Epigallocatechin-3-Gallate Reduces Fat Accumulation in Caenorhabditis Elegans

Jinning Liu
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EPIGALLOCATECHIN-3-GALLATE REDUCES FAT ACCUMULATION IN

CAENORHABDITIS ELEGANS

A Thesis Presented

By

JINNING LIU

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
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EPIGALLOCATECHIN-3-GALLATE REDUCES FAT ACCUMULATION IN CAENORHABDITIS ELEGANS

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ABSTRACT

EPIGALLOCATECHIN-3-GALLATE REDUCES FAT ACCUMULATION IN

CAENORHABDITIS ELEGANS

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Epigallocatechin gallate (EGCG), also known as epigallocatechin-3-gallate, is a polyphenol that is most abundant in tea. It has been shown from many studies that consumption of EGCG can contribute to weight loss, however, the underlying mechanism is not fully understood. To determine how EGCG acts to reduce fat, an organism model Caenorhabditis elegans (C. elegans) is introduced, which is a useful animal system in exploring crucial biological mechanisms that are readily applicable to humans. In this study, different strains were raised for two days on a diet with or without 100µM and 200µM EGCG treatment: N2 (i.e., wild type) and mutants (i.e., knockdown of fat metabolism related genes). EGCG’s effect on fat reduction was characterized by triglyceride content, food consumption and physiological behaviors. Our results showed that 100 and 200 µM EGCG significantly reduced the triglyceride content of wild type worms by 10% and 20%, respectively, without affecting its food intake and physiological behaviors. Additionally, EGCG could effectively reduce fat accumulation in C. elegans dependent on acs-2 and atgl-1.
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CHAPTER 1
INTRODUCTION

Obesity has become a major public concern causing chronic illnesses among over one third of the population in the world. It is well-known that obesity results from excessive energy intake and a lack of energy expenditure, such as physical activity. Treatment of obesity using drugs and noradrenergic agents is possible, but limited, and hard to reverse once obesity is established. By contrast, using food-based bioactive components to control and prevent obesity is more favorable.

Green tea is a popular beverage with a high content of flavonoids (a natural polyphenol) and has many desirable health benefits (anti-carcinogen, anti-inflammatory, and anti-oxidation). Consumption of green tea is very popular in Turkey, with an annual value of 12.54 kg (442 oz), while there is only 1.21 kg (43 oz) green tea consumption in the US. According to the database in World Health Organization, 33.9% of adults in the US are obese, whereas only 16.1% of adults in Turkey are obese. Epigallocatechin-3-gallate (EGCG) is a catechin that is most abundant in green tea. It is believed that this polyphenolic compound is responsible for those positive physiological bioactivities in green tea. EGCG has been reported to have fat reduction effects both in vitro (Moon et al., 2007) and in vivo (Kim et al., 2013), however, potential mechanism(s) are still unclear.

As a model system to test the effects of food components on fat content, Caenorhabditis elegans (C. elegans) was applied in the current study. C. elegans has been used extensively in biological and medical studies due to their short life span of ~20 days, a reproductive cycle of 3 days, and a large brood size of about 300 eggs by self-
fertilization (Gonzalez-Moragas et al., 2015). Moreover, it is a nematode that conserves 65% of genes related to human diseases (Baumeister & Ge, 2002), including those related to lipid metabolism, which makes it a great in vivo model for cellular and genetic studies.
2.1 Overview of obesity

Obesity is caused by a chronic imbalance between energy intake and energy expenditure. For the years 2013-2014, the overall prevalence of obesity was 37.7% in the United States (Flegal et al., 2016). According to the National Institutes of Health, obesity and being overweight together are the second leading cause of preventable death, close behind tobacco use. An estimated 300,000 deaths per year are due to obesity.

Many research projects, public health campaigns, and population interventions have been devoted to this epidemic disease. Four central acting noradrenergic agents: phentermine, diethylpropion, phendimetrazine and benzphetamine are currently FDA-approved for the short-term management of obesity (Yanovski & Yanovski, 2013). However, it is difficult to reverse it once obesity is established (Gortmaker et al., 2011). Thus, the focus of anti-obesity strategies should be on prevention to obtain long-term health gains. Many food and food bioactive components have effective anti-obesity functions which are more favorable than medical drugs. However, tests in cells and in animal models are required before application to patients. Thus, finding an appropriate testing system and model is indispensable.

2.2 Caenorhabditis elegans as a model for obesity research

*Caenorhabditis elegans* (*C. elegans*) is a nematode that has a short lifespan of 2-3 weeks, a reproductive cycle of 3 days and a large brood size of about 300 eggs by self-
fertilization. Moreover, it shares 65% of its genes with humans (Gonzalez-Moragas et al., 2015). Specifically, studies showed that the core mechanisms of energy homeostasis involved regulatory pathways that are largely shared between mammals and nematodes. Therefore, all these attractive advantages render *C. elegans* a popular and favorable experimental model for obesity research.

### 2.2.1 Characteristics of *Caenorhabditis elegans*

*C. elegans* is a eukaryotic and multi-organ animal, which does not require research approval by the Institutional Animal Care and Use Committees. With a small body size (1 mm in length in adult), a rapid life cycle, a short lifespan, a rapid reproductive cycle and a large brood size, *C. elegans* has been used as an organism, which can readily be cultured in the laboratory with a diet of *Escherichia coli* (E.coli), typically OP50, a non-pathogenic strain. These characteristics enable a large-scale production of millions of worms in a short time and make it easily observable under a microscope and provide convenient application for large experiments carried out in multi-well plates. The transparent body enables the fluorescence markers to be easily applied and observed from worms (Kaletta & Hengartner, 2006).

The genome sequence of *C. elegans* was first described in 1998 when the gene function was discovered and preserved for important physiological variables (Tabara et al., 1998). It is the first animal to have its genome completely sequenced and over 65% of the human disease genes have a counterpart in worms, including genes involved in lipid metabolic pathways. The high level of similarity in gene sequences in *C. elegans* facilitates genetic manipulations and promotes the acquisition of results of mechanistic information (Zheng & Greenway, 2012).
2.2.2 The insulin signaling pathway in C. elegans

The linking between nematodes and humans in obesity involved many components in the insulin/insulin-like growth factor-1 signaling (IIS) pathway (Ogg et al., 1997). This pathway is regulated by insulin-like peptide (ILPs) ligands in the presence of insulin or growth factors, which can bind to the insulin/Insulin-like growth factor-1 transmembrane receptor (IGFR) ortholog, DAF-2. DAF-2/IGFR activation recruits phosphoinositide 3-kinase (PI3K) to the cell membrane. PI3K generates phospholipid signals, PIPs: phosphatidylinositol 3,4,5-trisphosphate (PIP3) and phosphatidylinositol 4,5-bisphosphate (PIP2) (Gami & Wolkow, 2006). The DAF-18/PTEN lipid phosphatase negatively regulates AGE-1/PI3K signaling. So the inhibition of DAF-18/PTEN increases PIP3 levels (Ogg & Ruvkun, 1998). Successively, phosphoinositide-dependent kinase-1 (PDK-1), AKT serine/threonine kinase-1 (AKT-1), AKT-2 and serum glucocorticoid kinase (SGK-1) are activated, contributing to phosphorylation of the Forkhead family of transcription factor (DAF-16/FoxO). In turn, the phosphorylation of DAF-16 binds to Fourteen-Three-Three family proteins (FTT-2) and PARtitioning of cytoplasm (PAR-5), which results in the export of DAF-16 from the nucleus into the cytoplasm. This action of DAF-16 into the cytoplasm allows cell proliferation, stress sensitivity, and cell survival (Greer & Brunet, 2005). The serine/threonine phosphatase, PPTR-1, a regulatory subunit of the PP2A holoenzyme, acts by suppressing AKT-1 phosphorylation and as a result, inhibiting activity of DAF-16 (Figure 2.1) (Padmanabhan et al., 2010).
2.2.3 Lipogenesis in *C. elegans*

*C. elegans* acquires most of its fat from a bacterial diet directly, but is also able to synthesize palmitic acid (C16:0) *de novo* from acetyl-CoA (Watts, 2009). Specifically, for wild-type worms, approximately 7% of fat is gained from *de novo* synthesis, while the rest is obtained from diet (Perez & Gilst, 2008). For *de novo* synthesis (Figure 2.2), all enzyme activities that are necessary for the synthesis of palmitic acid (C16:0), are encoded by two multifunctional enzymes: acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS) (Rappleye et al., 2003). Palmitic acid can further be converted to...
polyunsaturated fatty acids (PUFAs) by various desaturases and elongases (Figure 2.2A). Seven fatty acid desaturases (FAT-1 through FAT-7), one 3-ketoacyl-CoA reductase (LET-767) and two fatty acid elongases (ELO-1 and ELO-2) have been suggested to be involved in PUFAs biosynthesis (Brock et al., 2006).

The monomethyl branched-chain fatty acids (mmBCFAs) are synthesized initially from isobutyryl-CoA, a bacterial degradation product from valine (Zheng & Greenway, 2012). It can then be elongated by FAS to form 11-methyl dodecanoic acid (C13:iso). The generation of 13-methyl tetradecanoic acid (C15:iso) and 15-methyl hexadecanoic acid (C17:iso) from C13:iso requires specialized elongases, ELO-5 and ELO-6, as well as LET-767 (Figure 2.2B). Enzyme ELO-5 and LET-767 are required for generation of both C15:iso and C17:iso, while ELO-6 is required for generation of C17:iso (Watts, 2009).
Figure 2.2 Summary of fatty acid synthesis in *C. elegans*. (A) Synthesis of polyunsaturated fatty acids. (B) Synthesis of monomethyl branched-chain fatty acids. Abbreviation: ACC (acetyl CoA carboxylase); FAS (fatty acid synthase); FAT (fatty acid desaturases); LET-767 (3-ketoacyl-CoA reductase); ELO (fatty acid elongases); 16:0 (palmitic acid); 18:0 (stearic acid); 18:1n-7 (vaccenic acid); 18:1n-9 (oleic acid); 18:2n-6 (linoleic acid); 18:3n-3 (alpha linolenic acid); 18:4n-3 (stearidonic acid); 20:3n-6 (dihomo gamma linolenic acid); 20:4n-3 (eicosatetraenoic acid); 20:4n-6 (arachidonic acid); 20:5n-3 (eicosapentaenoic acid); C13:iso (11-methyl dodecanoic acid); C15:iso (13-methyl tetradecanoic acid); C17:iso (15-methyl hexadecanoic acid).

### 2.2.4 Lipid metabolism related genes in *C. elegans*

#### 2.2.4.1 *sbp-1*

Sterol response element binding protein (SREBP) is a major transcriptional regulator of fat and sterol synthesis pathways in mammalian models (Eberlé et al., 2004). One ortholog of SREBP in *C. elegans* is known as SBP-1, which can be expressed in all metabolic related tissues and acts as a key regulator of fatty acid synthesis and fat homeostasis. Mutations in *sbp-1* in *C. elegans* result in significant reductions in fat level and intestinal lipid droplets biogenesis (Ashrafi, 2006). In addition, SBP-1 regulates the
expression of ELO-5 and ELO-6, which are two fatty acid elongation enzymes involved in branched-chain FA synthesis (Kniazeva et al., 2004).

2.2.4.2 nhr-49

Several nuclear hormone receptors (NHRs) in mammals act as essential regulators of energy homeostasis (Chawla, 2001). A large amount of NHRs are contained in the C. elegans genome, among which the function of NHR-49 is close to mammalian peroxisome proliferator-activated receptors (PPARs). In C. elegans, NHR-49 mediates β-oxidation and genes that are related to dietary intake. Knockout of nhr-49 will cause an increased fat deposition and reduced expression of genes involved in mitochondrial β-oxidation (Gilst et al., 2005).

2.2.4.3 tub-1

The TUBBY (TUB-1) is a member of the family of four homologous proteins, including TUB-1 and TULPs 1-3. These proteins are found in humans and other multicellular organisms, such as C. elegans. TUB-1 acts as a transcription regulator and is believed to be involved in the insulin signaling pathway (Santagata et al., 2001). In C. elegans, deletion of tub-1 increases fat content (Mukhopadhyay, 2005).

2.2.4.4 cebp-2

CCAAT/enhancer-binding proteins (C/EBPs) is a family of a highly conserved basic-leucine zipper (bZIP) transcription factors. These proteins are found in hepatocytes, adipocytes, the spleen, the kidney and many other organs. C/EBP proteins are involved in
different cellular responses, such as cellular proliferation, growth, and differentiation in metabolism and immunity. CEBP-2 is the ortholog of C/EBPs in *C. elegans*, which regulates body fat content by controlling fatty acid mitochondrial β-oxidation and desaturation. Mutations in *cebp-2* result in lower fat deposition (Xu et al., 2015).

### 2.2.4.5 *aak-2*

*aak-2* encodes one of two *C. elegans* orthologs of the catalytic alpha subunit of AMP-activated protein kinases (AMPKs). AMPK is an enzyme that plays a role in cellular energy homeostasis. *aak-2* activity is required, in parallel with *aak-1* and downstream of *daf-2, daf-7*, and *par-4*, for negative regulation of germline proliferation during dauer development (Narbonne & Roy, 2009). Mutations in *aak-2* result in higher fat content by inhibiting activation of the AMPK pathway which negatively regulates lipogenesis (Srivastava et al., 2012).

### 2.3 Studying obesity interventions using *C. elegans*

Compounds that potentially modulate body fat in *C. elegans* could be identified as active obesity intervention. In other words, *C. elegans* could be used for screening compounds that cause either fat loss or fat gain (Zheng et al., 2010). Some food and food bioactive components are already tested in *C. elegans* for the effect on fat content. Dietary fiber containing a high level of resistant starch reduced intestinal fat deposition in *C. elegans* (Zheng et al., 2010); legumes reduced intestinal fat deposition together with a decreased rate in food intake in *C. elegans* (Finley et al., 2013); oats had unique beneficial properties of reducing intestinal fat accumulation in *C. elegans* (Gao et al., 2010).
hesperidin, a commonly found flavanone glycoside in citrus fruits, exhibited the capacity of reducing fat content in *C. elegans* (Yang, 2016); and cranberry extract decreased fat deposition in *C. elegans* (Sun et al., 2016).

Compared with rodent models, *C. elegans* serves as a valuable model by saving the time in the laboratory. The results gained from *in vitro* could be tested in *C. elegans*, and studies conducted in *C. elegans* could enhance the understanding of the underlying mechanism on the effect of compounds and could suggest further study in humans. Thus, *C. elegans* is regarded as a great intermediate whole-animal model between *in vitro* and rodent animal experiments (Zheng & Greenway, 2012).

### 2.4 Current knowledge of epigallocatechin-3-gallate in obesity studies

During the last several decades, tea-derived bioactive components have gained considerable attention as cardio-protective agents (Lorenz et al., 2009). Tea contains large amounts of flavonoids, which are featured as containing two or more aromatic rings, each bearing more than one aromatic hydroxyl connected with a carbon bridge. The major flavonoids are catechins, which include epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epigallocatechin-3-gallate (EGCG) (Stangl et al., 2007). The naturally occurring compound EGCG is the major potent polyphenolic component and most abundant (50%-80%) of the total catechin content in green tea (*Camellia sinensis* L. Kuntze) (Khan et al., 2006). It has positive physiological bioactivities, such as anti-carcinogen (Morales & Haza, 2012), anti-inflammatory functions (Zhong et al., 2012), and anti-oxidation (Gao et al., 2016), as well as anti-obesity (Steinmann et al., 2013).
2.4.1 Effects of EGCG on energy balance

2.4.1.1 Inhibitory effects of EGCG on energy intake

Energy, obtained from food, is provided by digestion of carbohydrate, fat and protein. Excess energy intake will contribute to fat gain and furthermore, obesity. Kao et al. (2000) reported that EGCG could significantly reduce food intake in Sprague Dawley rats and obese male Zucker rats when injected intraperitoneally. Tho & Wolfram (2005) found the effect of dietary EGCG on obesity development and energy intake in a mouse model of diet-induced obesity by reducing nutrient absorption. However, others reported that EGCG was not affecting food intake when supplied orally due to its low bioavailability (Kao & Hiipakka, 2000).

2.4.1.1.1 Inhibitory effects of EGCG on α–amylase activity

α–amylase catalyzes the hydrolytic cleavage of starches in food to maltose and other low molecular weight sugars, which are easy to digest and absorb. Green tea has been shown to inhibit α–amylase activity in human saliva (Zhang & Kashket, 1998). To examine whether EGCG also has the same function, Forester et al. (2012) conducted an experiment reporting that EGCG inhibition of pancreatic α–amylase decreased postprandial blood glucose. Additionally, Zhan et al. (2016) suggested that EGCG could mitigate the risk of being overweight and obesity by suppressing the activity of α–amylase in vitro.

However, there are inconsistent reports on the relationship between obesity and α–amylase activity. Some researchers reported that inhibition of α–amylase activity attenuated the development of high-calorie diet-induced obesity (Zhan et al., 2016) and...
reduced blood glucose levels by inhibiting the ability to digest starch (Forester et al., 2012). Whereas others reported that people who have high $\alpha$–amylase activity adapt better to ingest starches, individuals with low activity may be at greater risk for insulin resistance and diabetes and furthermore obesity (Mandel & Breslin, 2012; Falchi et al., 2014; Mejía-Benítez et al., 2014). EGCG is confirmed to have an inhibitory effect on the activity of $\alpha$–amylase, even though the association with $\alpha$–amylase activity, starch digestion and obesity is unclear.

2.4.1.1.2 Inhibitory effects of EGCG on lipid digestion and absorption

The enzymes in the small intestine are responsible for almost all of the lipid digestion. Pancreatic lipase breaks down lipids into digestive products: free fatty acids and monoglycerides. Grove et al. (2012) reported that EGCG could inhibit lipid digestion by decreasing pancreatic lipase activity in high-fat-fed obese mice. Bose et al. (2008) found 16-week dietary EGCG treatment (3.2g/kg diet) could significantly decrease body weight and promote fecal lipids in comparison with high-fat-fed control mice, which indicated that EGCG could reduce lipid absorption resulting in reduced body weight.

Cholesterol, a hormone precursor and one of the components of plasma membranes, is primarily synthesized in the endoplasmic reticulum (ER). It is the initial molecule for synthesis of all steroid hormones and for several components of bile that assist digesting fats and lipids in the intestine. A previous report showed that EGCG inhibited the absorption of cholesterol at the level of cholesterol uptake in the intestine, and facilitated the excretion of serum cholesterol (Chisaka et al., 1988). Another experiment carried out by Raederstorff et al. (2003) further reported that EGCG influences the micellar
solubilization of cholesterol in the digestive tract, which then in turn reduced cholesterol absorption.

2.4.1.2 Effects of EGCG on stimulation of energy expenditure

In a human study, it was reported that acute intake of a green tea extract rich in catechin polyphenols increased 24-hour energy expenditure (Dulloo et al., 1999). Another human study showed that a mixture of EGCG and caffeine could increase energy expenditure in 24h (Bérubé-Parent et al., 2005). A study suggested green tea reduced body fat accumulation through β-adrenoceptor activation of thermogenesis in brown adipose tissue in rats (Choo, 2003). However, another study failed to observe EGCG’s effect on the uncoupling protein 1 (ucp-1) gene expression in brown adipose tissue, which indicated that EGCG did not influence brown fat thermogenesis (Tho & Wolfram, 2005). These inconsistent results may come from other components in green tea, such as EGC, ECG and EC.

2.4.2 Effects of EGCG on lipid metabolism

Lipid metabolism is a complicated process, including lipogenesis and lipolysis. Excessive fat turnover and oxidation might cause insulin resistance and, furthermore, obesity (Campbell et al., 1994). For example, release of free fatty acid is well regulated in normal-weight individuals; however, in upper-body obesity, excess lipolysis is commonly observed (Koutsari & Jensen, 2006). Several in vitro and in vivo studies reported that EGCG had a function of modulating lipid metabolism, which could consequently prevent and ameliorate obesity.
2.4.2.1 Inhibitory effects of EGCG on lipogenesis

It is found that green tea catechin could inhibit the activity and/or expression of lipogenic enzymes, such as ACC, FAS, malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PDH), glycerol-3-phosphate dehydrogenase (G3PDH), and stearoyl-CoA desaturase-1 (SCD-1) (Lin et al., 1999). Reports on in vivo studies found that tea extract could decrease the expression of genes that mediated lipogenesis, such as SREBP-1c, FAS, and SCD-1 in the liver (Chen et al., 2011) and adipose tissue (Hasegawa et al., 2003; Park et al., 2011). This finding suggested that tea might be a useful dietary strategy to mitigate obesity. Since EGCG is the most abundant catechin in tea, much research has been focused on the inhibitory effect on lipogenesis of EGCG. According to in vitro studies, EGCG could suppress lipogenesis in adipocytes (Hwang et al., 2005), and in hepatocytes (Huang et al., 2009; Kim et al., 2013).

2.4.2.2 Effects of EGCG on lipolysis

There has been growing evidence that EGCG activated lipid metabolism via improvement of lipolytic activities in 3T3-L1 adipocytes (Mochizuki & Hasegawa, 2004; Lee et al., 2009; Chen et al., 2015). However, Söhle et al. (2009) reported that EGCG had no contribution on the stimulation effect of white tea extract on lipolysis in vitro. The controversial statements, therefore, suggested that EGCG had inconsistent effects on lipolysis.
2.4.2.3 Effects of EGCG on stimulation of fat oxidation

Fat oxidation is a process requiring energy to break down lipids. Tho & Wolfram (2005) reported that dietary EGCG promoted fat oxidation in mice. Furthermore, a few pilot studies in overweight/obese men showed that EGCG alone had the potential to promote fat oxidation and might thereby render an anti-obesity effect (Boschmann & Thielecke, 2007; Thielecke et al., 2010). According to animal and human studies, EGCG could therefore stimulate fat oxidation. To further explore the mechanisms of EGCG increasing fat oxidation, reports showed it could be possible through the activation of the Liver Kinase B1(LBK1)/AMPK pathway (Murase et al., 2009).

2.4.3 Other potential mechanisms of EGCG in response to obesity

Obesity is now considered, partly, an inflammatory condition (Vick et al., 2007). Inflammation is a complex process involving various types of cells in the immune system. In a study, EGCG resulted in reduced inflammatory related proteins, such as IκB kinase 2 (IKK-2), tumor necrosis factor (TNF-α), and interleukin 6 (IL-6) in 3T3-L1 adipocytes (Bao et al., 2015). Therefore, it is indicated that EGCG might have potential anti-inflammatory effects.

Responses to immune and autoimmune systems are regulated by a balance between effector T cells (T_{eff}) that actively respond to stimuli, and regulator T cells (T_{reg}) (O'Garra & Vieira, 2004) that play a pivotal role in the maintenance of immune tolerance and suppression of autoimmunity (Vignali et al., 2008; Feuerer et al., 2009). A report showed that EGCG enhanced the number and function of T_{reg} isolated from obese subjects (Yun et al., 2010). In addition, Wong et al. (2011) reported that EGCG could induce Foxp3 (a
transcription factor of T$_{reg}$ cells) and IL-10 expression in CD4$^+$ Jurkat T cells, at physiologically relevant concentrations of EGCG (2–50 µM) in vitro. They further showed that EGCG could increase T$_{reg}$ frequencies and numbers in spleen and lymph nodes in vivo. These indicated that EGCG might be a promising compound to modulate the immune response to obesity.

2.5 Current studies on effects of EGCG in C. elegans

According to available publications, EGCG has been shown to display antioxidant activities (Brown et al., 2006; Mohri-Shiomi & Garsin, 2008), stress preventive effects (Zhang et al., 2009; Abbas & Wink, 2010; Bartholome et al., 2010), and significant longevity-extending effects (Zhang et al., 2009; Saul et al., 2009; Bartholome et al., 2010; Abbas & Wink, 2010) in C. elegans. However, there is no report focusing EGCG on the mechanism of anti-obesity effects in the nematode. Therefore, this study was to examine whether EGCG could play a role in reducing fat content or preventing obesity in C. elegans and to find out the underlying mechanism(s) involved.
CHAPTER 3
MATERIALS AND METHODS

3.1 Materials, reagents and strains

All the chemicals used were purchased from Thermo Fisher Scientific (Middletown, VA, USA). *C. elegans* strains and *Escherichia coli* OP50 used in this study were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, MN, USA), including N2, bristal (wildtype); CE541, *sbp-1 (ep79) III*; RB754, *aak-2 (ok524) X*; RB759, *akt-1 (ok525) V*; GR1307, *daf-16 (mgDf50) I*; RB1716, *nhr-49 (ok2165) I*; BX107, *fat-5 (tm420) V*; BX106, *fat-6 (tm331) IV*; BX153, *fat-7 (wa36) V*; OP50 and OP50-GFP *E. coli*. Household bleach (The Clorox company, Oakland, CA, USA) was used for bleaching the worms when synchronizing L1 worms.

3.2 Preparation of EGCG solution and *C. elegans* culture

The EGCG extract (purity >99%) was dissolved in sterilized water and filtered through a 0.22 µm-diameter membrane to remove potential contaminants. Previously, it was reported that 200 µM EGCG had beneficial effects on *C. elegans* (Abbas & Wink, 2009), however, it is assumed that with the higher concentration of the treatment, we might observe the adverse effect. Thus, we chose 100 µM and 200 µM EGCG as treatment groups to study the fat reducing effects in *C. elegans*.

M9 buffer, S-complete, and nematode growth media (NGM) agar were used in *C. elegans* culture. After synchronizing, all L1 worms were raised at 25°C in S-complete media supplemented with *E. coli* OP50 and treated with or without EGCG in 12-well
plates for 2 days.

3.3 Triglyceride quantification

At the end of the treatment, *C. elegans* were collected and washed twice with water to remove *E. coli* and EGCG. *C. elegans* samples were dissolved in 0.05% Tween 20 solution. After sonication, the samples were used for the triglyceride (TG) and protein measurements. TG assay was conducted with the Infinity™ Triglycerides Reagent and protein content was measured with the Coomassie Plus Protein Assay Reagent. TG content was then normalized with protein concentrations.

3.4 Measurement of growth rate

After the 2-day treatment, nematodes were transferred to new agar plates to measure the growth rate. The numbers of worms at different stages were counted under an optical microscope (Olympus Corporation, Tokyo, Japan).

3.5 Measurement of pharyngeal pumping, body size, and movement

After the 2-day treatment, nematodes were transferred to new plates with fresh *E. coli* OP50 for the measurement of pumping rate, body size, and locomotive activity. The pumping rate was measured by counting the rate of pharyngeal muscle contractions from *C. elegans* under the optical microscope. For body size and movement, a 30 s video was recorded and data for the length, width, and moving speed of worms were then analyzed by Wormlab tracking system (WormLab software version 3.1.0, MicroBrightField Inc., Williston, Vermont).
3.6 Measurement of food intake

To monitor food intake of the nematodes, age-synchronized N$_2$ nematodes were cultivated on *E. coli* OP50-GFP bacterial lawns on NGM plates with or without EGCG. After treatment for 2 days, nematodes were washed twice with water, placed and fixed onto slides which were prepared with fresh 5% agar pads, and then visualized under a fluorescent microscope. The integrated density was quantified using Image J software by determining the average pixel intensity.

3.7 mRNA expression analysis

Total RNA was extracted from *C. elegans* using TRIzol® reagent under RNase-free conditions. Total RNA was reverse transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed on a StepOne Plus real-time PCR system (Applied Biosystems). We used *cebp-2* (Ce02421574_g1), *hosl-1* (Ce02494529_m1), *atgl-1* (Ce02406733_g1), *mdt-15* (Ce02406575_g1), *pod-2* (Ce02427721_g1), and *acs-2* (Ce02486193_g1) for TaqMan gene expression assays. Threshold values were analyzed using the comparative CT method. The RNA polymerase II large subunit *ama-1* gene (Ce02462726_m1) was used as an internal standard.

3.8 Statistical analysis

Data are expressed as means ± standard errors. Statistical analysis for all data were performed by Statistical Analysis System (SAS version 9.4, SAS Institute, Cary, NC, USA). Differences between groups were assessed with one-way or two-way analysis of
variance (ANOVA), followed by Tukey’s multiple range test. Significance of differences was defined at the $P < 0.05$ level.
CHAPTER 4
RESULTS AND DISCUSSION

4.1 EGCG reduced fat accumulation in wild type C. elegans

Compared with the control, the triglyceride (TG) content decreased with the increased concentrations of EGCG (Figure 4.1). The results indicated that the TG content was significantly reduced by 10% (100 µM) and 20% (200 µM) in a dose-dependent manner. This is consistent with previous reports that EGCG treatments could inhibit lipogenesis in vitro (Moon et al., 2007) and in vivo (Wolfram et al., 2006; Richards et al., 2010). According to previous reports, EGCG might affect fat storage through the modulation of food intake, lipid digestion and absorption, lipogenesis, and the stimulation of energy expenditure, lipolysis, and fat oxidation. To examine the potential mechanism of EGCG in C. elegans, we further tested the effects of EGCG on food intake, energy expenditure, lipogenesis etc.

Figure 4.1 Effects of EGCG on triglyceride accumulation in wild type C. elegans. EGCG treatment of C. elegans started from L1 stage and the samples were analyzed after a 2-day treatment. Data are expressed as means ± S.E. (n=16). Values with * or ** show significant difference when compared with the control (P-value < 0.05 or < 0.01, respectively). Data were analyzed by two-way ANOVA.
4.2 EGCG did not affect food intake in *C. elegans*

It is well-known that reducing fat accumulation can be caused by decreasing food intake, but it is not clear if EGCG may have an effect on this in *C. elegans*. We counted pumping rate of N$_2$ worms’ pharynx with or without EGCG treatment and found that no significant difference was observed (Figure 4.2A). Pumping rate is a mechanical movement, which can be fluctuated by many factors, such as time, light and temperature (Wang et al., 2016). We conducted another experiment using *E. coli* OP50-GFP to feed the worms, and measured the integrated density as a marker of food intake. The images of *C. elegans* fed with *E. coli* OP50-GFP were showed in Figure 4.2B. The analysis of fluorescent intensity indicated that there was no significant difference between the treatment and control groups, which was consistent with the pumping rate. Overall, these results suggested that EGCG did not affect food intake in *C. elegans*.
Figure 4.2 Effects of EGCG on food intake in wild type *C. elegans*. (A) Pumping rate was measured by counting the rate of pharyngeal muscle contractions from *C. elegans* under the optical microscope; (B) Images of green fluorescence in *C. elegans* represented by *E. coli* OP50-GFP. Data are expressed as means ± S.E. (n=48). Data were analyzed by two-way ANOVA.

4.3 EGCG did not affect physical behavior and growth rate in *C. elegans*

EGCG contains a large number of bioactive functions that may have either benefit or adverse effects on *C. elegans*, in terms of growth rate and physical behavior, but these are almost unknown. Treatments of EGCG, at 100 µM or 200 µM, showed no significant difference on the effect of both growth rate (Figure 4.3A) and physical behavior (Figure 4.3B, C, &D). Taken collectively, these data indicated that EGCG has no effect on growth or physical behavior in *C. elegans*. 
Figure 4.3 Effects of EGCG on growth rate and physiological behavior in wild type *C. elegans*. (A) Percentage of worms from L1 to adult between treatment and control groups after the 2-day treatment of EGCG. Numbers represented mean values ± S.E. (n=12 plates, each plate had more than 100 worms); (B) Average body length of N2 worms after the 2-day treatment of EGCG. Numbers represent mean values ± S.E. (n>600); (C) Average body width of N2 worms after the 2-day treatment of EGCG. Numbers represent mean values ± S.E. (n>600); (D) Average locomotive activity of N2 worms after the 2-day treatment of EGCG. Numbers represent mean values ± S.E. (n>600). Data were analyzed by two-way ANOVA.

4.4 Effects of EGCG on fat accumulation in gene-knockdown mutants

Genetic factors are also known to affect overall fat accumulation (So et al., 2011). Thus, we completed gene-epistasis assays to determine if EGCG influences genes related to fat metabolism using various available mutants, including *sbp-1, nhr-49, aak-2, fat-5, fat-6,* and *fat-7*.

*sbp-1, fat-5, fat-6,* and *fat-7* are genes that are involved in fat synthesis pathways in *C. elegans* (Eberlé et al., 2004). Deletion of these will result in reduced fat content.
Significant differences between the TG level of control and EGCG treatment groups in *sbp-1, fat-5, fat-6, and fat-7* mutants were detected (Figure 4.4). This suggested that these genes might not be involved in EGCG’s effects on reducing fat storage in *C. elegans*.

*nhr-49* and *aak-2* are genes that are involved in energy homeostasis in *C. elegans*, and knockout of these genes may cause increased fat level. Significant decreases of TG in EGCG treatment groups were observed in both *nhr-49* and *aak-2* mutants, when compared to the control groups (Figure 4.4). This result indicated that EGCG might not act on *nhr-49* and *aak-2* to mediate fat reduction in *C. elegans*.

All the genes tested showed no involvement in EGCG’s fat reduction effect, which indicated that EGCG might not act via these genes in *C. elegans*.

![Figure 4.4 Effects of EGCG on tested genes. Numbers represent mean values ± S.E. (n=4). Values with *, ** or *** show significant difference when compared with control (P-value < 0.05, < 0.01 or < 0.001, respectively). *aak-2* (5'-AMP-activated protein kinase catalytic subunit alpha-2), *sbp-1* (sterol regulatory element binding protein), *nhr-49* (nuclear hormone receptor family), *fat-5* (∆9-fatty-acid desaturase fat-5), *fat-6* (∆9-fatty-acid desaturase fat-6), *fat-7* (∆9-fatty-acid desaturase fat-7). Data were analyzed by one-way ANOVA.](image-url)
4.5 Effects of EGCG on the expression of genes involved in lipid metabolism

Since no mutants provide potential mechanism of EGCG on reducing fat storage, we then determined other genes involved in lipogenesis and lipolysis: hosl-1, atgl-1, cebp-2, acs-2, mdt-15, and pod-2, as PCR target genes.

ATGL (adipose triglyceride lipase) and HOSL (a homolog of hormone-sensitive lipase) are responsible for more than 90% of triglyceride hydrolysis (Schweiger et al., 2006). However, ATGL is reported to be also induced during adipogenesis and remains highly expressed in mature adipocytes (Kershaw et al., 2007). Our results indicated that EGCG did not affect hosl-1 expression but significantly decreased the expression of atgl-1 by 14% and 23.7% at 100 and 200 µM compared to the control, respectively (Figure 4.5).

CEBP-2 is a homolog of the CCAAT/enhancer-binding protein, which is involved in adipocyte differentiation (Xu et al., 2015). acs-2 encodes an acyl-CoA synthetase, which catalyzes the conversion of a fatty acid to acyl-CoA for subsequent β-oxidation (Van Gilst et al., 2005). mdt-15 encodes a mediator sub-unit ortholog of human MED15, which is required for normal fat accumulation and for the expression of fatty acid desaturase genes (fat-5, fat-6, and fat-7) (Taubert et al., 2006). POD is a homolog of acetyl-CoA carboxylase alpha, which is predicted to catalyze the first step in de novo fatty acid biosynthesis (Ding et al., 2015). These genes are all involved in lipogenesis. Our results showed no significant difference in cebp-2, mdt-15, and pod-2 (Figure 4.5), while showing a significant decrease in acs-2 with a decreased rate of 17.5% and 25% at 100 and 200 µM in EGCG treatment groups, as compared to the control group, respectively (Figure 4.5).
This indicated that the fat reduction effect of EGCG in *C. elegans* might be independent of *cebp-2, hosl-1, mdt-15, and pod-2*, but dependent of *acs-2* and *atgl-1*, which are involved in lipogenesis and adipogenesis, respectively. Thus, EGCG might act on inhibiting lipogenesis and adipogenesis to reduce fat in *C. elegans*.

**Figure 4.5** Effects of EGCG on the expression of lipid metabolism-related genes in *C. elegans*. Numbers represent mean values ± S.E. (n=3). Values with * or ** show significant difference when compared with the control (*P*-value < 0.05 or < 0.01, respectively). Data were analyzed by one-way ANOVA.

### 4.6 Conclusion

In conclusion, the current results suggest that EGCG reduces fat accumulation in *C. elegans* without affecting food intake, physical behavior, and growth rate. This effect was independent of *sbp-1, nhr-49, aak-2, fat-5, fat-6, fat-7, cebp-2, hosl-1, mdt-15, and pod-2*, whereas dependent of *acs-2* and *atgl-1* that are genes related to lipogenesis and adipogenesis, respectively. Further investigation would be needed to confirm the significance of *acs-2* and *atgl-1* on EGCG’s effects on fat reduction by conducting gene-knockdown assays.
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