gene expression of nuclear respiratory factor 1 (Nrf1) and peroxisome proliferator-activator receptor coactivator-1a (Pgc-1α) in male C57BL/6J mice.

Mice were treated with 0, 250, 500 or 750 mg/kg b.w. EGCG once daily for 2 days. Gene expression was normalized to GAPDH for mice on HF diet and 18S for mice on LF diet. Each bar represents the mean of N = 8-18. Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) and t-test for diets.

Nuclear respiratory factor 1 is a key transcription factor in mitochondrial biogenesis. It regulates mitochondrial transcription factor A. Hepatic mRNA levels of Nrf1 followed an increasing trend as a function of EGCG dose up to 500 mg/kg, but decreased at 750 mg/kg dose in both obese and lean mice (Figure 4-8). There was no significant difference in Nrf1 expression between lean and obese mice. EGCG treatment caused a significant dose dependent decrease in Pgc-1α expression in both lean and obese mice treated with 500 and 750 mg/kg EGCG.

Mitochondrial DNA copy number is an indicator of mitochondrial biogenesis. Obese mice treated with EGCG showed a decreasing trend in mtDNA copy number from 0 to 500 mg/kg, but the change was not significant. Lean mice treated with 500 mg/kg EGCG showed a significant decrease in mtDNA copy number when compared to controls. No difference in Mt copy was seen at the other doses of EGCG when comparing lean and obese mice.
Figure 4-9. Effect of EGCG on mitochondrial copy number in male C57BL/6J mice.

Gene expression was normalized to GAPDH for mice on HF diet and 18S for mice on LF diet. Each bar represents the mean of N= 8-18. Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) and t-test for diets.

Effect of EGCG on factor involved in mitochondrial function

Mitochondrial transcription factor A (Tfam), is a transcription factor that initiates mitochondrial transcription during replication and is a regulator of MtDNA copy number. Tfam was significantly down regulated in lean and obese mice given 750 mg/kg EGCG when compared to controls (Figure 4-10). At the 250 and 500 mg/kg, EGCG appeared to increase the expression of Tfam, but this change was not significant.
Gene expression was normalized to GAPDH for mice on HF diet and 18S for mice on LF diet. Each bar represents the mean of N= 8-18. Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) and t-test for diets.

Mitochondrial transcription factors B1 and B2 (Figure 4-10) are dimethyltransferases that methylate mitochondrial ribosomal RNA. They are also involved in mitochondrial transcription, which is important for mitochondrial gene expression. EGCG treatment caused an increase in $Tfb1m$ mRNA levels at the 250 mg/kg dose level, although the effect was not statistically significant. At the higher dose levels, EGCG reduced the expression of $Tfb1m$. The effect reached statistical significance at 750 mg/kg. $Tfb2m$ showed a similar expression pattern to that seen for $Tfb1m$ and $Tfam$. The down regulation in $Tfb2m$ was significant at both the 500 and 750 mg/kg doses of EGCG for obese mice (P < 0.01 and P < 0.001), and at 750 mg/kg EGCG for lean mice (P < 0.05). Upregulation of $Tfb2m$ by EGCG was also observed at the 250 mg/kg dose. No difference in $Tfb2m$ expression between diets was seen at the different doses of EGCG, except for the 500 mg/kg dose, where LF mice had higher expression levels. This; however, was not significant.
Figure 4-11. Effect of EGCG on the hepatic gene expression of mitochondrial Complex I and mitochondrial complex III in male C57BL/6J mice.

Gene expression was normalized to GAPDH for mice on HF diet and 18S for mice on LF diet. Each bar represents the mean of N = 8-18. Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) and t-test for diets (# = P < 0.05, ## = P < 0.01, ### = P < 0.001).

Mitochondrial complex I and III are key parts of the mitochondrial electron transport chain, which are encoded in MtDNA and are important sites of electron leakage. The subunits analyzed were shown to be affected by EGCG in much the same way as other factors involved in mitochondrial replication, transcription and function. Gene expression of Ndusf 8, a subunit of mitochondrial complex I was significantly down regulated by EGCG treatment at the 500 and 750 mg/kg doses (Figure 4-11). At the 750 mg/kg dose, obese mice showed a significantly higher expression level of Ndusf 8 when compared to lean mice at the 750 mg/kg dose of EGCG (P < 0.05). Like Ndusf 8, Uqcre 1, a subunit of mitochondrial complex III also showed a decrease in gene expression. For lean mice, down regulation of Uqcre 1 was significant at 750 mg/kg EGCG, and at 500 mg/kg for obese mice. Additionally, obese mice also exhibited significantly higher levels of Uqcre 1 expression when given 750 mg/kg EGCG, compared to lean mice (P < 0.05).
**Figure 4-12.** Effect of EGCG on mitochondrial uncoupling protein 2 (*Ucp2*) in male C57BL/6J mice.

Gene expression was normalized to GAPDH for mice on HF diet and 18S for mice on LF diet. Each bar represents the mean of N= 8-18. Error bars represent SEM. Data were analyzed by one-way ANOVA based on dose with Dunnett’s post-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) and t-test for diets (# = P < 0.05, ## = P < 0.01, ### = P < 0.001).

The effects of EGCG treatment on gene expression of mitochondrial uncoupling protein 2 (*Ucp2*), which separates oxidative phosphorylation from ATP synthesis in an attempt to reduce ROS production, showed a dose dependent increase in lean and obese mice (Figure 4-12). Upregulation of *Ucp2* was most significant for obese mice that received 500 and 750 mg/kg EGCG. Additionally, obese mice showed significantly higher expression of Ucp 2 when given 250 and 500 mg/kg EGCG, compared to lean mice.
Discussion

In the previous chapter, we examined the effect of obesity on EGCG induced liver toxicity. We saw increases in markers of hepatotoxicity and oxidative stress. As stated previously, work coming out of our lab showed that EGCG induces mitochondrial ROS and subsequent dysfunction in cancer cells (Tao et al 2014). This suggests that the mitochondria may play a significant role in EGCG toxicity. In the current study we investigated the dose dependent effect of EGCG treatment on mitochondrial antioxidant responsive genes, mitochondrial biogenesis and mitochondrial function in C57BL/6J mice.

Figure 4-11. Scheme of mitochondrial antioxidant pathways and gene regulators.
Figure 4-13 shows the location of antioxidant genes and the regulatory transcription factors in the mitochondria. We examined the changes in mRNA, protein expression, and/or enzyme activity of these factors. Superoxide dismutase along with catalase and glutathione peroxidase is essentially the cell’s first line of defense against reactive oxygen species. In this study we determined the effect of EGCG treatment on gene expression, protein expression, and enzyme activity of these three enzymes; as well as the effect of diet. Mitochondrial superoxide dismutase (Sod2) has been shown to be down regulated in cases of hepatotoxicity or extreme oxidative stress. Compounds such as cadmium and acetaminophen, known to cause hepatotoxicity have been shown to cause a decrease in Sod2 expression [142], [143] and several other key antioxidant genes were shown to be significantly down regulated in cancer cells treated with EGCG, in which EGCG caused cytotoxicity (Tao et al. 2014).

The results of our study are consistent with the findings of these previous studies. Gene expression of Sod2 decreased in a dose dependent manner for mice in both diet groups. No difference in EGCG toxicity was seen for mice on either diet, from 0-500 mg/kg EGCG. At the 750 mg/kg dose of EGCG, a dose shown to cause hepatotoxicity, mice on LF diet showed significantly lower expression of Sod2 when compared to obese mice. Protein expression revealed something different from the gene expression data. For mice on HF diet there were no significant changes in expression for any of the doses of EGCG. LF mice on the other hand showed an increase in protein expression from 250 – 750 mg/kg EGCG. This increase in Sod2 expression we observed, maybe an effort by the cells to compensate for the increased oxidative environment. Superoxide dismutase activity was suppressed most significantly at the 750 mg/kg dose of EGCG for both diet groups. This could be as a result of changes to regulating factor Sirt3, which regulates Sod activity, where a decrease in Sirt3 expression was observed in mice treated with 750 mg/kg EGCG. The effect of EGCG on catalase gene expression was consistent with that
of Sod2, except for an increase at the 250 mg/kg dose. This increase was significant for obese mice and could be an adaptive mechanism by the cell to a change in the oxidative environment. As the dose of EGCG increases there is a significant down regulation of catalase for mice in both diet groups. This is consistent with data reported by Tao et al, where a down regulation of catalase was observed in cancer cells treated with EGCG [18]. Other hepatotoxins such as furan and methapyrilene have also been shown to down regulate catalase expression [144]. Protein expression of catalase though not significant supported gene expression data and is consistent with studies done using other hepatotoxins, namely acetaminophen and amiodarone, and showed that EGCG causes a decrease in catalase expression [145].

Glutathione peroxidase 1 (Gpx1), like catalase, is a peroxide scavenger and is the most abundant isozyme in the Gpx family [146]. In determining the effect of EGCG treatment on Gpx1 gene expression, protein expression and activity, we found no effect of EGCG at the gene level, as there were no changes in expression levels from dose to dose. This contrasts with previous work by Tao et al who had shown a down regulation of Gpx1 by EGCG treatment in cancer cells. Additionally, acetaminophen has been shown to downregulate Gpx1 [144]. As was not observed in gene expression, we found that protein expression of Gpx1 showed a significant decrease. This data is consistent with other studies on the impact of hepatotoxins on Gpx1 protein expression. Yamamoto et al., showed that acetaminophen, amiodarone and tetracycline all cause hepatotoxicity and treatment with these compounds cause a decrease in Gpx1 expression [145]. We also observed that at the higher doses of EGCG, expression of Gpx1 was significantly lower in obese mice compared to mice on LF diet. As was previously discussed, obesity causes oxidative stress, which results in depleted levels of reduced glutathione (GSH). Gpx1 forms part of the glutathione redox system where it used GSH to detoxify peroxides [147]. In our earlier results we saw that the levels of GSH in obese mice were significantly lower than LF mice; therefore this depletion of GSH can explain why Gpx1 expression is lower in HF mice. Further,
studies have shown that obesity causes reduced \( \text{Gpx1} \) expression [148]. A measure of GPx activity showed a decrease in enzyme activity. This decrease however, was not significant and there was no clear difference between diet groups.

Another component of the glutathione system is glutathione transferase which catalyzes the conjugation of GSH to xenobiotic compounds during phase II metabolism (Pickett 1989). We measured the gene expression of glutathione transferase zeta 1 (\( \text{Gstz1} \)) in this study, as it was shown to be down regulated by EGCG treatment in cancer cells [18]. We too saw a down regulation of \( \text{Gstz1} \) by EGCG treatment in this study, which confirms an increase in oxidative stress.

Mitochondrial localized peroxiredoxin 3 (\( \text{Prx3} \)) is a thiol containing endogenous antioxidant that scavenges up to 90% of the hydrogen peroxide produced in the mitochondria [146]. Peroxiredoxins are generally overexpressed under oxidative stress conditions [149], which makes them efficient peroxide scavengers; but they can become hyperoxidized under prolonged exposure to hydrogen peroxide. Hyperoxidation can inactivate \( \text{Prxs} \) and lead to the accumulation of the oxidized form [150]. \( \text{Prx3} \) has been shown to be hyperoxidized more slowly than other peroxiredoxins, but can be over oxidized under oxidative stress conditions [151], [152]. Our results show a downregulation of \( \text{Prx3} \) with increasing doses of EGCG. EGCG treatment was shown to cause prolonged oxidative stress in cells, and as a result hyperoxidation could be a possible explanation for the observed down regulation. Since \( \text{Prx3} \) is regulated at the gene expression level, another possible explanation for \( \text{Prx3} \) gene down regulation could be inactivation or down regulation of its transcription factors; \( \text{Foxo3a, Nrf2} \) and \( \text{Pgc1α} \), which we observed to be down regulated by EGCG [153]. There were no differential effects of diet on EGCG mediated hepatotoxicity.

Metallothioneins (MTs) are heavy metal binding proteins that traditionally have been associated with detoxification of heavy metals [154]. Recent studies have shown that
metallothioneins have other biological functions not related to metal detoxification and are also
induced by oxidative stress. MTs play a role in protecting the cell from ROS by scavenging
oxxygen free radicals [154], [155]. MT-I and MT-II are the inducible isoforms of MTs and they
are regulated by the transcription factor MTF-1 in response to oxidative stress [156], [157]. MTs
complement the GSH/GSSG redox cycle and are significantly upregulated in instances of GSH
depletion and superoxide dismutase down-regulation [155]. We investigated gene and protein
expression of MTs to determine the effect of EGCG treatment in mice fed HF and LF diets. MTF-
1 increased significantly for both diet groups from 0 – 750 mg/kg EGCG. This increase was dose
dependent for mice on a HF diet and supports previous data that EGCG induces oxidative stress.
For mice on LF diet the upregulation of MTF-1 was highest a 500 and 750 mg/kg doses, again
supporting our previous findings. MT-I expression increased with increasing doses of EGCG,
except at the 750 mg/kg dose, where a decrease in expression was observed and not a continuous
increase as would be expected. Down regulation of MT-I was seen to occur in hepatocellular
carcinoma cells lines when the MT promoter was extensively methylated [158]. Methylation of
the MT promoter could provide an explanation for the down regulation of MT-I seen at the 750
mg/kg dose in both diets. MT-II expression followed a similar trend to that of MTF-1, where
EGCG caused a significant dose dependent increase in expression for both diets. Protein
expression of MTs supported the data seen for both MTF-1 and MT-II. Other hepatotoxicants
such as acetaminophen have also been shown to increase metallothionein expression [159]. All
of these data suggest that EGCG causes a dose dependent increase in oxidative stress, which
correlates to a dose dependent increase in MT gene and protein expression and is consistent with
previous findings and studies.

Analysis of the metallothionein data showed that mice on a high fat diet consistently
expressed lower levels of MTs at the different doses of EGCG, when compared to LF fed mice.
In a study conducted by Waller-Evans et al [160] an analysis of gene expression responses to HF
feeding in C57BL/6J showed a downregulation of genes encoding for MT-I and MT-II. This response was specific to this mouse strain. These results can help explain the differences between diet groups that were observed in this study [160].

Mitochondrial NAD-dependent deacetylase sirtuin-3 (Sirt3) has been implicated in regulation of mitochondrial function and antioxidant activity [161]. Sirt3 is activated by Pgc-1α and through their combined efforts they modulate oxidative stress in the cell [129], [162], [163]. Sirt3 has been shown to regulate oxidative stress by activating mitochondrial antioxidant enzymes such as Sod2 and Gpx1 and possibly Prx 3; all of which showed significant down regulation by EGCG treatment [164]. Our data shows an initial up regulation of Sirt3, which can be attributed to the cell adapting to a change in the oxidative environment. This was followed by a significant down regulation of Sirt3 gene expression by EGCG treatment. Based on the fact that Sirt3 is regulated by PGC-1α, which was also down regulated by EGCG, the argument can be made that this contributed to the down regulation of Sirt3. Protein expression of Sirt3 from western blot analysis showed a significant decrease in expression in both lean and obese mice. This data supports what was seen at the gene level and in previous studies. For example, a study by Tao et al., showed that Sirt3 protein expression was down regulated in EGCG mediated cytotoxicity in cancer cells [165]. Cadmium, a known hepatotoxin was shown to decrease Sirt3 protein, which in turn decreased Sod2 activity [166]. Since EGCG has been shown to cause liver toxicity at the higher doses, this provides a possible explanation for the decrease in gene and protein expression seen for Sirt3. The data also revealed that obese mice had lower protein expression of Sirt3 when compared to lean mice, but the difference was not significant. One possible explanation for this is that decreased levels of Sirt3 have been associated with a high fat diet [167]. This effect combined with the toxic effect of EGCG may have made obese mice more susceptible, thus they expressed lower levels of Sirt3.
Forkhead box O3 transcription factor A (Foxo3a) is a transcription factor that forms part of the cell’s antioxidant defense system by regulating antioxidant genes [168]. Foxo3a can reduce the level of cellular oxidative stress by directly upregulating mRNA and protein levels of Sod2, catalase and peroxiredoxin 3 [168], [169]. Foxo3a like Nrf1 requires coactivator Pgc-1α to regulate oxidative stress [168]. Similar to the data seen for Pgc-1α a significant down regulation of Foxo3a was seen in lean and obese mice treated with EGCG. This data supports what has been seen based on the effect of EGCG on other transcription factors. Additionally, Foxo3a has been shown to be regulated by Sirt3 and this provides an explanation to support the down regulation of Foxo3a seen [164]. In obese mice given 500 mg/kg EGCG, a significantly higher expression of Foxo3a was seen in lean mice fed the same dose. One possible explanation for this is the fact that Foxo3a has been shown to be upregulated by a high fat diet [170]. In this case the effects of EGCG treatment were seen, but compared to LF mice the effects were not as severe in obese mice probably due to them having higher initial levels of Foxo3a.

Another important mitochondrial regulator is transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Nrf2 has been implicated in the cell’s antioxidant defense system with its ability to activate genes involved in detoxification of ROS through the antioxidant response element (ARE) and is essential in cellular response to oxidative stress [171], [172]. Nrf2, like key the antioxidant enzyme that it regulates, glutathione-S-transferases, have been shown to be down regulated in aggressive prostate cancer tissue [173]. Our analysis of Nrf2 expression after treatment in obese and lean mice showed that there was an upregulation from the 0 – 500 mg/kg dose of EGCG in both diet groups, followed by a down regulation at the toxic 750 mg/kg dose, also in both diet groups. Our results are consistent with data from previous studies. In a study conducted by Wang et al., EGCG was shown to upregulate Nrf2 at non-lethal doses, but down regulated Nrf2, as well as superoxide dismutase, catalase and glutathione peroxidase at a toxic dose [174].
We analyzed nuclear respiratory factor 1 (Nrf1) expression. Nrf1 is a transcription factor located in the nucleus, and is involved in nuclear regulation of mitochondrial transcription, translation and replication [175], [176]. Nrf1 along with co-activator Pgc-1α is an important regulator of mitochondrial biogenesis [177], [178]. Our data showed an upregulation of Nrf1 from 0 mg/kg to 500 mg/kg which was significant at 500 mg/kg EGCG. Studies have shown that Nrf1 is induced by compounds that cause damage to mitochondria in liver tissue, through ROS generation, GSH depletion, and mtDNA damage [179]. EGCG has been shown to generate ROS and deplete GSH [18], [105], so it is reasonable to assume that like these other compounds, EGCG can induce Nrf1. As the dose of EGCG increased to 750 mg/kg, a known toxic dose, a decrease in Nrf1 expression was seen in lean and obese mice. This could be explained by an increase in cellular apoptosis and tissue necrosis seen at that dose. Also, Nrf1 is regulated by Pgc-1α, and this too was down regulated, which could cause a down regulation of Nrf1. Additionally, since Nrf1 is located in the nucleus, where an increase on DNA strand breaks were seen for 750 mg/kg EGCG, this could also explain the down regulation of Nrf1 seen here.

As was previously stated, Pgc-1α is a regulator of mitochondrial biogenesis [180][91]. Pgc-1α has also been shown to be a powerful regulator of ROS removal by increasing the expression of numerous ROS-detoxifying enzymes thereby being effective in the cell’s antioxidant capabilities [177]. These antioxidants include Sod2, Gpx and Prx3 just to name a few; all of which were measured in our study and shown to be significantly down regulated. We saw a significant decrease in Pgc-1α gene expression at 500 and 750 mg/kg EGCG in lean and obese mice. Other hepatotoxins such as CCL4 have been shown to depress Pgc-1α in rats in which it induced hepatic injury [181]. Pgc-1α has been shown to be associated with adaptive thermogenesis, and is elevated in response to oxidative stress and under obese conditions [182], [183]. At the 750 mg/kg dose of EGCG, though not significant, obese mice had even lower expression levels than lean mice.
The data obtained so far, based on the changes seen in expression of antioxidant enzymes, regulators of antioxidant genes and mitochondrial biogenesis indicates that EGCG induced changes in genes and proteins associated with or localized in the mitochondria. This is consistent with previous work done by Tao et al., where EGCG was shown to induce mitochondrial ROS and subsequent mitochondrial dysfunction [18]. We investigated the effect of EGCG genes involved in mitochondrial function.

Mitochondrial DNA copy number is a marker of mitochondrial biogenesis and was determined as the ratio between mitochondrial encoded gene, cytochrome c oxidase subunit 3 (CoxIII) and nuclear endogenous gene 18S [184], [185]. Mitochondrial DNA is highly susceptible to oxidative damage as the mitochondria are an endogenous source of ROS in the cell due to the electron transport chain (ETC) [186]. A decrease in mtDNA copy number is associated with many pathologies such as liver disease and type II diabetes among others. Mitochondrial dysfunction reduces the contents of mitochondria, which is expressed as a decreased mtDNA copy number [185]. Our results show that mtDNA copy number decreased in all treatment groups compared to control. This data is consistent with previous studies. Pieters et al., showed that polycyclic aromatic hydrocarbons (PAHs), which are known to induce oxidative stress, were shown to decrease mtDNA copy number, further supporting our findings, as EGCG also induces mitochondrial oxidative stress [187]. Aging, which increases oxidative stress, also decreased mtDNA copy in rat liver tissue [188]. The decrease in mtDNA copy number observed here can also be attributed to the changes seen in transcriptional co-activator Pgc-1α. Pgc-1α, along with nuclear respiratory factor 1 (Nrf1) and mitochondrial transcription factor A (Tfam), regulate mitochondria biogenesis [177], [189]. EGCG treatment caused significant down regulation of Pgc-1α, which could explain the observed decrease in mtDNA copy number. Many studies have also shown that obesity decreases mtDNA copy number. However, no significant differences as a direct consequence of obesity were observed in this study [190].
We measured mitochondrial transcription factor A (Tfam) based on changes seen in Pgc-1α, Nrf1 and mtDNA copy number. Tfam is a key activator of mitochondrial transcription as well as a participant in mitochondrial genome replication and consequently mtDNA copy number [191], [192]. Tfam is regulated by Nrf1 in collaboration with Pgc-1α, and is upregulated by oxidative stress [189], [193], [194]. Similar to data obtained for Nrf1, we saw an initial upregulation of Tfam (0 – 500 mg/kg EGCG) followed by a significant down regulation at the 750 mg/kg dose. The data obtained can be explained by the fact that Tfam is regulated by Nrf1 and the changes seen are consistent with those of Nrf1. It is also regulated by Pgc-1α, which was significantly down regulated by EGCG. Obese mice given 250 mg/kg EGCG expressed higher levels of Tfam then lean mice at that dose. This could be explained by the fact that as previously indicated, Tfam is induced by oxidative stress, and also as previously stated, higher levels of oxidative stress are associated with obese mice compared to lean mice, so obese mice can potentially express higher levels of Tfam than lean mice.

In addition to Tfam we investigated other mitochondrial transcription factors; mitochondrial transcription factor B1 (Tfb1m), and mitochondrial transcription factor B2 (Tfb2m). Tfb1m and Tfb2m are dimethyltransferases encoded in the nucleus and essential for mitochondrial DNA replication in the presence of TFAM and mitochondrial RNA polymerase (POLYRMT) [192], [195]. Both transcription factors are regulated by Nrf1 and Pgc-1α [178]. Tfb1m has been shown to regulate mitochondrial translation and loss of Tfb1m can result in mitochondrial dysfunction [196]–[198]. Analysis of Tfb1m data showed an upregulation of the transcription factor in lean and obese mice given 250 mg/kg EGCG. At this dose, though not significant obese mice expressed higher levels of Tfb1m compared to lean mice. Like Tfam, Tfb1m is induced by oxidative stress and obesity has been shown to facilitate an oxidative environment, so this could explain the differences seen between the diets at this dose of EGCG. A down regulation of Tfb1m
was observed at the other doses, and this could be as a result of the down regulation of transcription factor \textit{Nrf1} and co-activator \textit{Pgc-1a}, which are regulators of \textit{Tfb1m}.

Whereas \textit{Tfb1m} regulates mitochondrial translation, \textit{Tfb2m} has been shown to regulate mitochondrial transcription, making it have a more direct effect on mtDNA copy number [199]–[201]. Gene expression of \textit{Tfb2m} is consistent with data seen for \textit{Tfb1m} and \textit{Tfb2m}, and the expression pattern can be explained in the same way. Obese mice expressed lower levels of \textit{Tfb2m} compared to lean mice, although this was not significant, one possible explanation for this is that \textit{Tfb2m}, because of its role in mitochondrial transcription has been associated with metabolic functions, and shown to be essential [199]. Obesity is a metabolic disorder that has been shown to increase oxidative stress and reduce mtDNA copy number [202], which could explain why obese mice had lower expression of \textit{Tfb2m}.

Complex I and III of the mitochondrial electron transport chain (ETC) are major sites of ROS generation in the cell. They are exclusively encoded by mitochondrial DNA and as such are makers for mtDNA oxidative damage [128], [131], [186]. Our study showed significant down regulation of both complexes by EGCG treatment. This data is consistent with other studies that show ROS generating compound, capsaicin decreases complex I and complex III [203]. This data also supports our previous findings of the effect of EGCG on genes involved in mitochondrial replication, transcription and function. The changes seen in complex I and III expression can be attributed to down regulation of transcription factor \textit{Nrf1}, which has been shown to regulate these complexes, and was down regulated in the study [177]. Additionally, both complex I and III are activated by \textit{Sirt3} [164]. In our study \textit{Sirt3} was also significantly down regulated by EGCG treatment, and this could account for the down regulation seen in the complexes.

Mitochondrial uncoupling proteins separate oxidative phosphorylation from ATP synthesis with energy dissipated as heat. UCPs facilitate the transfer of anions from the inner to the outer mitochondrial membrane and the return transfer of protons from the outer to the inner
mitochondrial membrane. *Ucp2* has been implicated in the control of mitochondria-derived reactive oxygen species. The uncoupling activity of *Ucp2* increases the respiratory rate and thus lowers the mitochondrial production of reactive oxygen species (ROS) [204]. In fact, increased *Ucp2* expression has been shown in tumor cells and have been associated with lower ROS levels, indicating that cancer cells have adapted the over expression of *Ucp2* as a mechanism to maintain homeostasis, thus preventing mitochondrial superoxide generation and subsequent apoptosis [205], [206]. *Ucp2* has been shown to be highly inducible under conditions of oxidative stress generated by agents such as H$_2$O$_2$, lipopolysaccharide, TNFα, free fatty acids, irradiation, as well as high-fat diet both *in vitro* and *in vivo*. This suggests that a major function of *Ucp2* is to lessen mitochondrial production of ROS and like in cancer cells this too can be an adaptive response to oxidative stress [143].

*Ucp2* has also been shown to be upregulated by ROS in other tissue as well. Tsuboyama-Kasaoka *et al.* saw a significant upregulation of liver *Ucp2* in C57BL/6J mice fed a high safflower and a high fish oil diet for 5 months, compared to the high carbohydrate fed mice [207]. Our results showed a dose dependent increase in *Ucp2* in both lean and obese mice. These data are consistent with previous studies showing that *Ucp2* is expressed under conditions of oxidative stress. EGCG treatment has been shown to increase *Ucp2* expression in obese mice [30], [31]. Additionally, significant differences in *Ucp2* gene expression levels were observed between lean and mice treated with 250 and 500 mg/kg EGCG. Fulop *et al.*, showed that *Ucp2* is highly induced in hepatocytes under obese conditions [208]. Rashid *et al.*, also showed increased expression of *Ucp2* in 2 mice models of fatty liver [209]. Evaluation of *Ucp2* expression in livers of male obese mice showed an increase in both gene and protein expression of *Ucp2*, compared to their lean counterparts where no expression was observed. The study also showed that the increased *Ucp2* expression corresponded with depleted ATP levels, which increased hepatocyte sensitivity to necrosis [210].
Based on these results, it can be seen that EGCG treatment lead to significant decreases in antioxidant response in liver tissue of male C57BL/6J. Changes were seen in gene, protein and activity levels. A corresponding decrease in regulators of antioxidant genes was also observed due to EGCG treatment. EGCG treatment also significantly upregulated markers of oxidative stress which coincided with reduced antioxidant activity. EGCG resulted in a decrease in mitochondrial biogenesis as evidenced by decreased mtDNA copy and gene expression of mitochondrial transcription factors. Overall EGCG was able to induce mitochondrial dysfunction by inhibiting the gene, protein and/or activity of many key targets.
Chapter 5
Conclusions and Recommendation for Future Work

Conclusions

Here, I tested the hypothesis that dietary pretreatment with EGCG markedly decreases EGCG bioavailability and can potentially mitigate the toxic potential of EGCG, and that ORFLD increases susceptibility to high dose EGCG-mediated hepatotoxicity. In addition, the underlying mechanism by which EGCG mediates hepatotoxicity is through induction of mitochondrial oxidative stress and increased mitochondrial dysfunction.

To determine the effect of dietary pretreatment with EGCG on the bioavailability and hepatotoxicity of subsequent oral bolus dosing with EGCG in mice.

I found that dietary pretreatment with EGCG can significantly increase the gene expression of antioxidant response genes in liver tissue, as well as reduce makers of liver injury and oxidative stress. The decrease in oxidative stress and subsequent liver injury can be interpreted as direct result of the increased antioxidant enzyme activity, which was able to mitigate the toxic potential of high dose EGCG. My results also showed that dietary pretreatment with EGCG was able to reduce the bioavailability of EGCG, which corresponded to decreased plasma and tissue concentrations of EGCG; and consequently reduced the hepatotoxic potential of subsequent high dose oral bolus EGCG. Essentially dietary pre-treatment was able to exert a protective effect on subsequent high dose oral bolus EGCG. In terms of translation to human consumption patterns, the results are analogous to comparing exposure of a non-tea
consumer (NP) to a regular tea consumer (EP), and suggest that regular consumers of green tea beverage may be at decreased risk of hepatotoxicity associated with use of green tea based dietary supplements.

To determine the effect of pre-existing obesity and ORFLD on EGCG-mediated hepatotoxicity in mice.

Obesity and obesity related fatty liver disease have been shown to adversely affect the liver in many different ways, including but not limited to; increasing sensitivity to drug induced liver injury, decreasing the antioxidant capacity of the liver and increasing hepatic oxidative stress. These effects can severely disrupt liver function and consequently make the liver more prone to damage from toxic compounds. I found that pre-existing obesity and ORFLD increased susceptibility to EGCG mediated hepatotoxicity. The effects observed occurred in a dose dependent manner, based on increasing dose of EGCG. Obese mice had lower survival rates and exhibited higher levels of oxidative stress when compared to lean mice. However, the extent of liver tissue damage was not significantly different between lean and obese mice. The effect of high dose EGCG in lean mice was in some instances comparable to obese mice, when examined in the context of overall tissue damage. No stark differences were seen when liver tissue from mice in both diet groups treated with 500 and 750 mg/kg EGCG were evaluated for changes in tissue necrosis, inflammation, hepatic lipidosis, hemorrhaging and apoptosis following EGCG treatment. This observation could be attributed to the high carbohydrate content of the low fat diet. Mice fed a high carbohydrate diet have been shown to accumulate lipid in the liver and potentially exhibit signs of non-alcoholic fatty liver disease. EGCG treatment was able to exacerbate tissue damage in lean mice treated with 500 and 750 mg/kg EGCG, causing the extent of tissue damage to be similar to that seen in obese mice at those doses.
To examine the effect of EGCG on hepatic markers of antioxidant response and mitochondrial biogenesis/function in lean and obese mice.

Previous work in our lab showed that EGCG induced mitochondrial dysfunction in cancer cells treated with varying doses of EGCG. This data suggested that the direct effects on EGCG on the mitochondria observed, was a possible mechanisms by which EGCG can mediate hepatotoxicity. I examined the gene expression, protein expression and/or activity of hepatic markers of mitochondrial antioxidant response, biogenesis/function in a mouse model. I found that EGCG treatment significantly reduced the antioxidant response and mitochondrial biogenesis and function in the livers of lean and obese mice. Those effects occurred in a dose dependent manner, with high doses of EGCG having the lowest gene expression, protein expression and/or activity. EGCG treatment significantly reduced the gene and protein expression and activity of endogenous antioxidants, as well as the gene expression of their regulators. EGCG treatment significantly decreased the gene expression of transcription factors that regulate mitochondrial biogenesis and genes involved in mitochondrial function in both lean and obese mice. These results supported the hypothesis that the mitochondria are a target for EGCG induced mitochondrial dysfunction in vivo, based on data obtained from previous in vitro studies, and provide a mechanistic explanation for the observed hepatotoxic effects of EGCG.

Overall, the results of my study support the hypothesis that dietary pretreatment with EGCG can mitigate the toxic effects of high oral bolus EGCG and that obesity increases susceptibility to EGCG mediated liver toxicity. Through the inhibition of antioxidant response and mitochondrial biogenesis/function EGCG reportedly increased mitochondrial oxidative stress and induced mitochondrial dysfunction, which resulted in subsequent hepatotoxicity.
The results of this work highlighted the potential of EGCG, a compound that is generally regarded as safe and healthy when consumed in its more common form of a beverage to become toxic when taken as a dietary supplement, where the doses are higher. These results can be extrapolated to highlight the potential of many other compounds which are assumed to be safe because they are natural, to be harmful when taken in more a concentrated form like that of a dietary supplement as opposed to the more traditional forms, most commonly, beverages (teas). Many dietary supplements are marketed with an emphasis on the fact that they are natural. As we see in the results of this study, in the case of EGCG, this can be very misleading, and this could be the case for many other supplements containing natural compounds. This exposes consumers to risks that they are unaware of. It can also be seen from the data that more of a compound is not necessarily more effective or safer. Again, dietary supplements expose persons to a higher dosage form of a compound and based on how they are taken it is easy for persons to go from a safe dose to a potentially toxic dose. From a public health perspective, this research sheds light on the need to better evaluate compounds that are generally thought of as safe and used as dietary supplements and to take into consideration lifestyle factors that affect the safety of these compounds.
Future Work

Effect of Other Lifestyle Factors on EGCG Mediated Liver Toxicity

In the present study I examined two aspects of the effect of lifestyle on the safety of taking dietary supplement; diet and obesity. However many other lifestyle factors can potentially increase the risks associated with taking supplements. It would be worthwhile to examine the effects of other lifestyle factors, for example alcohol consumption, and how this impacts the consumption of green tea supplements. Many people consume alcohol and take supplements. Alcohol is a known hepatotoxin and the combined effect of alcohol and green tea supplement warrants further investigation. A previous study in mice showed that green tea catechins can exacerbate acetaminophen induced liver toxicity when taken together. [211]. The ability of green tea catechins to potentiate the toxicity of acetaminophen indicates the ability of catechins to interact with other compounds and impact their toxicity. Alcohol has also been shown to enhance the toxicity of acetaminophen. Several studies report the toxic effect of consuming alcohol with acetaminophen [212]. It has been established that alcohol induces cytochrome P450 enzymes, which can metabolize acetaminophen into the toxic metabolite, NAPQI, which can bind to cellular proteins and nucleic acids and induce toxicity. Additionally, alcohol has been shown to reduce glutathione levels, which in turn can contribute to the increased toxicity of acetaminophen, as the toxic metabolite is detoxified by glutathione conjugation [213]. Alcohol has also been shown to affect the ability of the liver to metabolized compounds, by inhibiting the production of UDP-glucuronic acid, another major detoxification pathway in the liver [214].

Based on this, it would be meaningful to investigate the potential of alcohol to impact EGCG mediated liver toxicity. As was seen in previous results in this study, reduced glutathione levels were associated with EGCG induced hepatotoxicity. With the ability of alcohol to also
reduce glutathione levels, the possibility of high levels of oxidative stress is increased as a result of these reduced GSH levels and can liver to further liver damage. EGCG was shown to be extensively glucuronidated when metabolized in the liver. Alcohol was shown to reduce UDP-glucuronic acid, which is needed for glucuronidation. This could lead to reduced EGCG glucuronidation, increased bioavailability of EGCG, and possibly increased EGCG mediated liver toxicity.

**Determination of Hepatotoxicity of Green Tea Extract**

In the present study we only focused on the effects of EGCG, as studies show that it is the most active green tea polyphenol. With regards to dietary supplements, many contain green tea extract as opposed to just EGCG. Now that we have established high dose EGCG mediated liver toxicity, further studies on the potential toxicity of the whole extract should be conducted, and the results compare it that of only EGCG. This would provide more insight into the safety of green tea supplements.

One possible way to evaluate this, based on experiments conducted in this study, would be perform *in vivo* studies, where mice are treated with only EGCG or with GTE containing the same concentration of EGCG as the other treatment. Briefly, mice will be separated into 3 treatment groups. One group will receive a 750 mg/kg b.w. dose of EGCG, which in previous studies was shown to induce hepatotoxicity. Another group will receive a dose of GTE solution which has been prepared to contain 750 mg/kg EGCG. The last group will serve as controls and not be treated with any EGCG. Mice will be treated with the respective solutions for 2-3 days. Post treatment the plasma and liver tissue of the various treatment groups will be analyzed and assessed for changes/differences in liver tissue damage, markers of liver toxicity and oxidative
stress. The results of this study could potentially determine if supplements containing GTE are toxic, and how much more or less toxic they compared to pure EGCG containing supplements.

**Inhibition of Mitochondrial Proteins Involved in Mitochondrial Antioxidant Response and Function by EGCG**

Our data confirmed that EGCG treatment disrupted mitochondrial function and corroborated previous data showing that EGCG induced mitochondrial dysfunction *in vitro*. These results provided some evidence that the underlying mechanism of EGCG mediated liver toxicity is mitochondrial dysfunction. However, further studies to generate more mechanistic data are warranted. Following oxidation, EGCG had been shown to bind to cellular proteins like cysteine and glutathione [4] [215]. As we have seen, glutathione is a key antioxidant in the cell and plays an important role in the detoxification of compounds in the liver. Glutathione has been shown to conjugate compounds like EGCG in order to eliminate them and can itself become oxidized in order to remove reactive species. Reduced levels of glutathione have been observed following high dose EGCG treatment, indicating the potential of EGCG to bind to this antioxidant protein and deplete its levels.

EGCG has been shown to interact with a number of cytosolic and nuclear proteins in the cell including enzymes, growth factors and their receptors, for MAP kinases, phosphatases, and DNA methyltransferase. Some of these interactions have been determined to be inhibitory interactions [216]. Inhibition of some of these proteins has been implicated in the chemopreventative actions of EGCG and shown to affect signal transduction pathways, transcription factors, DNA methylation, and mitochondrial function [217].

Having shown that mitochondrial dysfunction is the underlying mechanism of high dose EGCG mediated hepatotoxicity, and that EGCG can interact with and inhibit several nuclear
proteins, the potential of EGCG to bind to and directly inhibit key mitochondrial proteins should be investigated. The results of these studies will provide further mechanistic data on the toxicity of high dose EGCG. Tao et al showed the significance of mitochondria localized protein, Sirtuin 3 in mitochondrial response to EGCG treatment. The data showed that EGCG suppressed Sirtuin 3 mRNA and protein expression, as well as, SIRT3 activity in human oral squamous carcinoma cells, where EGCG treatment had induced mitochondrial dysfunction [165]. Research into the ability of EGCG to bind to and inhibit Sirtuin 3, needs to be investigated, as this protein has been shown to be instrumental in mitochondrial response to oxidative stress, since it activates mitochondrial antioxidant enzymes such as Sod2 and Gpx1 and Prx 3.

Results from the previous study shows that EGCG reduced mRNA and protein expression, as well as activity of several mitochondria antioxidant enzymes. Decreases in levels Sod2, Cat and Gpx1 were observed in the study. The effect of EGCG on these antioxidant enzymes contributed to the observed increase in oxidative stress in the mice following EGCG treatment. Investigating the interaction between EGCG and these antioxidant enzymes will provide data on the ability of EGCG to induce oxidative stress and subsequent mitochondrial dysfunction by directly inhibiting antioxidant enzymes in the mitochondria. A study by Pal et al showed that EGCG had the highest binding affinity to and inhibition of pure and cellular catalase in K562 cancer cells, which resulted in significant increases in cellular ROS and suppression of cell viability [218]. However, the inhibition of mitochondrial superoxide dismutase (Sod2) and glutathione peroxidase 1 (Gpx1) have not been very well studied. Additionally many of these studies have been carried in vitro, in cancer cell lines. To get a clearer understanding of the direct inhibiting effects of EGCG on these antioxidant enzymes as it relates to their in vivo activities and EGCG mediated hepatotoxicity, binding and inhibition studies can be carried out on primary hepatocytes of cells isolated from mice treated with varying doses of EGCG. Data from these
studies will provide addition evidence for EGCG induced mitochondrial oxidative stress and dysfunction.

Effect of EGCG Metabolites on EGCG Mediated Liver Toxicity

EGCG has been shown to undergo significant biotransformation \textit{in vivo} (Figure 1-3). EGCG is readily methylated, sulfated, glucuronidated and undergoes ring-fission metabolism, with glucuronidation being the predominant form of transformation in humans, rats, and mice [8]. EGCG has also been shown to form thiol conjugates following treatment with toxic doses of EGCG. For example EGCG-cysteine conjugates were detected in urine of mice following treatment with 200–400 mg/kg, i.p. or 1,500 mg/kg, i.g. EGCG [215]. EGCG biotransformation has been shown to generally occur in liver microsomes and cytosol, as well as small intestines. Although EGCG is greatly biotransformed, EGCG frequently appears in the unmetabolized form in the plasma.

Recent studies have shown the EGCG and other polyphenols can also be metabolized by gut microbiota, which can impact their bioavailability and subsequent activities [219][220]. It has been shown that approximately 70% of green tea catechins pass from the small intestines to the large intestines, where about 33% is in the parent form and undergoes microbial metabolism [219]. The metabolites are then absorbed in the liver were they are metabolized by phase II conjugating enzymes and then eliminated in the urine. The unconjugated metabolites are eliminated in the feces. These microbial metabolites have also been shown to exert biological activity. EGCG microbial metabolites have been shown to have antioxidant and anti-inflammatory effects, and inhibit cell growth.
With such a large amount of catechin being biotransformed by microbiota, and these metabolites also having significant bioactivity, it raises the question of whether the effects of EGCG are a direct result of the parent compound or the metabolites. The results of this study focused on the toxicity of the EGCG parent compound, but did not take the metabolites into consideration. In order to establish the toxicity of EGCG and not have these results be confounded by the possible activity of microbial metabolites, EGCG studies should be repeated and carried out in germ-free mice. Conducting studies in germ-free mice will eliminate the microbially generated metabolites and determine toxicity of EGCG following liver and small intestine metabolism where the parent compound has been shown to be active. The results of these study can then be compared to the studies carried out in normal mice and be evaluated to determine if EGCG metabolites impact the EGCG mediated liver toxicity.
References


[32] S. Sae-Tan, K. a Grove, M. J. Kennett, and J. D. Lambert, “(−)-Epigallocatechin-3-gallate
increases the expression of genes related to fat oxidation in the skeletal muscle of high fat-fed mice,” *Food Funct.*, vol. 2, no. 2, pp. 111–6, Feb. 2011.


K. B. Scribner, D. B. Pawlak, and D. S. Ludwig, “Hepatic steatosis and increased adiposity in mice consuming rapidly vs. slowly absorbed carbohydrate,” *Obesity (Silver Spring)*, vol. 15, no. 9, pp. 2190–9, 2007.


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