

# PREVENTING AND TREATING OBESITY AND METABOLIC DYSFUNCTIONS WITH ANTIOXIDANTS

by

SARY IBRAHIM ALSANEA

(Under the Direction of Dexi Liu)

## ABSTRACT

Because of excessive energy intake and lack of sufficient physical activity, obesity prevalence has been increasing worldwide. In the United States, more than a third of the population is obese, which has brought about the recrudescence of obesity-associated diseases such as cardiovascular diseases, fatty liver diseases, type 2 diabetes, and cancer. The underlying mechanism of obesity, especially diet-induced obesity, is a low-grade inflammation in adipose tissue initiated by the high level of reactive oxygen species generated by the oxidation process of energy metabolism. In fact, human and animal studies have linked obesity with oxidative stress and inflammation. This dissertation project is designed to explore the possible use of antioxidants to obstruct diet-induced obesity and to study the underlying mechanisms in an animal model. The rationale for this research is that reactive oxygen species generated by excessive energy intake are directly responsible for a low-level and chronic inflammation, leading to weight gain, insulin resistance, and fatty liver. Scavenging the excess amount of reactive oxygen species generated by overeating through supplying the appropriate antioxidants should suppress obesity development and alleviate obesity-associated metabolic problems. This dissertation encompasses five chapters. **Chapter 1** is a literature review

about the roles of reactive oxygen species in obesity and applications of antioxidants for obesity management. **Chapters 2** and **3** are published research articles on obesity prevention with antioxidants. **Chapter 4** summarizes the work with intention to reverse obesity and obesity-induced metabolic disorders using antioxidants. The last chapter, **Chapter 5**, discusses future perspectives and the remaining challenges of antioxidant-based approaches for the management of obesity.

INDEX WORDS: Obesity, Fatty liver, Insulin resistance, Inflammation, Antioxidant, Phloretin, Benzyl isothiocyanate, *S*-carvone, Catechin, EGCG

PREVENTING AND TREATING OBESITY AND METABOLIC DYSFUNCTIONS  
WITH ANTIOXIDANTS

by

SARY IBRAHIM ALSANEA

B.S., College of Pharmacy, King Saud University, Riyadh, Saudi Arabia, 2010

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial  
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2017

© 2017

SARY IBRAHIM ALSANEA

All Rights Reserved

PREVENTING AND TREATING OBESITY AND METABOLIC DYSFUNCTIONS  
WITH ANTIOXIDANTS

by

SARY IBRAHIM ALSANEA

Major Professor:	Dexi Liu
Committee:	Mandi M. Murph Shelley B. Hooks James L. Franklin Joan G. Fischer

Electronic Version Approved:

Suzanne Barbour  
Dean of the Graduate School  
The University of Georgia  
December 2017

## DEDICATION

I would like to dedicate this dissertation to my lovely parents: my mother, Mashail Alsaidan, and my father, Ibrahim Alsanea. You have been supporting and encouraging me toward success since I was born. I also would like to dedicate this dissertation to my terrific wife, Arwa Alkhulaifi, and to my handsome son, Ibrahim. Without your extreme patience and emotional motivation, I could not have earned this degree.

To my cooperative and kind siblings: Yaser, Hadel, Fahad, and Monerah. To my teachers who have taught me throughout elementary, intermediate, high-school, undergraduate degree, English as a second language institute, and graduate school. I would not be able to be in the scientific and academic fields without your education. A special dedication to my high school mathematics teacher, Mr. Khaled Montaser, from whom I have learned a lot about self-discipline and sincerity.

## ACKNOWLEDGEMENTS

All Praise is for God (Allah) by whose favor good works are accomplished. I would like to thank my advisor Dr. Dexi Liu for accepting me in his lab and sharing his scientific and philosophical wisdom. Thanks, Dr. Liu, for your time, effort, and patience until I reached this stage and I am looking forward to going further. Thanks for all that you have done for me. You have truly made a difference to my life. I am very proud and pleased that I graduated from your lab and under your guidance. In addition, I would like to thank my advisory committee members, Dr. Murph, Dr. Hooks, Dr. Franklin, and Dr. Fischer for their valuable comments, feedback, and advice. Thanks to all current and previous members of Dr. Liu's laboratory for their collaboration and help. Many thanks to Dr. Mingming Gao and Dr. Yongjie Ma for their technical support and valuable suggestions for my experiments. Special thanks to my labmates and friends, Dr. Yahya Alhamhoom and Dr. Mohammad Alsaggar. The time we spent together and the efforts you made during this special period of my career development are extremely valuable and greatly appreciated. I thank Dr. Megan Morgan for proofreading and editing the published manuscripts and her useful English writing tips and comments. I would like to thank Mr. Robby Nadler (the Assistant Director of UGA Writing Center) and Mrs. Judy Milton (the Assistant Dean of the Graduate School) for their effort and cooperation in the Dissertation and Thesis Writing Course (GRSC 8300W). Many thanks to Dr. Ruchi Yadav, Dr. Hardik Mody, Ali Altharawi, Omar Alsaidan, Faris Almutairi, Ali Alshamrani, Mohammed Alqinyah, Ibrahim Asiri, Dr. Rashed Alhotan, Dr. Mohammed

Alasmrai, Esam Qattan, Hamad Alenezi, Dr. Fahaad Alenazi, Dr. Ahmad Albassam, Abdulrahman Alwhaibi, Fahad Almughem, and other friends for their support. I would like to thank the Department of Pharmaceutical and Biomedical Sciences for their support, and the animal facilities at the University of Georgia for their wonderful work and cooperation during my dissertation research. Many thanks go to the funding agencies for financial support. Special thanks go to my sponsor, the King Saud University, for my scholarship. And finally, I would like to extend my thanks to the Saudi Arabian Cultural Mission (SACM) to the U.S. for their support during the last seven years.



## TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS .....	v
LIST OF TABLES .....	ix
LIST OF FIGURES .....	x
CHAPTER	
1 INTRODUCTION AND LITERATURE REVIEW ON OBESITY AND REACTIVE OXYGEN SPECIES .....	1
Introduction.....	1
Contributing Factors to Obesity .....	3
Obesity Pathogenesis .....	8
Obesity-associated Metabolic Diseases .....	16
Blocking Obesity by Neutralizing ROS.....	18
Obesity Animal Models .....	21
Antioxidants Used in Dissertation Research.....	24
Objectives of Dissertation Research .....	30
References.....	39
2 PHLORETIN PREVENTS HIGH-FAT DIET-INDUCED OBESITY AND IMPROVES METABOLIC HOMEOSTASIS .....	71
Abstract.....	72
Introduction.....	73

	Material and Methods .....	75
	Results.....	78
	Discussion.....	82
	References.....	93
3	BITC AND <i>S</i> -CARVONE RESTRAIN HIGH-FAT DIET-INDUCED OBESITY AND AMELIORATE HEPATIC STEATOSIS AND INSULIN RESISTANCE .....	100
	Abstract.....	101
	Introduction.....	103
	Material and Methods .....	105
	Results.....	108
	Discussion.....	111
	References.....	122
4	ANTIOXIDANTS REVERSE OBESITY-ASSOCIATED INSULIN RESISTANCE WITHOUT REDUCING OBESE MOUSE WEIGHT.....	127
	Introduction.....	127
	Material and Methods .....	128
	Results.....	131
	Discussion.....	135
	References.....	147
5	CONCLUSION AND FUTURE PERSPECTIVES .....	150
	References.....	154

## LIST OF TABLES

	Page
Table 1.1: Structures, sources, and characteristics of reactive oxygen species. ....	31
Table 1.2: Basic antioxidant mechanisms with chemical functional groups .....	32
Table 1.3: Summary of antioxidants prevention studies conducted on animal obesity models .....	33
Table. 1.4 Summary of antioxidants impact on body weight of overweight and obese subjects.....	34
Table 2.1: Primers used for quantitative real time PCR analysis of gene expression .....	86
Table 3.1: Primers used for gene expression analysis by real time PCR.....	115

## LIST OF FIGURES

	Page
Figure 1.1: ROS generation and obesity promotion mechanism .....	35
Figure 1.2: Mechanistic illustration of association between obesity and other diseases ...	37
Figure 1.3: Chemical structures of antioxidants used in dissertation research .....	38
Figure 2.1: Phloretin inhibits body weight gain in mice fed an HFD.....	87
Figure 2.2: Impacts of phloretin treatment on adipose tissues of animals.....	88
Figure 2.3: Phloretin blocked HFD-induced inflammation .....	89
Figure 2.4: Phloretin prevented HFD-induced fatty liver .....	90
Figure 2.5: Phloretin improved glucose homeostasis and hyperinsulinemia in HFD-fed mice.....	91
Figure 2.6: Effects of phloretin treatment on obese mice fed an HFD .....	92
Figure 3.1: BITC and <i>S</i> -carvone inhibit body weight gain in mice fed an HFD .....	116
Figure 3.2: Impacts of BITC and <i>S</i> -carvone treatment on adipose tissues of animals ....	117
Figure 3.3: BITC and <i>S</i> -carvone suppressed HFD-induced inflammation .....	118
Figure 3.4: BITC and <i>S</i> -carvone blocked HFD-induced fatty liver.....	119
Figure 3.5: Impacts of BITC and <i>S</i> -carvone treatment on mRNA levels of genes responsible for hepatic lipid metabolism .....	120
Figure 3.6: BITC and <i>S</i> -carvone improved glucose homeostasis and hyperinsulinemia in HFD-fed mice .....	121
Figure 4.1: Catechin and EGCG inhibit body weight gain in mice fed an HFD .....	138

Figure 4.2: Impacts of catechin and EGCG treatment on adipose tissues of animals .....	139
Figure 4.3: Catechin and EGCG suppressed HFD-induced hepatic lipid accumulation .	140
Figure 4.4: Catechin and EGCG improved hyperinsulinemia and insulin resistance in HFD-fed mice .....	141
Figure 4.5: EGCG did not induce weight loss in obese mice .....	142
Figure 4.6: Impacts of EGCG treatment on adipose tissues of animals .....	143
Figure 4.7: EGCG failed to restore HFD-induced fatty liver in obese mice .....	144
Figure 4.8: EGCG slightly improved insulin sensitivity in obese mice .....	145
Figure 4.9: Antioxidant combination treatment study .....	146

## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW ON OBESITY AND REACTIVE OXYGEN SPECIES

#### **Introduction**

Obesity is defined by the World Health Organization as “abnormal or excessive fat accumulation that may impair health” and is also defined medically as a body mass index (BMI) greater than 30 kg/m<sup>2</sup>. A recent research article published in the *New England Journal of Medicine* reported that the world’s obese population in 2015 totaled 107.7 million for children and 603.7 million for adults. High BMI accounted for 4 million global deaths in 2015 with nearly 70% of those deaths due to cardiovascular diseases. About 50 different diseases have been linked to obesity, including cardiovascular diseases, diabetes, hypertension, liver and kidney diseases, and many types of cancer. The United States has the highest total number of obese adults with about 79 million individuals (1). Obesity has become the most severe disease in the United States, and the trend continues to rise (2). In 2016, the total percentage of overweight and obese subjects among American population was 64.8% (3). Economically, the estimated direct costs for obesity-associated diseases range from US\$147 billion to \$210 billion per year (4). The estimated productivity-related cost is \$4.3 billion per year, which is considered as indirect cost due to job absenteeism (5).

Current obesity treatments can be divided as either pharmacological or surgical in nature, but effectiveness is limited in both cases. Medication-based obesity therapy is

limited in efficacy ( $\approx 5.8 - 10.2$  kg weight loss) and accompanied by many side effects. In 2016, there were only six drugs (Orlistat [Alli<sup>®</sup>/Xenical<sup>®</sup>], Lorcaserin [Belviq<sup>®</sup>], Phentermine/Topiramate [Qsymia<sup>®</sup>], Naltrexone/Bupropion [Contrave<sup>®</sup>], and Liraglutide [Saxenda<sup>®</sup>]) approved as anti-obesity drugs by the Food and Drug Administration. The side effects of these drugs include: steatorrhea, nausea, vomiting, diarrhea, dizziness, dry mouth, headache, hypoglycemia, paresthesia, insomnia, and flatulence (6). On the other hand, bariatric surgeries (e.g., gastric bypass, sleeve gastrectomy, adjustable gastric band, and biliopancreatic diversion with duodenal switch) are expensive, have complications, and are recommended only for severe cases of obesity (Body mass index  $\geq 40$  kg/m<sup>2</sup>) (7). More importantly, some bariatric surgeries lose their effectiveness and patients regain weight after 2 years (8).

Among the many factors that are inherent and/or environmental in nature, the increased availability, accessibility and affordability of energy-dense foods, along with the marketing of such foods, play a critical role in promoting excess caloric consumption and increasing obesity prevalence. Significant measures have been taken to curb obesity in recent years, including restriction of advertisements of unhealthy foods to children, as well as improvements of school meals to reduce consumption of unhealthy foods and promote healthy food. Low-income families also received offers for free cooking classes to encourage healthy home-cooked meals (9). The public campaign “Change4Life” has helped the establishment of various education programs focusing on “Eat Well, Move More, and Live Longer.” Despite these and many other interventions, some of which are very effective for people and display significant benefits such as increasing physical activity and reducing screen watching time (9, 10), the overall trend of obesity is

increasing (11). Between 1990 and 2015, the prevalence of obesity in the US increased by 264% (2).

## **Contributing Factors to Obesity**

### *Genetic*

Human biology and behavior are considered major contributing factors to the obesity epidemic. Individuals with obese family members, the children of obese parents, and obese children are more likely to become obese adults (12). Genetic influence on obesity can be monogenic or polygenic, involving mutations of genes that play an important role in controlling appetite and/or energy homeostasis. Examples of these genes are melanocortin-4 receptor (MC-4R), leptin receptor, leptin, and fat mass and obesity associated gene (FTO) (13-15). MC-4R, a G-protein-coupled receptor, regulates feeding behavior and energy metabolism upon binding to its ligand  $\alpha$ -melanocyte stimulating hormone. As a result, certain mutations in MC-4R have been shown to lead to the rapid onset of obesity (16, 17). Leptin, an important hormone secreted by adipocytes, regulates food intake centrally and energy expenditure peripherally. Mutations in leptin gene or its receptor gene can lead to obesity due to overfeeding caused by a low satiety feeling (18). Moreover, FTO mutations were proposed as one of the most critical gene mutations linked to obesity. It was reported that certain FTO mutations lead to lipolysis suppression and food intake stimulation; the mechanism in question suggested to occur through oxoglutarate-dependent nucleic acid demethylase inhibition (19-21). However, genetic obesity is often caused by polygenic mutations rather than monogenic mutation. Over a hundred gene variants with a connection to



obesity have been identified (22, 23). As far as the treatment of genetic obesity is concerned, monogenic obesity is believed to be easier to treat since it is caused by single gene mutation, and may be corrected using the principles and techniques of gene therapy currently available (24).

### *Positive energy-balance*

Positive caloric intake represented by sugar-sweetened beverage consumption and a high-energy density diet has been linked to obesity (25, 26). Sweetened beverages are considered one of the largest sources of calories in the United States for children and adults (27). The weak satiety and compensatory dietary responses associated with sugar-sweetened beverage consumption is the proposed mechanism for the sugar-sweetened beverage-obesity relationship (28). Alternatively, diet, especially a fat-enriched diet or high-fat diet (HFD) (because the caloric content per gram is more than doubled of carbohydrates or proteins), plays a critical role in weight gain compared to other contributing factors for obesity. In fact, an HFD with 30-78% of total energy from fat is commonly used in animal studies to induce obese animal model (29, 30). HFD leads to leptin resistance and attenuates satiation feeling (31, 32). Similar to drug addiction, HFD eating is often addictive, exhibiting anxiety-like behavior with increased corticosterone levels and motivation for food (33, 34).

### *Social factors*

Eating is often part of social events and has always been an important part of our daily routine. Eating at social events is different from eating alone. For example,

individuals tend to eat more when they join people who eat more (35). Therefore, excess food consumption at social events has been identified as a contributing factor for obesity, as food-serving social events or parties are becoming increasingly popular (36). The influence of social events as a contributing factor of obesity will be greater after alcohol is consumed with fatty food because the combination induces the release of hypothalamic peptides such as galanin, orexin, and dynorphin that have been found to promote obesity (37).

### *Stress*

Stress has been given distinctive attention and studied during the last 30 years. The PubMed search for the keyword “stress” publication increased ten fold between 1986 and 2016. The American Psychological Association (APA) reports that 75% of Americans experienced at least one stress symptom in the last month (38). Given that 23% of Americans report consuming more food in response to stress, mental health plays an important role in understanding obesity’s mechanisms. It appears that the pleasurable feeling associated with eating can suppress stress or at least reduce negative feelings. The elevated glucocorticoid and insulin levels associated with stress have been indicated as the mechanism behind stress-triggered eating behaviors (39, 40). Additionally, tasty food activates reward center via stimulation of endogenous opioid release (41). This might account for the correlation between stress prevalence and increased obesity prevalence, where women have higher stress and obesity rates than men (11, 38, 42).

### *Lifestyle*

Lifestyle, including a lack of regular physical activities, poor eating habits (e.g., frequency and eating time), tobacco smoking, circadian rhythm (the “biological clock”) disruption, and adipogenic chemical exposure contribute significantly to the obesity epidemic (43-46). These unhealthy habits could influence the expression of genes that favor adipogenesis and weight gain. Unfortunately, the impact of affected gene expression might persist for long periods. For example, after smoking cessation, former heavy smokers have a higher obesity risk than former light smokers (47).

### *Sleep deprivation*

Inadequate sleep is considered as another risk factor for obesity (48). It was reported that the number of sleep hours is negatively correlated with BMI (48, 49). Sleeping less than 6 hours/day has been associated with obesity, independently of work and health-related factors (50). A meta-analysis of global studies has demonstrated the relationship between short sleep and obesity in both children and adults (51). The mechanism involves a hormonal imbalance between grehlin and leptin that results in hunger stimulation and energy expenditure inhibition (52).

### *Socioeconomic*

Socioeconomic status has been linked with obesity prevalence (53). The relationship between obesity and greater socioeconomic status corresponds with higher obesity rates in developing countries (54). In contrast, developed countries (e.g., the United States) have different patterns where a higher socioeconomic status corresponds

to lower obesity rates compared with low-income families (children and adults) (55). Beside education differences, there are different economic factors that participate in this phenomenon. People with good socioeconomic status can afford healthy food and access to physical activity facilities. On the other hand, lower-income families choose calorically-dense food because it is cheaper (54, 55).

### *Viral infection*

Viral infection can cause weight gain and obesity (56). Human adenovirus-36 has been reported to cause obesity in mice, chicken, and humans through the direct increase in the adipocytes size (hypertrophy) and number (hyperplasia) (57). Other viruses such as Canine distemper virus, Rous-associated virus, and Borna disease virus, have been linked with obesity as well (58). The proposed mechanism includes adipogenesis stimulation and hormonal dysregulation after viral-induced hypothalamic disruption (57, 59).

### *Endocrine diseases*

Hormonal imbalance occurs in endocrine diseases causing fat accumulation and obesity development. Low levels of thyroid hormone (i.e., hypothyroidism patients) suppress metabolism rate and thermogenesis, leading to increased storage of fat (60). On the other hand, raised levels of glucocorticoids, as in Cushing's syndrome patients, increase appetite and cause weight gain through activation of the central stress-response pathways (61, 62). Regulation these hormones reverses hormonal imbalance-induced obesity (63).

## *Medications*

Numerous medications have been reported to induce weight gain (64, 65). These medications include antipsychotics, antidepressants, antiepileptics, steroids, contraceptives, antihistamines, protease inhibitors, antihypertensives, and antidiabetics (49, 66). While the mechanisms of weight gain induction are different from each other and the mechanisms are not entirely illustrative, dose and treatment duration are the main players that impact the weight gain of these medications. Alternatives with lower impact on weight gain should be considered, especially for people who are at high risk of obesity and obesity-associated complications.

## **Obesity Pathogenesis**

Since several factors are involved in obesity development, obesity pathogenesis is complex and multifactorial. Two key tissues, gut and adipose tissue, play a crucial role in obesity development. They produce hormones and peptides that are responsible for the hypothalamus's regulation of hunger and satiety (67). While the circulating peptide hormone ghrelin is secreted from the stomach to stimulate hunger, all other gastrointestinal tract peptides activate satiety pathways. Examples of gastrointestinal tract peptides, which inhibit food intake, are glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), and peptide YY3-36 (PYY3-36) (68). Any dysregulation, downregulation, or inactivation of these peptides might continuously stimulate hunger or inhibit the satiety signals, and consequently lead to high caloric consumption and obesity development (69-71).

Adipose tissue is an endocrine organ that maintains and regulates metabolism via secretion of adipokines (72). Leptin is an anorexigenic hormone secreted from white adipose tissue. Circulating leptin can cross the blood-brain barrier and bind to leptin receptors in the hypothalamus. Upon activation, the appetite signaling pathway is inhibited through the downregulation of neuropeptide Y (NPY) and agouti-related peptide (AgRP) (68). Genetic defects of a leptin gene (*ob/ob*) or leptin receptor gene (*db/db*) has been shown to lead to obesity in mice and children (13, 73). The lack of leptin signaling pathway efficiency leads to elevation of leptin plasma levels in obese subjects that ends with leptin resistance. Another adipokine that is secreted from white adipose tissue is adiponectin, which is also known as adipocyte complement-related protein of 30 kDa (Acrp30). The plasma level of adiponectin was downregulated in obese subjects and resorted after body weight loss (74). Adiponectin plays a critical role in metabolism through regulating insulin sensitivity, glucose transportation, gluconeogenesis, and fatty acid oxidation (75). Other fatty tissues, such as beige and brown adipose tissues, also play an important role in regulating thermogenesis, insulin sensitivity, and metabolic homeostasis (76).

#### *Obesity and chronic inflammation*

Obesity is correlated with low-grade chronic inflammation in adipose tissues that play a critical role in adipokine dysregulation (77, 78). Adipose tissue with inflammation releases free fatty acid (FFA) and pro-inflammation mediators, such as resistin, IL-1, IL-6, IL-12, TNF $\alpha$  and IFN $\gamma$ , that contribute to obesity development and metabolic dysfunction (79-81). Several human studies support a chronic inflammation-obesity association (82-86). It was observed that calorie restriction for 8 weeks with seafood

consumption significantly reduced inflammatory markers (high-sensitivity C-reactive protein, IL-6, prostaglandin  $EF2\alpha$ ) in overweight/obese subjects (82). Comparable observation was perceived with longer calorie restriction diets (one year) in overweight/obese postmenopausal women (83). Similarly, another study showed that reduction in percent body fat percentage and waist circumference were also associated with a drop in the plasma level of inflammation marker high-sensitivity C-reactive protein (84). At the tissue level, adipose tissue inflammatory markers (IL-1- $\beta$ , TNF- $\alpha$ , CD11B, CD11C) were suppressed in women with gestational diabetes mellitus after dietary weight loss (86). All the previous studies serve as epidemiological evidence for the association between chronic inflammation and overweight/obese BMI ( $\geq 25$  kg/m<sup>2</sup>).

Anti-inflammatory medications have shown different response patterns depending on the inflammatory condition. Tumor necrosis factor  $\alpha$  blockers showed better anti-inflammatory effects on patients who had lost weight compared with overweight and obese patients with inflammatory diseases (e.g., psoriatic arthritis) (87). A similar phenomenon was observed with salicylates (known as non-steroidal anti-inflammatory drugs or NSAID). Salicylates resulted in lower circulatory inflammatory markers (c-peptide and IL-1 $\beta$ ) that were associated with other beneficial outcomes such as increasing insulin secretion or decreasing insulin clearance rate (88-90). In addition, salicylates can restore the plasma level of anti-inflammatory adipokines (91, 92) and reduce oxidative stress in overweight obese subjects (93).

*Biochemical and cell biological basis of ROS in obesity*

Reactive oxygen species (ROS) (**Table 1.1**) are produced as byproducts of natural metabolic reactions in cells. Despite the fact that NADPH oxidase (NOX), peroxisomes, and endoplasmic reticulum can generate ROS, the major source of ROS production is mitochondria during energy production (94). In oxidative phosphorylation, transferred electrons are required for establishing a proton gradient and adenosine triphosphate (ATP) synthesis. Biochemically, electrons are passed through a series of proteins via oxidative-reductive reactions in which each acceptor protein along the chain has greater reduction potential than the previous, with oxygen being the destination molecules of electron transfer. In normal states, oxygen through several reactions will be converted eventually to water ( $O_2 + 1e \rightarrow \text{Superoxide} + 1e$  [SOD]  $\rightarrow H_2O_2 + 1e$  [CAT]  $\rightarrow$  hydroxyl radical + 1e [GSH]  $\rightarrow H_2O$ ); yet,  $\leq 2\%$  of electrons are added to oxygen resulting in incomplete reduction and superoxide radical ( $O_2^{\bullet-}$ ) formation. Superoxide anions can lead to the propagation of free radicals, causing tissue damage by attacking unsaturated fatty acyl chains in the lipid bilayer of cell membranes, proteins, and even DNA in the nucleus. Accumulating oxidative damage in cells can then affect mitochondrial function and further induce the rate of ROS production, immune reactions, and inflammation (95, 96).

The antioxidant defense system exists in various tissues and can be classified as non-enzymatic and enzymatic. The non-enzymatic antioxidant defense system includes cofactors (e.g., coenzyme Q10), organosulfur compounds (e.g., glutathione), retinol (vitamin A), and nitrogen non-protein compounds (e.g., uric acid). Alternatively, the endogenous enzymatic antioxidants are glutathione peroxidase, catalase, and superoxide dismutase (SOD) (97). Glutathione peroxidase (GPx) reduces hydrogen peroxide ( $H_2O_2$ )



by transferring the energy of the reactive peroxides to glutathione (GSH). Catalase (CAT) is another primary antioxidant enzyme, which is located in peroxisomes near mitochondria, reacts with the hydrogen peroxide ( $H_2O_2$ ) to produce water and oxygen. Another major antioxidant enzyme is superoxide dismutase (SOD), which catalyzes the dismutation of superoxide into hydrogen peroxide. There are three forms of superoxide dismutase exist: SOD1 (CuZn-SOD) in cytoplasm, SOD2 (Mn-SOD) in mitochondria, and SOD3 (EC-SOD) in extracellular spaces (98, 99). Glutathione reductase and glucose-6-phosphate dehydrogenase are considered as secondary enzymatic antioxidants because they do not scavenge ROS directly. Instead, they reactivate other antioxidants through regenerating the reducing coenzymes, e.g., nicotinamide adenine dinucleotide phosphate (NADPH) (97).

### *ROS in obesity*

Oxidative stress is associated with obesity as well as many different diseases, including Alzheimer's disease, Parkinson's disease (100), cancer, asthma, joint diseases (101), diabetes, cardiovascular diseases (102), mitochondria disease (103). Oxidative stress generated by accumulated ROS was proposed as one of the contributing factors for chronic inflammation during obesity development (104-106). In the process of weight gain, the excess intake of nutrition or energy results in accumulation of triglyceride (TG) in adipose tissues. An increased lipid accumulation and possibly lipid metabolism elevates ROS level in adipose tissue (104). The elevated ROS and hypoxia (due to the rapid expansion of the size and number of adipocytes) induce cell damage. The adipocyte damage functions as a signal for infiltration of macrophages, which, upon activation, release pro-inflammation cytokines and establish inflammation in adipose

tissue. Consequently, the proteases released by the activated macrophages digest proteins in the intracellular space of adipocytes, creating room for each adipocyte to expand its size and help store the excess energy acquired from eating.

#### *Supportive studies about the role of ROS in obesity development*

Evidence that supports the obesity and oxidative stress relationship can be seen in several studies (104-106). Furukawa *et al.* (104) showed that oxidative stress markers (plasma TBARS and urinary 8-epi-PGF2 $\alpha$ ) of obese subjects were positively correlated with BMI and waist circumferences. The elevated oxidative stress markers (H<sub>2</sub>O<sub>2</sub> and thiobarbituric acid reactive substances [TBARS]) were observed in plasma of three obese mice models (KKAy, *db/db*, diet-induced obesity). At the tissue level, the oxidative stress marker TBARS was selectively elevated in adipose tissue but not liver or muscle. In white adipose tissue of obese mice, the proinflammatory gene (TNF- $\alpha$ ) was upregulated. Both gene expression and activity of antioxidant enzymes (SOD and GPx) were inhibited in white adipose tissue but not muscle or liver. In *in vitro* mechanistic studies, ROS-associated adipogenesis in 3T3-L1 was blocked with *N*-acetyl-cysteine (NAC) antioxidant. NAC also reversed H<sub>2</sub>O<sub>2</sub>-induced elevation of adiponectin, plasminogen activator inhibitor-1, and PPAR $\gamma$ . The second supportive study was conducted by Tormos *et al.* (105). They showed that a mitochondrial targeted antioxidant (MitoCP) reduced mitochondrial H<sub>2</sub>O<sub>2</sub> levels and diminished differentiation of primary human mesenchymal stem cells to adipocytes. Exogenous ROS (H<sub>2</sub>O<sub>2</sub>) restores adipocyte differentiation in the presence of mitochondrial antioxidant. The third supportive study was offered by Higuchi and colleagues (106). They showed that ROS

and Forkhead box O (FOXO) transcription factor are involved in adipocyte differentiation from preadipocytes, which is human adipose-derived stem cells. Scavenging ROS with NAC or EUK-8 (an antioxidant that mimics SOD and CAT) inhibited adipocyte differentiation.

#### *Sources of adipose ROS*

Sources of ROS in accumulated fat can be varied. A study showed that the main source of ROS during adipocyte differentiation was NADPH oxidase because differentiation suppression was observed with NADPH oxidase inhibitors (diphenyleneidonium, apocynin) but not xanthine oxidase inhibitor (oxypurinol), mitochondrial electron transport chain complex I inhibitor (rotenone), or mitochondrial electron transport chain complex II inhibitor (thenoyltrifluoroacetone) (104). In contrast, another study demonstrated that mitochondria, specifically mitochondrial electron transport chain complex III, are the main source of ROS that regulate adipocyte differentiation through mammalian target of rapamycin complex 1 (mTORC-1) mediated mechanism (105). Taken together, the ROS elevated level was detected *in vitro* and *in vivo*, where it is not entirely understood which part of the adipocyte produces the most ROS.

ROS are believed to be part of the low-grade inflammation commonly seen in obese patients, a benchmark of adipose tissue expansion and remodeling. A histological landmark of adipose tissue inflammation is the presence of crown-like structures (CLS), consisting of many macrophages surrounding one adipocyte (107, 108). At the molecular level, the high levels of ROS can be generated from mitochondrial superoxide

overproduction and NADPH oxidase (NOX) activation (**Fig. 1.1**). The mitochondrial superoxide overproduction is triggered by high oxygen consumption, hyperglycemia, and high free fatty acid levels. High glucose levels can also stimulate ROS production through protein kinase C in the short-term and advanced glycation-end production in long-term hyperglycemia. The high free fatty acids levels lead to ROS through different mechanisms including mitochondrial superoxide overproduction and NOX stimulation via the toll-like receptor and diacylglycerol-mediated protein kinase C activation. The high levels of ROS in cells activate the redox-sensitive transcriptional factor NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells), triggering expression of monocyte chemoattractant protein-1 (MCP-1) and macrophage migration inhibitory factor (MIF) genes and the release of pro-inflammatory cytokines (TNF- $\alpha$  and IL-6) and growth factors (connective tissue growth factor and insulin-like growth factor-1) (109, 110). After these sequences, pro-inflammatory macrophage (M1) is infiltrated and causes extracellular matrix (ECM) remodeling through protease secretion that leads to degradation of the connective tissue and basement membrane proteins. As a result, remodeling of adipose tissue ECM facilitates angiogenesis and adipogenesis.

#### *Diet might affect ROS production*

The idea of altering ROS production through diet was supported by Kitabchi and colleagues (111), whose study showed that a high-protein diet (30% protein) showed better improvement in oxidative stress markers (dichlorofluorescein and malondialdehyde) and inflammatory markers (c-reactive protein, TNF- $\alpha$ , and IL-6) compared to high carbohydrate diets (55% carbohydrate) in non-diabetic obese

premenopausal women. Although the study was relatively long (six months), the study population was relatively small (n=24) and included only one gender. Indeed, a different study showed that a high-carbohydrate meal (76.1% carbohydrate) reduced plasma and muscle level of the SOD antioxidant enzyme significantly compared with a high-fat meal (76.7% fat) (112). The reduction of the endogenous enzyme activity can be explained by the sharp increase in the blood glucose and serum insulin levels, which were reported only with the high-carbohydrate meal. Moreover, the decrease of proinflammatory cytokine (IL-18) was reported only with the high-fat meal.

## **Obesity-associated Metabolic Diseases**

### *Cardiovascular disease*

The relationship between obesity and cardiovascular disease is well established (113). In obese patients, left ventricular hypertrophy occurs to increase cardiac output and maintain supply of blood to the whole body. Excess fat, especially visceral fat, increases peripheral resistance. Furthermore, adipose tissue produces inflammatory mediators such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$  that contribute to plaque formation and pathogenesis of atherosclerosis (114).

### *Insulin resistance*

Obesity-associated insulin resistance and hyperglycemia are well established (104, 110, 115, 116). Nutritional overload tends to saturate the electron transport chain in mitochondria and generates excess ROS, leading to inhibition of FFA oxidation and production of diacylglycerol (DAG). DAG activates several signaling pathways,

including protein kinase C (PKC), c-Jun N-terminal kinase (JNK), and inhibitor of nuclear factor  $\kappa$ B kinase  $\beta$  (IKK- $\beta$ ), to inhibit insulin receptor substrate -1 (IRS-1) and thus attenuate glucose uptake. Under this circumstance, the pancreatic  $\beta$  cells produce more insulin, leading to hyperinsulinemia and insulin resistance (117) (**Fig. 1.2**).

#### *Nonalcoholic fatty liver disease*

Nonalcoholic fatty liver disease (NAFLD) is strongly connected with obesity (118). The association between NAFLD and obesity involves multiple factors such as insulin resistance, hyperlipidemia, and oxidative stress (**Fig. 1.2**). Insulin resistance with hyperinsulinemia activates the hormone-sensitive lipase to induce lipolysis and release of free fatty acids (FFA) from adipose tissue to liver plus other tissues. On the other hand, oxidative stress associated with obesity can suppress FFA exportation from the liver and promote fatty liver pathogenesis, triggering endoplasmic reticulum stress that causes inhibition of apolipoprotein synthesis. The accumulated lipids further induce cytochrome P450 to produce more ROS (118-121).

#### *Cancer*

Many types of cancer have been associated with obesity (122). Clinical studies have shown that higher BMI is associated with higher cancer prevalence and tumor recurrence after chemotherapy (123, 124) while decreasing BMI was associated with favorable cancer prognoses (125). Obesity-associated oxidative stress, low-grade chronic inflammation, adipocyte progenitors, dysregulated hormones, adipokines, and lipid mediators (126, 127) are directly involved in cancer development. For example, oxidative

stress at the early stage of obesity development suppresses critical enzymes in autophagy (128, 129). Moreover, obesity-associated ROS interact with DNA and proteins involved in transcription and regulation of gene expression (130, 131). DNA damage is a direct cause of cell transformation (132). Oxidative stress also contributes to cancer progression and metastasis (133-135).

### **Blocking Obesity by Neutralizing ROS**

The established connection between ROS and obesity has inspired efforts to block obesity by neutralizing the effects of ROS. Both preclinical and clinical studies have been conducted in different models and dealing with various stages of obesity and with obesity-associated metabolic symptoms. Four approaches have been taken (**Table 1.2**), each employing different mechanisms including: (1) enzyme-based systems (SOD, CAT, and GPx), (2) oxidant chelating reagents (transferrin), (3) electron donor molecules (vitamins, GSH, resveratrol), or (4) activators to enhance the overall capacity of endogenous antioxidant (136-138).

Since GPx and SOD levels were significantly lower in obese subjects (139), Cui and colleagues have employed a gene transfer approach to overexpress SOD3 in mice and demonstrated effective blockage of diet-induced weight gain, insulin resistance, and fatty liver (140). However, the major limitation for an enzyme-based approach is that these enzymes have limited activity in scavenging the hydroxyl radical ( $\bullet\text{OH}$ ), the most reactive radical (141). For this reason, most studies in the literature have focused on the use of molecules with high reduction potential, more commonly called antioxidants. Both

preclinical and clinical studies have been conducted to characterize the effects of antioxidants on prevention or treatment of obesity and other metabolic disorders.

#### *Preclinical studies*

Preclinical studies often employ diet-induced obesity in mice as a model, although transgenic *db/db* or *ob/ob* mice are also used (140, 142-150) (**Table 1.3**). Diet-induced obesity is more relevant to human obesity. It appears that antioxidant treatment does not affect food intake, with the exception of galangin (146) and carnosic acid (147). However, the impact of the treatments on body weight observed varies, likely due to the differences in various studies of the injection dose, route of administration, injection frequency, and the physiological status of animals. Regardless, data collected so far support the notion that some antioxidants may be effective in blocking weight gain of animals fed an HFD.

#### *Clinical studies*

Most clinical investigations have focused on the use of antioxidants to induce weight loss and/or improve metabolic syndrome (151-159). Antioxidants are often provided as food supplements or included in drinks. In many cases, vegetables or fruits rich in antioxidants were directly employed. Different from preclinical studies, antioxidant-based treatments of oxidative stress-induced diseases have resulted in some controversial observations in clinical trials (160). As summarized in **Table 1.4**, three out of ten studies demonstrated weight loss in antioxidant-treated patients. Weight loss was reported in some studies (152, 157, 159) using green tea (152), *Nigella Sativa* (157), or pomegranate extract (159) but not others. Improvement in metabolic markers was also



reported (153, 158). A similar observation was also made in cancer clinical studies, where antioxidants showed positive (161), negative (162), or no effects on patients (163) in contrast to more consistent and positive conclusions derived from animal studies. Overall, the clinical studies conducted so far have not provided convincing evidence in support of beneficial effects of antioxidants in obese patients or patients with other metabolic diseases such as diabetes or cardiovascular diseases.

*Why did antioxidants fail in clinical studies?*

Multiple reasons exist for such a discrepancy. First of all, the time of antioxidant administration could be critical. A synchronized physiological condition in patients does not exist in clinical trials, as there is no way to select enough patients with an identical pathophysiology. Previous studies have shown that the effects of antioxidant treatment in animals are time-dependent (142, 145). It is more effective if the treatment starts at the early stage of chronic inflammation and obesity development, where oxidative stress dominates the pathophysiology. Secondly, the effective dose of antioxidants could be significantly different from one patient to another and between animals and humans. As naturally occurring nutrients in human diet, antioxidants are readily metabolized by liver and other tissues. A dose of antioxidants effective in animals may not be sufficient in humans with a higher metabolic and clearance rate. Also, the components mediating antioxidant activities could also be different between human and animals and may vary from one patient to another. Despite these and other unmentioned possibilities, additional work will help identify factors that influence the outcome of antioxidant treatments and bring about the beneficial effects against obesity and obesity-associated diseases.

## **Obesity Animal Models**

Several animal obesity studies are offered here. Every model serves different research perspectives. Based on the main question of obesity research of interest, the appropriate obesity model can be selected. Below I will mention and discuss some of the existing obesity models (164-166)

### *Diet-induced obesity*

Diet-induced obesity is a widely used animal model for obesity. The diet-induced obesity model mimics human obesity that is caused mainly by over-caloric intake. There are two types of diet-induced obesity model. The first diet is the high-carbohydrate diet (60% calories from carbohydrate) and can produce obesity within 8 weeks and 10 weeks in the rat and mouse, respectively (167). High carbohydrate diet also can generate other obesity complications such as hyperglycemia and hypertension. The second diet used in animal obesity model is high-fat diet (40-60% calories from fat). The high-fat diet develops obesity as well as other associated complications such as fatty liver, hyperglycemia, and insulin resistance faster than a high carbohydrate diet. Consequently, the high-fat diet model is used more frequently than the high-carbohydrate diet in obesity research. Moreover, the male C57BL/6 black mouse is commonly used and is more sensitive to high-fat diet-induced obesity than female mouse (29, 164, 168). Therefore, the C57BL/6 black male mouse fed 60% high-fat was utilized in the entire obesity experiments of this dissertation study.

### *Genetic model of obesity*

Genetic mutation-induced obesity models have either one or multiple gene mutations. These genetic mutations result in appetite activation and/or lower energy expenditure. The net result of these mutation(s) is positive caloric balance that leads to weight gain and obesity development. Examples of obesity genetic models are the *ob/ob* mouse (leptin deficient), *db/db* mouse (leptin receptor deficient), *s/s* mouse (disrupted STAT3 signal of leptin receptor), Zucker rat (mutated leptin receptor), proopiomelanocortin (POMC) knockout mouse, POMC/Agouti related protein double knockout mouse, melanocortin receptor 4 (MC4R) knockout mouse, MC4R knockout rat, MC3R knockout mouse, MC4/MCR receptor double knockout mouse, corticotrophin releasing factor (CRF)-transgenic mouse, glucose transporter subtype 4-overexpression mouse, melanin concentrating hormone transgenic mouse,  $\beta$ -3 adrenergic receptor knockout mouse, serotonin-2c (5-hydroxytryptamine-2c, 5HT-2c) receptor knockout, neuropeptide-Y 1 receptor knockout mouse, neuropeptide-Y 2 receptor knockout mouse, bombesin 3 receptor knockout mouse, neuronal insulin receptor knockout mouse, and 11 $\beta$ -HSD-1 overexpression mouse (164, 166, 169).

### *Surgical lesion-induced obesity*

Surgical lesion-induced obesity has a result similar to the previous genetic obesity model. Compared with other obesity models, surgical lesion-induced obesity model is used less frequently. Surgical interference represents the main disadvantage of this model. Nevertheless, surgical lesion-induced obesity is still used to mimic post-surgical obesity. In this model, the central and peripheral surgical lesions lead to obesity through

continuous over energy intake activation and/or energy expenditure suppression. The surgical lesion also causes hyperphagia, insulin induction, glucagon reduction, and other hormonal changes. Examples of surgical lesions that cause obesity are the ventromedial hypothalamus lesion, hypothalamic paraventricular nucleus lesion, hypothalamic arcuate nucleus lesion, ovariectomy of female mouse/rat and brown adipose tissue ablation (164, 166).

#### *Age-induced obesity*

Age-related process of overweight and obesity development has become a concern in the last few years (170, 171). Therefore, an age-induced obesity model was developed. Similar to human, the obesity is developed slowly over a period of time due to gradual fat accumulation even with a regular chow diet. In addition to weight gain, other obesity metabolic complications develop with age such as hyperglycemia, hyperinsulinemia, and hyperleptinemia. Two age obesity models are represented here; age-related obesity in the mouse (late-onset obesity or LOO mouse) and age-related obesity in macaque monkeys. While age-related obesity development takes several months in LOO mouse, it can take up to 15 years in macaque monkeys (166).

#### *Seasonal models of obesity*

Adiposity and body weight are affected by the day-time and weather temperature through changing the circadian rhythm and melatonin release. There was a need for developing a unique model that represents seasonal obesity. Two commonly used seasonal models of obesity are developed, the Syrian hamster (*Mesocricetus auratus*) and

Siberian hamster (*Phodopus sungorus*). They are used since they are sensitive to seasonal variations. They have opposed responses regarding the seasonal changes (i.e. long-day vs. short-day photoperiods), independently of energy intake. For instance, the Siberian hamster loses weight with short-days while the Syrian hamster gains weight with short-days (164, 166).

### **Antioxidants Used in Dissertation Research**

#### *Phloretin*

Phloretin's nomenclature is 3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)propan-1-one. The chemical formula is C<sub>15</sub>H<sub>14</sub>O<sub>5</sub> with a molecular weight of 274.272 g/mol. As shown in the chemical structure of phloretin (**Fig. 1.3a**), phloretin has dihydrochalcone backbone and belongs to flavonoids. Phloretin can be found in apple fruit and apple leaves (*Malus pumila*). Phloretin is easily derived from the hydrolysis of the sugar moiety of its glycoside (phloretin-2'- $\beta$ -glucopyranoside, also called phlorizin or phloridzin) (172, 173). Based on a pharmacokinetic study in rats (174), both phloretin and phlorizin can be orally absorbed, and phlorizin will eventually be converted to phloretin by circulatory esterase enzymes.

Different beneficial properties of phloretin were found against inflammation (175), cancer (176), skin diseases, neuron diseases, and bone diseases (172, 177). In recent years, a patent on phloretin was granted regarding potential anti-aging properties (patent number US20090286874 A1, 2009) (178). Since phloretin has an inhibitory effect on transporters (179), phloretin is commonly used in *in vitro* studies and becomes a candidate drug for transporter-dependent diseases (180).

Studies have supported the protective activity of phloretin against obesity through its antioxidant/anti-inflammatory properties. The antioxidant activity of phloretin was demonstrated by an increase in the activity of antioxidant enzymes (SOD, CAT, GPx, thioredoxin reductase), the content of endogenous antioxidants (GSH, Nrf2, heme oxygenase-1) or direct scavenging effect on ROS ( $\text{ONOO}^-$ ,  $\text{O}_2^{\bullet-}$ ) (172). Recently, Behzad *et al.* (181) reviewed phloretin's favorable effects on inflammation and immune cell modulation, which demonstrated suppression impacts against fat accumulation and weight gain (182-184). Phlorizin, as a phloretin precursor, has shown a slight reduction in body weight of streptozotocin-induced diabetic rats with a significant reduction in food intake (180). Phloretin has shown lipolysis activation and inflammation inhibition in adipocyte/macrophage co-cultured murine cells (185).

#### *Benzyl isothiocyanate*

Benzyl isothiocyanate (BITC) (**Fig. 1.3b**) is isothiocyanatomethyl benzene. The chemical formula is  $\text{C}_8\text{H}_7\text{NS}$  with a molecular weight of 149.211 g/mol. BITC is a natural isothiocyanate that belongs to *Brassicaceae* (also called *Cruciferae*). Glucosinolates (BITC precursor) is hydrolyzed by the plant enzyme myrosinase to form active molecules such as BITC and other indole molecules (186).

BITC has shown antibacterial, especially against Gram-negative bacteria, as well as anticancer properties (187, 188). The anticancer mechanism includes carcinogen activation inhibition or acceleration of carcinogen deactivation through the cytochrome P450 enzymatic system (188, 189). In human pancreatic cancer cells, BITC treatment showed G2/M cell cycle arrest and apoptosis (190). The role of BITC against

inflammation-related carcinogenesis was associated with superoxide generation inhibition (191). Another anticancer mechanism of BITC was reported by epigenetic inactivation (histone deacetylase inhibition) of NF- $\kappa$ B expression in human pancreatic cancer (192). In the last five years, a patent on BITC was received for treating prostatic diseases and skin cancer (patent number EP1961418 B1, 2012) (193).

Concerning obesity, sulforaphane is another isothiocyanate (ITC) that suppressed weight gain and fat accumulation in HFD induced obesity animal model. The mechanism included the inhibition of adipogenesis-related genes (PPAR $\gamma$ , CCAAT/enhancer binding protein  $\alpha$ ) and activation of the AMP-activated protein kinase  $\alpha$  (AMPK $\alpha$ ) pathway, which leads to inhibition of lipogenesis-related genes (acetyl-CoA carboxylase, hydroxyl-3-methylglutaryl coenzyme A reductase) (194).

### *S-carvone*

*S*-carvone (**Fig. 1.3c**) is 2-Methyl-5-(prop-1-en-2-yl) cyclohex-2-en-1-one. The molecular formula is C<sub>10</sub>H<sub>14</sub>O with a molecular weight of 150.221 g/mol. *S*-carvone is a monoterpene that can be found naturally in caraway seed oil (*Carum carvi*) and dill (*Anethum graveolens*). Caraway grows naturally in Asia, North Africa, Northern and Central Europe and has been cultivated in North America and Australia. Recently, *S*-carvone is synthesized with reaction yield up to 93% (195-197).

*S*-carvone has several manufacturing, agricultural, and medical uses. It is used as a food additive, flavor agent (e.g., bread, cheese, meat, sauces), cosmetic, fragrance, medicine (e.g., antispasmodic, carminative, expectorant), potato sprouting inhibitor, insecticide, and antimicrobial agent (198). In 2014, a patent on *S*-carvone was granted for

an antiviral combination (patent number US8883859 B1, 2014) (199). *S*-carvone also has anticancer activity through modulating carcinogen activation, inflammation, and the immune system (195-197).

Few *in vitro* and *in vivo* studies have shown the antioxidant activity of *S*-carvone (200-202). Regarding obesity, current data are very limited. Only one study has been conducted when Kazemipour *et al.* investigated the anti-obesity effect of caraway extract on overweight and obese women in a randomized, triple-blind, placebo-controlled clinical trial. After 90 days of daily ingestion of caraway extract, the body weight, body mass index, body fat percentage, and waist-to-hip ratio were significantly reduced compared with the placebo group. The proposed mechanism of caraway extract body weight reduction included balancing gut microbiota, inhibiting inflammation, stimulation of preadipocyte apoptosis, and enhancement of adipocytes lipolysis (203).

### *Catechin*

(+)-Catechin (**Fig. 1.3d**) is (2*R*,3*S*)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2*H*-chromene-3,5,7-triol. The chemical formula is C<sub>15</sub>H<sub>16</sub>O<sub>7</sub> with a molecular weight of 308.286 g/mol. (+)-Catechin is a polyphenolic compound that belongs to flavan-3-ols (also called flavanols), a subgroup of flavonoid family. The first extraction of catechin was in 1902 from *Gambier catechu* and *Acacia catechu*. Other sources of (+)-Catechin are apple, strawberries, green tea, and black tea. (+)-Catechin usually presents with other catechins such as (-)-gallocatechin gallate, (-)-catechin gallate, (+)-gallocatechin, (-)-epigallocatechin, (-) epicatechin, (-)-epicatechin gallate, and (-)-epigallocatechin gallate



(EGCG) (204, 205). Recently, a patent on catechin was received regarding its synthesis (patent number EP2557079 A1, 2013) (206).

Catechins target several cellular components and pathways. These include glutamate release, mitochondrial proton gradient, protease enzymes, nitric oxide production, apoptosis, lipid peroxidation, and inflammatory mediators (207). These multiple targets allow catechin to function as antioxidant, anti-inflammatory, anticancer, antimicrobial, antiviral, and antiparasitic agent (204). A recently published systemic review and meta-analysis study of green tea catechins in randomized controlled trials showed that green tea consumption significantly reduced systolic and diastolic blood pressure, total cholesterol, and low-density lipoprotein cholesterol (208). Catechin can also protect against common carotid artery ischemia via enhancing the ability of the brain to scavenge ROS such as superoxide (207, 209).

Green tea catechin has been shown to reduce body weight in animal and human studies (152, 210). Body weight reduction was associated with a statistically significant reduction in intraabdominal fat area and waist circumference of obese human subjects (211). Feeding mice with tea catechin with HFD prevented weight gain, hyperleptinemia, hyperinsulinemia, and fat accumulation in the liver and visceral areas. The gene expression of  $\beta$ -oxidation genes (acyl-CoA oxidase and medium-chain acyl-CoA dehydrogenase) were significantly increased in the tea catechins-treated mice group (212). Several mechanisms were suggested including, but not limited to, increased energy expenditure and fat oxidation, inflammation reduction, appetite suppression, and diet absorption inhibition (213).

### *Epigallocatechin gallate*

(-)-Epigallocatechin gallate (EGCG) (**Fig. 1.3e**) is (2*R*,3*R*)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2*H*-1-benzopyran-3-yl 3,4,5-trihydroxybenzoate. The chemical formula is C<sub>22</sub>H<sub>18</sub>O<sub>11</sub> with a molecular weight of 458.375 g/mol. EGCG is the ester of epigallocatechin and gallic acid. EGCG is the most abundant and important polyphenol in *Camellia sinensis*, which produces green tea, black tea, and white tea. The EGCG content in green tea is higher than (+)-catechin (up to 65% epicatechin). In contrast, EGCG can be found in lower concentrations in other fruits such as berries, strawberries, cherries, lemons, oranges, and black currants (204, 214, 215). While EGCG has a poor bioavailability, several factors (e.g., fasting, take it with piperine, fish oil, albumin, vitamin C, or water) can be taken to increase EGCG absorption in the small intestine (215).

Green tea, one of the most popular beverages globally, and EGCG have been given close scholarly attention due to their multiple health benefits, which have been reported on since 1940. Beside EGCG's antioxidant property, EGCG has anti-inflammatory, antihyperlipidemic, antimutagenic, antiproliferative, antibacterial, and antiviral properties (204, 214-216). This year, a patent on EGCG was received regarding the effect on mitigating skeletal muscle loss (patent number US9579347 B2, 2017) (217). The numerous benefits of EGCG can be achieved through the abundant molecular targets across the cell membrane, cytoplasm, and nucleus. EGCG can inhibit inflammatory cytokines (e.g., interleukin-8), inflammatory growth factors (e.g., insulin-like growth factor 1), inflammatory mediators (e.g., cyclooxygenase-2 or COX2), and inducible nitric oxide synthase (iNOS) (218).

Green tea extract studies have shown body weight reductions (212-214). The studies suggested that EGCG intake suppressed diet-induced obesity due to reduced fat absorption and increased fat oxidation, with no impact on food intake (219, 220). The inhibition of salivary  $\alpha$ -amylase activity was also a reported mechanism as an anti-obesity effect by EGCG (221). EGCG has been shown to induce anti-inflammatory effects and ameliorates obesity through induction of regulatory T cells and reducing IL-17, IL-6, IL-1 $\beta$ , and TNF- $\alpha$  (222). A study has recently shown that EGCG supplementation provided a protective effect against HFD-induced colon DNA damage and gut microbiota imbalance (223). Obesity-associated complications such as insulin resistance and non-alcoholic fatty liver were also prevented with green tea extract in leptin-deficient transgenic obese mice and diet-induced obese mice (212, 224).

### **Objectives of Dissertation Research**

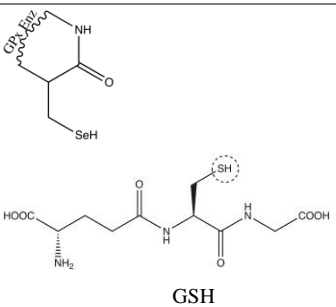
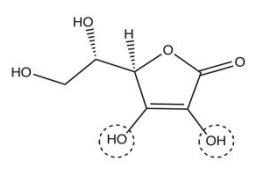
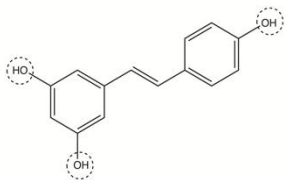
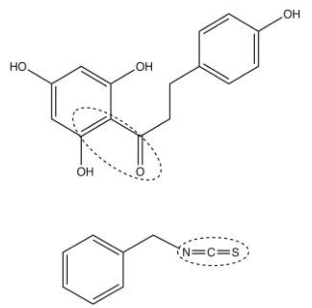
This dissertation aims to expand our knowledge about the relationship between ROS and the development of obesity and obesity-associated insulin resistance and fatty liver. The study employed HFD-induced obesity in mice as a disease model. Using selected compounds known to have antioxidant activity, including phloretin, BITC, and *S*-carvone, the study systematically examined their effects on prevention of diet-induced weight gain, insulin resistance, and fatty liver on improvement of metabolic homeostasis in animals with preconditions of obesity and insulin resistance. The results obtained set a solid foundation for the use of these compounds to prevent obesity and provide direct evidence in support of clinical investigations into the use of antioxidants to prevent obesity.

**Table 1.1 Structures, sources, and characteristics of reactive oxygen species (ROS)**

(225, 226).

Type	Name	Structure	Generation Reaction	ROS Characteristics
Radical	Superoxide anion	$O_2^{\bullet -}$	- Adding electron to oxygen $O_2 + e^- \rightarrow O_2^{\bullet -}$	- One unpaired electron - Less reactive - Limited membrane permeability - React selectively with HOCl, or NO• of iron or sulfur proteins.
	Hydroxyl radical	$\bullet OH$	- Adding electron to Hydrogen peroxide (Fenton's reaction): $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$	- One unpaired electron - The most reactive ROS
	Peroxyl radical	$ROO\bullet$	- Organic peroxide (ROOH) degradation: $ROOH + Fe^{3+} \rightarrow ROO\bullet + Fe^{2+} + H^+$	- One unpaired electron - Aliphatic peroxyl tends to be more reactive than aromatic - React with NADH $\rightarrow NAD^+ + O_2^{\bullet -}$
	Alkoxy radical	$RO\bullet$	- ROOH degradation: $ROOH + Fe^{2+} \rightarrow RO\bullet + Fe^{3+} + OH^-$	- One unpaired electron - Aliphatic alkoxy tends to be more reactive than aromatic
	Hydroperoxyl	$HOO\bullet$	1- $H + O_2 \rightarrow HOO\bullet$ 2- $H^+ + O_2^{\bullet -} \rightarrow HOO\bullet$	- One unpaired electron - The simplest form of peroxyl radical
Non-Radical	Hydrogen peroxide	$H_2O_2$	1- Dismutase (SOD) $O_2^{\bullet -} \rightarrow H_2O_2$ 2- Monoamine oxidase (MAO) $RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3$	- Selective and limited reactivity e.g. reacts slowly with -SH - Membrane permeable - Works as signaling molecule
	Hypochlorous acid	HOCl	Myeloperoxidase (MPO): $H_2O_2 + Cl^- \rightarrow HOCl + OH^-$	- Highly reactive - Two electrons oxidizing agent - Attacks membrane lipids and proteins. - Permeable
	Singlet oxygen	$^1O_2$	$O_2^{\bullet -} + H_2O_2 \rightarrow ^1O_2 + HO^- + HO^-$	- More oxidizing ability than oxygen since $^1O_2$ does not have spine restriction.
	Ozone	$O_3$	$H_2O + ^1O_2 \rightarrow H_2O_3 + ^1O_2 \rightarrow H_2O_2 + O_3$	- Triatomic and has two oxygen-oxygen bonds. - Powerful oxidizing agent that can oxidized lipids double bonds to produce ozonides, OH•, $^1O_2$ , and $H_2O_2$
	Peroxynitrite	$ONOO^-$	1- $NO\bullet + O_2^{\bullet -} \rightarrow ONOO^-$ 2- $O_2 + NO^- \rightarrow ONOO^-$	- Very strong and leads to depletion of antioxidants, nitration of lipids, proteins, and DNA bases.

**Table 1.2 Approaches taken to neutralize ROS<sup>a</sup>**

Antioxidant Mechanism	Example	Chemical Structure and Antioxidant Functional Group(s)
<p>1 – Enzyme mediated: The enzyme carries the electron and adds it to the ROS. For example, one electron is donated from the glutathione thiol group (SH) to GPx-Selenium (Se) to neutralize H<sub>2</sub>O<sub>2</sub> as the following: GSH + GPxSe → 1/2 GSSG + GPxSeH H<sub>2</sub>O<sub>2</sub> + GPxSeH → H<sub>2</sub>O + GPxSeOH GSH + GPxSeOH → H<sub>2</sub>O + GPxSeSG</p>	Glutathione peroxidase (GPx)	 <p style="text-align: center;">GSH</p>
<p>2 – Chelating pro-oxidants metals: Metal ions, e.g., Fe<sup>2+</sup>, trigger ROS production via Fenton's reaction: Fe<sup>2+</sup> + H<sub>2</sub>O<sub>2</sub> → Fe<sup>3+</sup> + •OH + OH Ascorbic acid can chelate such pro-oxidant metals (besides its direct electron donating feature). Note. Some antioxidant proteins follow the same mechanism, e.g., transferrin chelates iron ions.</p>	Ascorbic acid (Vitamin C)	
<p>3 – Direct electron donation: Electron donating functional groups such as OH, SH, and SeH can donate one electron (H) to scavenge ROS, as shown in the following equation: R• + Ar-OH + → RH+ Ar•-O (non-reactive)</p>	Resveratrol	
<p>4- Activating endogenous antioxidant system through Nrf2 signaling pathway: Antioxidant containing α,β- unsaturated carbonyl or isocyanate reacts with critical cysteine of Keap1 and detached Nrf2, which will be phosphorylated and translocated to the nucleus for gene expression activation of phase II antioxidant enzymes.</p>	<p>Phloretin</p> <p>Benzyl isothiocyanate</p>	

<sup>a</sup> Combined from references (136-138, 227)

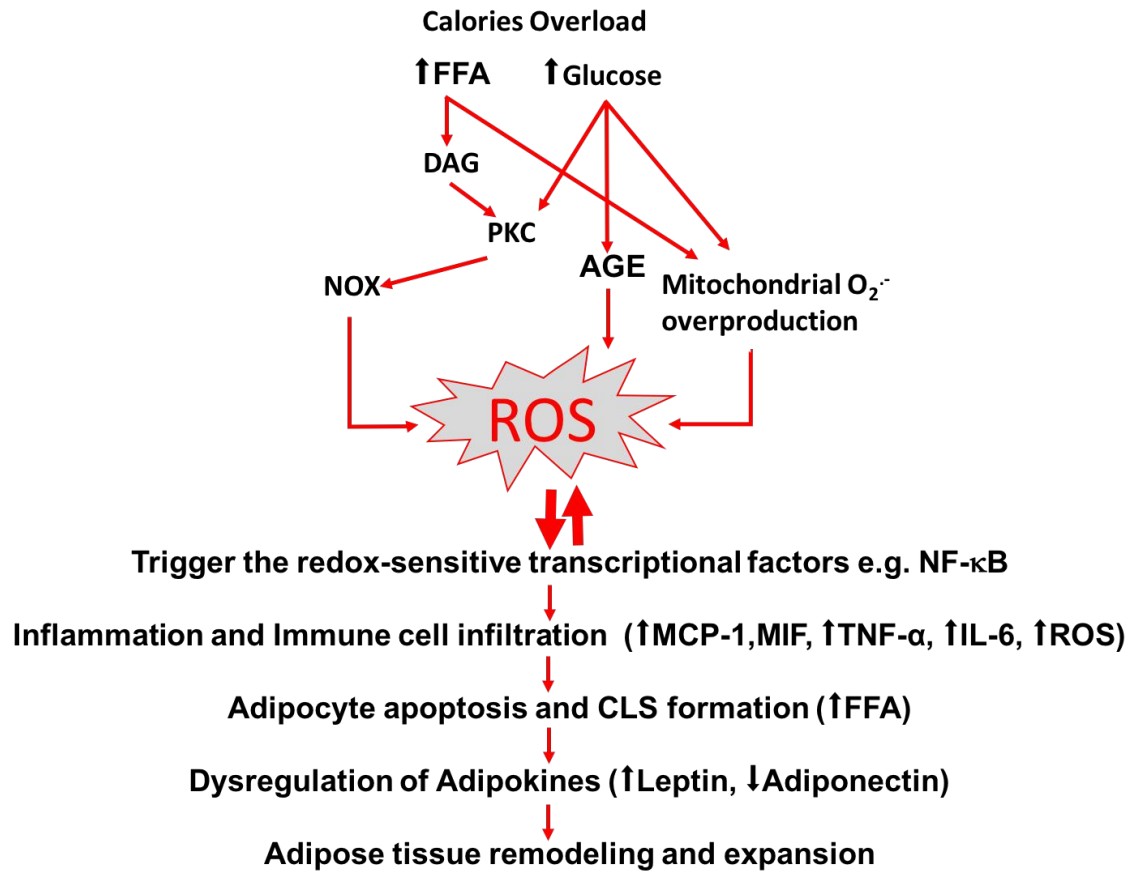
**Table 1.3 Selected preclinical studies for obesity prevention with antioxidants.**

Antioxidant	Treatment Regimen	Obesity Model	Impact on Body Weight (BW)	Food Intake	Ref.
<b>Phloretin</b>	10 mg/kg <i>i.p.</i> twice weekly for 12 weeks	Male C57BL/6 mice + 60% HFD	Obesity prevention with 10 g (24%) difference in BW	No change	(142)
<b>Benzyl isothiocyanate</b>	12.5 mg/kg <i>i.p.</i> twice weekly for 8 weeks	Male C57BL/6 mice + 60% HFD	Obesity prevention with 11 g (27%) difference in BW	No change	(143)
<b>S-carvone</b>	175 mg/kg <i>i.p.</i> twice weekly for 8 weeks	Male C57BL/6 mice + 60% HFD	Obesity prevention with 9 g (23%) difference in BW	No change	(143)
<b>Rutin</b>	50 mg/kg <i>i.p.</i> twice weekly for 8 weeks	Male C57BL/6 mice + 60% HFD	Obesity prevention with 9.8 g (25%) difference in BW	No change	(144)
<b>Chlorogenic acid</b>	100 mg/kg <i>i.p.</i> twice weekly for 15 weeks	Male C57BL/6 mice + 60% HFD	Obesity prevention with 16 g (34%) difference in BW	No change	(145)
<b>Galangin</b>	50 mg/kg <i>p.o.</i> for 6 weeks	Female Wistar rats + Cafeteria diet*	Obesity prevention with 62 g (20%) difference in BW	Decreased	(146)
<b>Carnosic acid</b>	0.02% (w/w) in diet for 4 weeks	Male <i>ob/ob</i> mice	Obesity prevention with 3 g (27%) difference in weight gain	Decreased	(147)
<b>Resveratrol</b>	0.005% (w/w) in diet for 6 weeks	Male C57BL/6 mice + 20% HFD	Obesity prevention with 5 g (14%) difference in BW	No change	(148)
<b>Quercetin</b>	0.1% (w/w) in diet for 12 weeks	Male C57BL/6 mice + 45% HFD	Obesity prevention with 4 g (10%) difference in BW	No change	(149)
<b>Bardoxolone methyl</b>	10 mg/kg in drinking water for 21 weeks	Male C57BL/6 mice + 40% HFD	Obesity prevention with 13 g (31%) difference in BW	Not reported	(150)
<b>SOD3 overexpression</b>	One hydrodynamic plasmid injection (10 µg/mice) for the study period (8 weeks)	Male C57BL/6 mice + 60% HFD	Obesity prevention with 11.5 g (34%) difference in weight gain	No change	(140)

\*Cafeteria diet consists of condensed milk, bread, chocolate, biscuit, dried coconut, cheese, and boiled potato.

**Table 1.4 Summary of antioxidants impact on body weight of overweight and obese subjects.**

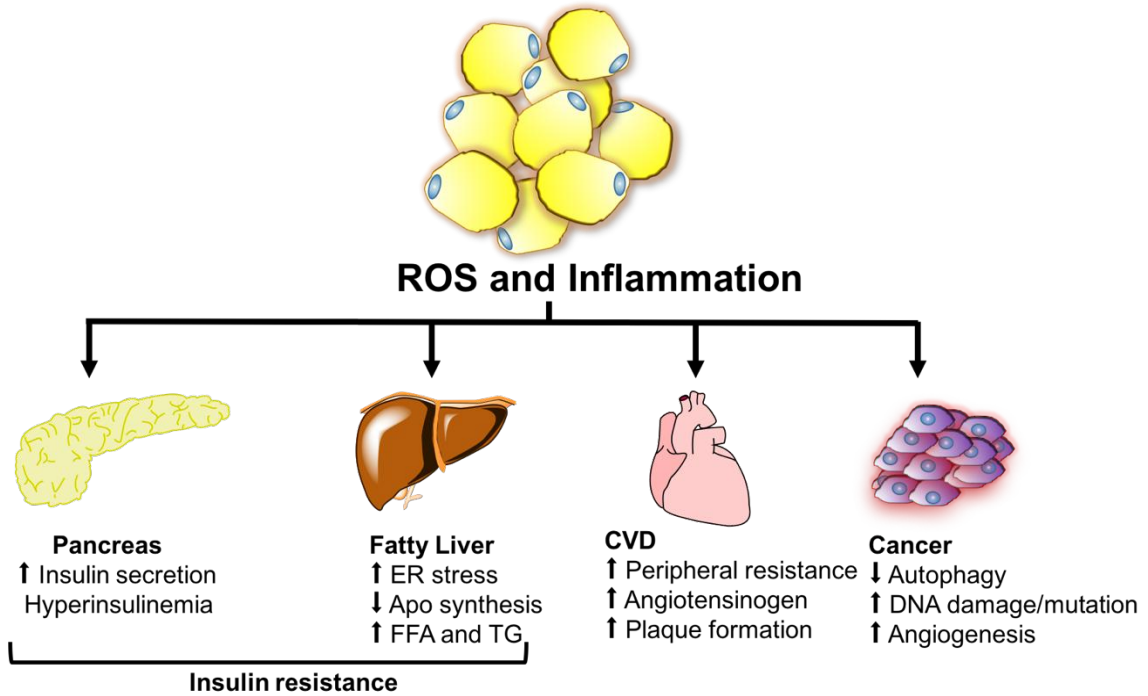
Antioxidant	Daily Dose	Study Design and Population	Study Duration	Impact on BW	Ref.
<b>Epigallocatechin-3-gallate (EGCG)</b>	300 mg/kg	Randomized double-blind placebo-controlled trial 83 Obese premenopausal women	12 Weeks	No change	(151)
<b>Green tea (GT)</b>	4 cups (928 mg catechins)	Randomized controlled trial 35 Obese subjects with metabolic syndrome	8 Weeks	-2.5 kg	(152)
<b>GT extract (GTE)</b>	2 capsules (870 mg catechins)			-1.9 kg	
<b>Antioxidants rich diet</b>	Equivalent to 383-1093 mg of Vitamin C	Uncontrolled observational trial 35 Overweight and obese postmenopausal women	9 Months	No change	(153)
<b><math>\alpha</math>-Lipoic acid</b>	1 g	Randomized controlled trial 24 Obese subjects with impaired glucose tolerance	12 Weeks	No change	(154)
<b>Green vegetables</b>	2 serving (130 g) 5 serving (287 g) 10 serving (614 g)	Crossover feeding trial 49 Overweight postmenopausal women	3 Weeks (4 weeks washout)	No change	(155)
<b>Pine bark extract</b>	200 mg	Randomized double-blind placebo-controlled trial 130 Overweight and obese subjects	12 Weeks	No change	(156)
<b>Nigella Sativa</b>	3 g (1.5g twice daily)	Randomized double-blind placebo-controlled trial 39 Central obese men	3 Months	-4.5 kg	(157)
<b>Red orange juice</b>	750 ml	Uncontrolled observational trial 35 Clinically healthy subjects (normal weight, overweight, obese)	8 Weeks	No change	(158)
<b>Pomegranate extract</b>	1 g	Randomized double-blind placebo-controlled trial 42 Overweight and obese	30 days	- 2.2 kg	(159)



**Fig. 1.1 ROS generation and obesity promotion mechanism.** Excess energy intake leads to ROS accumulation through several mechanisms. The high glucose level activates glycolysis and mitochondrial superoxide production. The PKC-mediated NOX activation is also triggered by high glucose level. Long-term hyperglycemia leads to formation of AGE of lipids and proteins that trigger ROS production. The high FFA level can activate mitochondrial fatty acid oxidation and ROS production. Moreover, FFA can be converted to DAG and activate NOX and promote ROS production via PKC-mediated mechanism. The accumulated ROS will activate the redox-sensitive transcriptional factors and inflammation, and immune cell infiltration and activation will take place. Eventually, macrophage infiltration and inflammation lead to remodeling and expansion of adipose tissue. AGE, advanced glycation end products; CLS, Crown-like

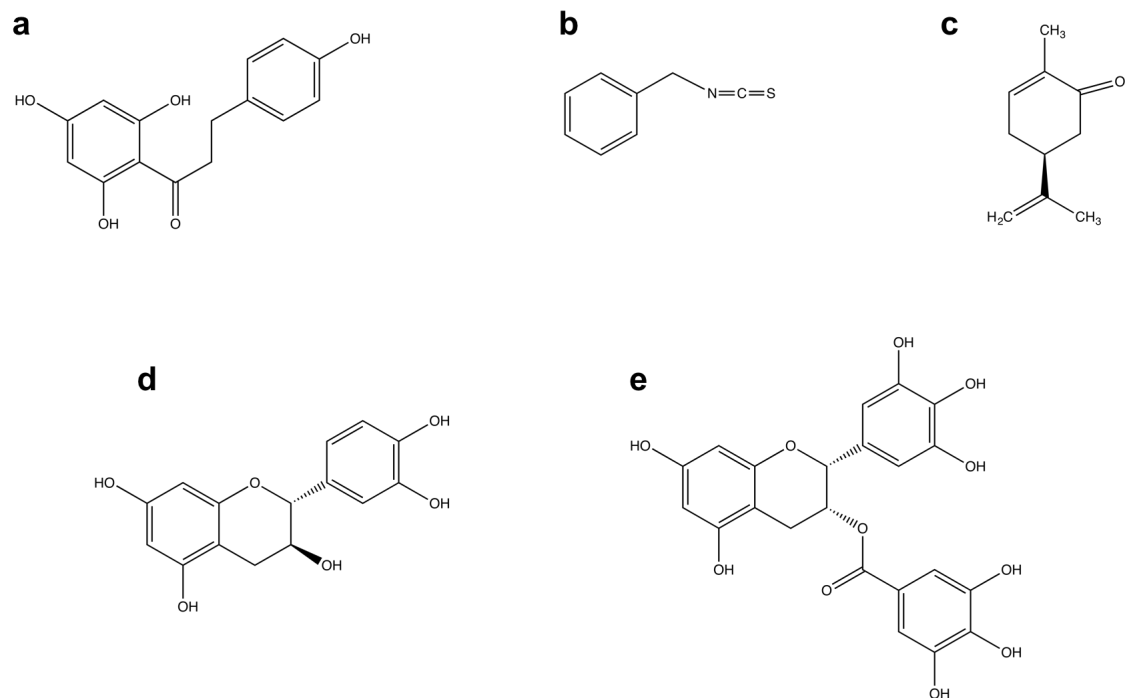


structures; DAG, Diacylglycerol; FFA, Free fatty acids; IL-6, Interleukin 6; MCP, monocyte chemotactic protein; NOX, Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase;  $O_2^-$  Superoxide; PKC, Protein kinase C; TNF, tumor necrosis factor.



**Fig. 1.2 Mechanistic illustration of association between obesity and other diseases.**

Chronic inflammation represents the connection between oxidative stress in obesity and obesity-associated diseases such as insulin resistance, fatty liver, CVD, and cancer. Apo, Apolipoprotein; CVD, Cardiovascular Diseases; ER, Endoplasmic Reticulum; FFA, Free Fatty Acids ROS, Reactive Oxygen Species; TG, Triglycerides.



**Fig. 1.3 Chemical structures of antioxidants used in dissertation research. (a)** Phloretin; **(b)** benzyl isothiocyanate (BITC); **(c)** *S*-(+)-carvone; **(d)** (+)-catechin; **(e)** (-)-epigallocatechin gallate (EGCG). Chemical structures were generated by ChemDraw Professional<sup>®</sup> 16.0 software (PerkinElmer Informatics, Inc).

## References

1. Collaborators GBDO. Health Effects of Overweight and Obesity in 195 Countries over 25 Years. *N Engl J Med*. 2017.
2. The State of Obesity. Adult Obesity in the United States 2015 [updated 9-1-2016. Available from: <http://stateofobesity.org/adult-obesity/>.]
3. Centers for Disease Control and Prevention. National Center for Chronic Disease Prevention and Health Promotion, Division of Nutrition, Physical Activity, and Obesity. Data, Trend and Maps [online] [accessed Oct 18, 2017] [Available from: <https://www.cdc.gov/nccdphp/dnpao/data-trends-maps/index.html>.]
4. Hammond RA, Levine R. The economic impact of obesity in the United States. *Diabetes Metab Syndr Obes*. 2010; 3: 285-95.
5. Cawley J, Rizzo JA, Haas K. Occupation-specific absenteeism costs associated with obesity and morbid obesity. *J Occup Environ Med*. 2007; 49: 1317-24.
6. Daneschvar HL, Aronson MD, Smetana GW. FDA-Approved Anti-Obesity Drugs in the United States. *Am J Med*. 2016; 129: 879 e1-6.
7. Ryan DH. BMI guidelines for bariatric surgery in diabetes: how low can we go? *Diabetes Care*. 2012; 35: 1399-400.

8. Magro DO, Geloneze B, Delfini R, Pareja BC, Callejas F, Pareja JC. Long-term weight regain after gastric bypass: a 5-year prospective study. *Obes Surg.* 2008; 18: 648-51.
9. Walls HL, Peeters A, Proietto J, McNeil JJ. Public health campaigns and obesity - a critique. *BMC Public Health.* 2011; 11: 136.
10. Chan RS, Woo J. Prevention of overweight and obesity: how effective is the current public health approach. *Int J Environ Res Public Health.* 2010; 7: 765-83.
11. Flegal KM, Kruszon-Moran D, Carroll MD, Fryar CD, Ogden CL. Trends in Obesity Among Adults in the United States, 2005 to 2014. *JAMA.* 2016; 315: 2284-91.
12. Wardle J, Carnell S, Haworth CM, Plomin R. Evidence for a strong genetic influence on childhood adiposity despite the force of the obesogenic environment. *Am J Clin Nutr.* 2008; 87: 398-404.
13. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature.* 1998; 395: 763-70.
14. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, *et al.* Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell.* 1997; 88: 131-41.

15. Dina C, Meyre D, Gallina S, Durand E, Korner A, Jacobson P, *et al.* Variation in FTO contributes to childhood obesity and severe adult obesity. *Nat Genet.* 2007; 39: 724-6.
16. Shawky RM, Sadik DI. Genetics of obesity. *Egyptian Journal of Medical Human Genetics.* 2012; 13: 11-7.
17. Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature.* 1997; 385: 165-8.
18. Friedman JM. Obesity: Causes and control of excess body fat. *Nature.* 2009; 459: 340-2.
19. Wahlen K, Sjolin E, Hoffstedt J. The common rs9939609 gene variant of the fat mass- and obesity-associated gene FTO is related to fat cell lipolysis. *J Lipid Res.* 2008; 49: 607-11.
20. Gerken T, Girard CA, Tung YC, Webby CJ, Saudek V, Hewitson KS, *et al.* The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. *Science.* 2007; 318: 1469-72.
21. Herrera BM, Keildson S, Lindgren CM. Genetics and epigenetics of obesity. *Maturitas.* 2011; 69: 41-9.
22. Choquet H, Meyre D. Genetics of Obesity: What have we Learned? *Curr Genomics.* 2011; 12: 169-79.

23. Yazdi FT, Clee SM, Meyre D. Obesity genetics in mouse and human: back and forth, and back again. *PeerJ*. 2015; 3: e856.
24. Gao M, Liu D. Gene therapy for obesity: progress and prospects. *Discov Med*. 2014; 17: 319-28.
25. Hu FB. Resolved: there is sufficient scientific evidence that decreasing sugar-sweetened beverage consumption will reduce the prevalence of obesity and obesity-related diseases. *Obes Rev*. 2013; 14: 606-19.
26. Drewnowski A. Obesity and the food environment: dietary energy density and diet costs. *Am J Prev Med*. 2004; 27: 154-62.
27. Malik VS, Hu FB. Sweeteners and Risk of Obesity and Type 2 Diabetes: The Role of Sugar-Sweetened Beverages. *Curr Diab Rep*. 2012.
28. Caprio S. Calories from soft drinks--do they matter? *N Engl J Med*. 2012; 367: 1462-3.
29. Hariri N, Thibault L. High-fat diet-induced obesity in animal models. *Nutr Res Rev*. 2010; 23: 270-99.
30. Gao M, Ma Y, Liu D. High-fat diet-induced adiposity, adipose inflammation, hepatic steatosis and hyperinsulinemia in outbred CD-1 mice. *PLoS One*. 2015; 10: e0119784.

31. de Lartigue G, Barbier de la Serre C, Espero E, Lee J, Raybould HE. Diet-induced obesity leads to the development of leptin resistance in vagal afferent neurons. *Am J Physiol Endocrinol Metab.* 2011; 301: E187-95.
32. de Git KC, Adan RA. Leptin resistance in diet-induced obesity: the role of hypothalamic inflammation. *Obes Rev.* 2015; 16: 207-24.
33. Avena NM, Rada P, Hoebel BG. Sugar and fat bingeing have notable differences in addictive-like behavior. *J Nutr.* 2009; 139: 623-8.
34. Sharma S, Fernandes MF, Fulton S. Adaptations in brain reward circuitry underlie palatable food cravings and anxiety induced by high-fat diet withdrawal. *Int J Obes (Lond).* 2013; 37: 1183-91.
35. Cruwys T, Bevelander KE, Hermans RC. Social modeling of eating: A review of when and why social influence affects food intake and choice. *Appetite.* 2015; 86: 3-18.
36. Higgs S, Thomas J. Social influences on eating. *Current Opinion in Behavioral Sciences.* 2016; 9: 1-6.
37. Leibowitz SF. Overconsumption of dietary fat and alcohol: mechanisms involving lipids and hypothalamic peptides. *Physiol Behav.* 2007; 91: 513-21.
38. American Psychological Association. Stress in America 2015 [Available from: <https://www.apa.org/news/press/releases/stress/2014/stress-report.pdf>.]



39. Dallman MF. Stress-induced obesity and the emotional nervous system. *Trends Endocrinol Metab.* 2010; 21: 159-65.
40. Patterson ZR, Abizaid A. Stress induced obesity: lessons from rodent models of stress. *Front Neurosci.* 2013; 7: 130.
41. Adam TC, Epel ES. Stress, eating and the reward system. *Physiol Behav.* 2007; 91: 449-58.
42. Memish ZA, El Bcheraoui C, Tuffaha M, Robinson M, Daoud F, Jaber S, *et al.* Obesity and associated factors--Kingdom of Saudi Arabia, 2013. *Prev Chronic Dis.* 2014; 11: E174.
43. Nguyen DM, El-Serag HB. The epidemiology of obesity. *Gastroenterol Clin North Am.* 2010; 39: 1-7.
44. Thayer KA, Heindel JJ, Bucher JR, Gallo MA. Role of environmental chemicals in diabetes and obesity: a National Toxicology Program workshop review. *Environ Health Perspect.* 2012; 120: 779-89.
45. Hatori M, Vollmers C, Zarrinpar A, DiTacchio L, Bushong EA, Gill S, *et al.* Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. *Cell Metab.* 2012; 15: 848-60.
46. Tuovinen EL, Saarni SE, Mannisto S, Borodulin K, Patja K, Kinnunen TH, *et al.* Smoking status and abdominal obesity among normal- and overweight/obese adults: Population-based FINRISK study. *Prev Med Rep.* 2016; 4: 324-30.

47. Dare S, Mackay DF, Pell JP. Relationship between smoking and obesity: a cross-sectional study of 499,504 middle-aged adults in the UK general population. *PLoS One*. 2015; 10: e0123579.
48. Gangwisch JE, Malaspina D, Boden-Albala B, Heymsfield SB. Inadequate sleep as a risk factor for obesity: analyses of the NHANES I. *Sleep*. 2005; 28: 1289-96.
49. Wright SM, Aronne LJ. Causes of obesity. *Abdom Imaging*. 2012; 37: 730-2.
50. Di Milia L, Vandelanotte C, Duncan MJ. The association between short sleep and obesity after controlling for demographic, lifestyle, work and health related factors. *Sleep Med*. 2013; 14: 319-23.
51. Cappuccio FP, Taggart FM, Kandala NB, Currie A, Peile E, Stranges S, *et al*. Meta-analysis of short sleep duration and obesity in children and adults. *Sleep*. 2008; 31: 619-26.
52. Spiegel K, Tasali E, Penev P, Van Cauter E. Brief communication: Sleep curtailment in healthy young men is associated with decreased leptin levels, elevated ghrelin levels, and increased hunger and appetite. *Ann Intern Med*. 2004; 141: 846-50.
53. McLaren L. Socioeconomic status and obesity. *Epidemiol Rev*. 2007; 29: 29-48.
54. Bhurosy T, Jeewon R. Overweight and obesity epidemic in developing countries: a problem with diet, physical activity, or socioeconomic status? *ScientificWorldJournal*. 2014; 2014: 964236.

55. Ogden CL, Lamb MM, Carroll MD, Flegal KM. Obesity and socioeconomic status in children and adolescents: United States, 2005-2008. NCHS Data Brief. 2010: 1-8.
56. Ponterio E, Gnessi L. Adenovirus 36 and Obesity: An Overview. *Viruses*. 2015; 7: 3719-40.
57. Atkinson RL, Dhurandhar NV, Allison DB, Bowen RL, Israel BA, Albu JB, *et al*. Human adenovirus-36 is associated with increased body weight and paradoxical reduction of serum lipids. *Int J Obes (Lond)*. 2005; 29: 281-6.
58. Vasilakopoulou A, le Roux CW. Could a virus contribute to weight gain? *Int J Obes (Lond)*. 2007; 31: 1350-6.
59. Atkinson RL. Viruses as an etiology of obesity. *Mayo Clin Proc*. 2007; 82: 1192-8.
60. Reinehr T. Obesity and thyroid function. *Mol Cell Endocrinol*. 2010; 316: 165-71.
61. Dallman MF, Pecoraro NC, La Fleur SE, Warne JP, Ginsberg AB, Akana SF, *et al*. Glucocorticoids, chronic stress, and obesity. *Prog Brain Res*. 2006; 153: 75-105.
62. Hewagalamulage SD, Lee TK, Clarke IJ, Henry BA. Stress, cortisol, and obesity: a role for cortisol responsiveness in identifying individuals prone to obesity. *Domest Anim Endocrinol*. 2016; 56 Suppl: S112-20.
63. Weaver JU. Classical endocrine diseases causing obesity. *Front Horm Res*. 2008; 36: 212-28.

64. Aronne LJ, Segal KR. Weight gain in the treatment of mood disorders. *J Clin Psychiatry*. 2003; 64 Suppl 8: 22-9.
65. Grundy A, Cotterchio M, Kirsh VA, Kreiger N. Associations between anxiety, depression, antidepressant medication, obesity and weight gain among Canadian women. *PLoS One*. 2014; 9: e99780.
66. Jallon P, Picard F. Bodyweight gain and anticonvulsants: a comparative review. *Drug Saf*. 2001; 24: 969-78.
67. Kaila B, Raman M. Obesity: a review of pathogenesis and management strategies. *Can J Gastroenterol*. 2008; 22: 61-8.
68. Zhang Y, Liu J, Yao J, Ji G, Qian L, Wang J, *et al*. Obesity: pathophysiology and intervention. *Nutrients*. 2014; 6: 5153-83.
69. Madsbad S. The role of glucagon-like peptide-1 impairment in obesity and potential therapeutic implications. *Diabetes Obes Metab*. 2014; 16: 9-21.
70. Duca FA, Zhong L, Covasa M. Reduced CCK signaling in obese-prone rats fed a high fat diet. *Horm Behav*. 2013; 64: 812-7.
71. le Roux CW, Batterham RL, Aylwin SJ, Patterson M, Borg CM, Wynne KJ, *et al*. Attenuated peptide YY release in obese subjects is associated with reduced satiety. *Endocrinology*. 2006; 147: 3-8.
72. Coelho M, Oliveira T, Fernandes R. Biochemistry of adipose tissue: an endocrine organ. *Arch Med Sci*. 2013; 9: 191-200.

73. Farooqi IS, O'Rahilly S. Recent advances in the genetics of severe childhood obesity. *Arch Dis Child*. 2000; 83: 31-4.
74. Calvani M, Scarfone A, Granato L, Mora EV, Nanni G, Castagneto M, *et al*. Restoration of adiponectin pulsatility in severely obese subjects after weight loss. *Diabetes*. 2004; 53: 939-47.
75. Nigro E, Scudiero O, Monaco ML, Palmieri A, Mazzarella G, Costagliola C, *et al*. New insight into adiponectin role in obesity and obesity-related diseases. *Biomed Res Int*. 2014; 2014: 658913.
76. Sidossis L, Kajimura S. Brown and beige fat in humans: thermogenic adipocytes that control energy and glucose homeostasis. *J Clin Invest*. 2015; 125: 478-86.
77. Skurk T, Alberti-Huber C, Herder C, Hauner H. Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab*. 2007; 92: 1023-33.
78. Maury E, Brichard SM. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Mol Cell Endocrinol*. 2010; 314: 1-16.
79. Kahn BB, Flier JS. Obesity and insulin resistance. *J Clin Invest*. 2000; 106: 473-81.
80. Stepan CM, Lazar MA. Resistin and obesity-associated insulin resistance. *Trends Endocrinol Metab*. 2002; 13: 18-23.
81. Vachharajani V, Granger DN. Adipose tissue: a motor for the inflammation associated with obesity. *IUBMB Life*. 2009; 61: 424-30.

82. Ramel A, Martinez JA, Kiely M, Bandarra NM, Thorsdottir I. Effects of weight loss and seafood consumption on inflammation parameters in young, overweight and obese European men and women during 8 weeks of energy restriction. *Eur J Clin Nutr.* 2010; 64: 987-93.
83. Imayama I, Ulrich CM, Alfano CM, Wang C, Xiao L, Wener MH, *et al.* Effects of a caloric restriction weight loss diet and exercise on inflammatory biomarkers in overweight/obese postmenopausal women: a randomized controlled trial. *Cancer Res.* 2012; 72: 2314-26.
84. Imayama I, Alfano CM, Mason C, Wang C, Duggan C, Campbell KL, *et al.* Weight and metabolic effects of dietary weight loss and exercise interventions in postmenopausal antidepressant medication users and non-users: a randomized controlled trial. *Prev Med.* 2013; 57: 525-32.
85. Shelton RC, Pencina MJ, Barrentine LW, Ruiz JA, Fava M, Zajecka JM, *et al.* Association of obesity and inflammatory marker levels on treatment outcome: results from a double-blind, randomized study of adjunctive L-methylfolate calcium in patients with MDD who are inadequate responders to SSRIs. *J Clin Psychiatry.* 2015; 76: 1635-41.
86. Hernandez TL, Van Pelt RE, Anderson MA, Reece MS, Reynolds RM, de la Houssaye BA, *et al.* Women With Gestational Diabetes Mellitus Randomized to a Higher-Complex Carbohydrate/Low-Fat Diet Manifest Lower Adipose Tissue Insulin Resistance, Inflammation, Glucose, and Free Fatty Acids: A Pilot Study. *Diabetes Care.* 2016; 39: 39-42.

87. Di Minno MN, Peluso R, Iervolino S, Russolillo A, Lupoli R, Scarpa R, *et al.* Weight loss and achievement of minimal disease activity in patients with psoriatic arthritis starting treatment with tumour necrosis factor alpha blockers. *Ann Rheum Dis.* 2014; 73: 1157-62.
88. Fernandez-Real JM, Lopez-Bermejo A, Ropero AB, Piquer S, Nadal A, Bassols J, *et al.* Salicylates increase insulin secretion in healthy obese subjects. *J Clin Endocrinol Metab.* 2008; 93: 2523-30.
89. Penesova A, Koska J, Ortega E, Bunt JC, Bogardus C, de Courten B. Salsalate has no effect on insulin secretion but decreases insulin clearance: a randomized, placebo-controlled trial in subjects without diabetes. *Diabetes Obes Metab.* 2015; 17: 608-12.
90. Alderete TL, Sattler FR, Richey JM, Allayee H, Mittelman SD, Sheng X, *et al.* Salsalate treatment improves glycemia without altering adipose tissue in nondiabetic obese hispanics. *Obesity (Silver Spring).* 2015; 23: 543-51.
91. Fleischman A, Shoelson SE, Bernier R, Goldfine AB. Salsalate improves glycemia and inflammatory parameters in obese young adults. *Diabetes Care.* 2008; 31: 289-94.
92. Ohashi K, Shibata R, Murohara T, Ouchi N. Role of anti-inflammatory adipokines in obesity-related diseases. *Trends Endocrinol Metab.* 2014; 25: 348-55.
93. Pierce GL, Lesniewski LA, Lawson BR, Beske SD, Seals DR. Nuclear factor- $\kappa$ B activation contributes to vascular endothelial dysfunction via oxidative

- stress in overweight/obese middle-aged and older humans. *Circulation*. 2009; 119: 1284-92.
94. Holmstrom KM, Finkel T. Cellular mechanisms and physiological consequences of redox-dependent signalling. *Nat Rev Mol Cell Biol*. 2014; 15: 411-21.
  95. Krumova K, Cosa G. Overview of Reactive Oxygen Species. *Singlet Oxygen: Applications in Biosciences and Nanosciences, Vol 1*. 132016. p. 3-21.
  96. Le Lay S, Simard G, Martinez MC, Andriantsitohaina R. Oxidative stress and metabolic pathologies: from an adipocentric point of view. *Oxid Med Cell Longev*. 2014; 2014: 908539.
  97. Carocho M, Ferreira IC. A review on antioxidants, prooxidants and related controversy: natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food Chem Toxicol*. 2013; 51: 15-25.
  98. Johnson F, Giulivi C. Superoxide dismutases and their impact upon human health. *Mol Aspects Med*. 2005; 26: 340-52.
  99. Wang X, Tao L, Hai CX. Redox-regulating role of insulin: the essence of insulin effect. *Mol Cell Endocrinol*. 2012; 349: 111-27.
  100. Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr Neuropharmacol*. 2009; 7: 65-74.



101. Afonso V, Champy R, Mitrovic D, Collin P, Lomri A. Reactive oxygen species and superoxide dismutases: role in joint diseases. *Joint Bone Spine*. 2007; 74: 324-9.
102. Santilli F, D'Ardes D, Davi G. Oxidative stress in chronic vascular disease: From prediction to prevention. *Vascul Pharmacol*. 2015; 74: 23-37.
103. Videla LA, Rodrigo R, Orellana M, Fernandez V, Tapia G, Quinones L, *et al*. Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients. *Clin Sci (Lond)*. 2004; 106: 261-8.
104. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, *et al*. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest*. 2004; 114: 1752-61.
105. Tormos KV, Anso E, Hamanaka RB, Eisenbart J, Joseph J, Kalyanaraman B, *et al*. Mitochondrial complex III ROS regulate adipocyte differentiation. *Cell Metab*. 2011; 14: 537-44.
106. Higuchi M, Dusing GJ, Peshavariya H, Jiang F, Hsiao ST, Chan EC, *et al*. Differentiation of human adipose-derived stem cells into fat involves reactive oxygen species and Forkhead box O1 mediated upregulation of antioxidant enzymes. *Stem Cells Dev*. 2013; 22: 878-88.
107. Choe SS, Huh JY, Hwang IJ, Kim JI, Kim JB. Adipose Tissue Remodeling: Its Role in Energy Metabolism and Metabolic Disorders. *Front Endocrinol (Lausanne)*. 2016; 7: 30.

108. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest.* 2003; 112: 1796-808.
109. Marseglia L, Manti S, D'Angelo G, Nicotera A, Parisi E, Di Rosa G, *et al.* Oxidative stress in obesity: a critical component in human diseases. *Int J Mol Sci.* 2014; 16: 378-400.
110. Lionetti L, Mollica MP, Lombardi A, Cavaliere G, Gifuni G, Barletta A. From chronic overnutrition to insulin resistance: the role of fat-storing capacity and inflammation. *Nutr Metab Cardiovasc Dis.* 2009; 19: 146-52.
111. Kitabchi AE, McDaniel KA, Wan JY, Tylavsky FA, Jacovino CA, Sands CW, *et al.* Effects of high-protein versus high-carbohydrate diets on markers of beta-cell function, oxidative stress, lipid peroxidation, proinflammatory cytokines, and adipokines in obese, premenopausal women without diabetes: a randomized controlled trial. *Diabetes Care.* 2013; 36: 1919-25.
112. Gregersen S, Samocha-Bonet D, Heilbronn LK, Campbell LV. Inflammatory and oxidative stress responses to high-carbohydrate and high-fat meals in healthy humans. *J Nutr Metab.* 2012; 2012: 238056.
113. Wilson PW, D'Agostino RB, Sullivan L, Parise H, Kannel WB. Overweight and obesity as determinants of cardiovascular risk: the Framingham experience. *Arch Intern Med.* 2002; 162: 1867-72.

114. Bastien M, Poirier P, Lemieux I, Despres JP. Overview of epidemiology and contribution of obesity to cardiovascular disease. *Prog Cardiovasc Dis.* 2014; 56: 369-81.
115. Ceriello A, Motz E. Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. *Arterioscler Thromb Vasc Biol.* 2004; 24: 816-23.
116. Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature.* 2006; 440: 944-8.
117. Rains JL, Jain SK. Oxidative stress, insulin signaling, and diabetes. *Free Radic Biol Med.* 2011; 50: 567-75.
118. Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. *Hepatology.* 2010; 51: 679-89.
119. Harrison SA, Day CP. Benefits of lifestyle modification in NAFLD. *Gut.* 2007; 56: 1760-9.
120. Liu Q, Izamis ML, Xu H, Berendsen T, Yarmush M, Uygun K. Strategies to rescue steatotic livers before transplantation in clinical and experimental studies. *World J Gastroenterol.* 2013; 19: 4638-50.
121. Zeeshan HM, Lee GH, Kim HR, Chae HJ. Endoplasmic Reticulum Stress and Associated ROS. *Int J Mol Sci.* 2016; 17: 327.

122. Lauby-Secretan B, Scoccianti C, Loomis D, Grosse Y, Bianchini F, Straif K, *et al.* Body Fatness and Cancer--Viewpoint of the IARC Working Group. *N Engl J Med.* 2016; 375: 794-8.
123. Meyerhardt JA, Tepper JE, Niedzwiecki D, Hollis DR, McCollum AD, Brady D, *et al.* Impact of body mass index on outcomes and treatment-related toxicity in patients with stage II and III rectal cancer: findings from Intergroup Trial 0114. *J Clin Oncol.* 2004; 22: 648-57.
124. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. *Lancet.* 2008; 371: 569-78.
125. Bastarrachea J, Hortobagyi GN, Smith TL, Kau SW, Buzdar AU. Obesity as an adverse prognostic factor for patients receiving adjuvant chemotherapy for breast cancer. *Ann Intern Med.* 1994; 120: 18-25.
126. Roberts DL, Dive C, Renehan AG. Biological mechanisms linking obesity and cancer risk: new perspectives. *Annu Rev Med.* 2010; 61: 301-16.
127. Park J, Morley TS, Kim M, Clegg DJ, Scherer PE. Obesity and cancer--mechanisms underlying tumour progression and recurrence. *Nat Rev Endocrinol.* 2014; 10: 455-65.
128. Yang L, Li P, Fu S, Calay ES, Hotamisligil GS. Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. *Cell Metab.* 2010; 11: 467-78.

129. Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, *et al.* Autophagy-deficient mice develop multiple liver tumors. *Genes Dev.* 2011; 25: 795-800.
130. Vincent HK, Taylor AG. Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans. *Int J Obes (Lond).* 2006; 30: 400-18.
131. Perse M. Oxidative stress in the pathogenesis of colorectal cancer: cause or consequence? *Biomed Res Int.* 2013; 2013: 725710.
132. Guyton KZ, Kensler TW. Oxidative mechanisms in carcinogenesis. *Br Med Bull.* 1993; 49: 523-44.
133. Rhyu DY, Yang Y, Ha H, Lee GT, Song JS, Uh ST, *et al.* Role of reactive oxygen species in TGF-beta1-induced mitogen-activated protein kinase activation and epithelial-mesenchymal transition in renal tubular epithelial cells. *J Am Soc Nephrol.* 2005; 16: 667-75.
134. Katiyar SK, Meeran SM. Obesity increases the risk of UV radiation-induced oxidative stress and activation of MAPK and NF-kappaB signaling. *Free Radic Biol Med.* 2007; 42: 299-310.
135. Sutti S, Jindal A, Locatelli I, Vacchiano M, Gigliotti L, Bozzola C, *et al.* Adaptive immune responses triggered by oxidative stress contribute to hepatic inflammation in NASH. *Hepatology.* 2014; 59: 886-97.

136. Forman HJ, Davies KJ, Ursini F. How do nutritional antioxidants really work: nucleophilic tone and para-hormesis versus free radical scavenging in vivo. *Free Radic Biol Med.* 2014; 66: 24-35.
137. Queiroz AN, Gomes BA, Moraes WM, Jr., Borges RS. A theoretical antioxidant pharmacophore for resveratrol. *Eur J Med Chem.* 2009; 44: 1644-9.
138. Limon-Pacheco J, Gonsebatt ME. The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. *Mutat Res.* 2009; 674: 137-47.
139. Ozata M, Mergen M, Oktenli C, Aydin A, Sanisoglu SY, Bolu E, *et al.* Increased oxidative stress and hypozincemia in male obesity. *Clin Biochem.* 2002; 35: 627-31.
140. Cui R, Gao M, Qu S, Liu D. Overexpression of superoxide dismutase 3 gene blocks high-fat diet-induced obesity, fatty liver and insulin resistance. *Gene Ther.* 2014; 21: 840-8.
141. Bergamini CM, Gambetti S, Dondi A, Cervellati C. Oxygen, reactive oxygen species and tissue damage. *Curr Pharm Des.* 2004; 10: 1611-26.
142. Alsanea S, Gao M, Liu D. Phloretin Prevents High-Fat Diet-Induced Obesity and Improves Metabolic Homeostasis. *AAPS J.* 2017; 19: 797-805.

143. Alsanea S, Liu D. BITC and S-Carvone Restrain High-Fat Diet-Induced Obesity and Ameliorate Hepatic Steatosis and Insulin Resistance. *Pharm Res.* 2017; 34: 2241-9.
144. Gao M, Ma Y, Liu D. Rutin suppresses palmitic acids-triggered inflammation in macrophages and blocks high fat diet-induced obesity and fatty liver in mice. *Pharm Res.* 2013; 30: 2940-50.
145. Ma Y, Gao M, Liu D. Chlorogenic acid improves high fat diet-induced hepatic steatosis and insulin resistance in mice. *Pharm Res.* 2015; 32: 1200-9.
146. Kumar S, Alagawadi KR. Anti-obesity effects of galangin, a pancreatic lipase inhibitor in cafeteria diet fed female rats. *Pharm Biol.* 2013; 51: 607-13.
147. Park MY, Sung MK. Carnosic acid attenuates obesity-induced glucose intolerance and hepatic fat accumulation by modulating genes of lipid metabolism in C57BL/6J-ob/ob mice. *J Sci Food Agric.* 2015; 95: 828-35.
148. Cho SJ, Jung UJ, Choi MS. Differential effects of low-dose resveratrol on adiposity and hepatic steatosis in diet-induced obese mice. *Br J Nutr.* 2012; 108: 2166-75.
149. Dong J, Zhang X, Zhang L, Bian HX, Xu N, Bao B, *et al.* Quercetin reduces obesity-associated ATM infiltration and inflammation in mice: a mechanism including AMPK $\alpha$ 1/SIRT1. *J Lipid Res.* 2014; 55: 363-74.

150. Dinh CH, Szabo A, Camer D, Yu Y, Wang H, Huang XF. Bardoxolone methyl prevents fat deposition and inflammation in the visceral fat of mice fed a high-fat diet. *Chem Biol Interact.* 2015; 229: 1-8.
151. Mielgo-Ayuso J, Barrenechea L, Alcorta P, Larrarte E, Margareto J, Labayen I. Effects of dietary supplementation with epigallocatechin-3-gallate on weight loss, energy homeostasis, cardiometabolic risk factors and liver function in obese women: randomised, double-blind, placebo-controlled clinical trial. *Br J Nutr.* 2014; 111: 1263-71.
152. Basu A, Sanchez K, Leyva MJ, Wu MY, Betts NM, Aston CE, *et al.* Green Tea Supplementation Affects Body Weight, Lipids, and Lipid Peroxidation in Obese Subjects with Metabolic Syndrome. *Journal of the American College of Nutrition.* 2010; 29: 31-40.
153. Wang Y, Yang M, Lee SG, Davis CG, Koo SI, Fernandez ML, *et al.* Diets high in total antioxidant capacity improve risk biomarkers of cardiovascular disease: a 9-month observational study among overweight/obese postmenopausal women. *Eur J Nutr.* 2014; 53: 1363-9.
154. McNeilly AM, Davison GW, Murphy MH, Nadeem N, Trinick T, Duly E, *et al.* Effect of alpha-lipoic acid and exercise training on cardiovascular disease risk in obesity with impaired glucose tolerance. *Lipids Health Dis.* 2011; 10: 217.
155. Crane TE, Kubota C, West JL, Kroggel MA, Wertheim BC, Thomson CA. Increasing the vegetable intake dose is associated with a rise in plasma carotenoids



- without modifying oxidative stress or inflammation in overweight or obese postmenopausal women. *J Nutr.* 2011; 141: 1827-33.
156. Drieling RL, Gardner CD, Ma J, Ahn DK, Stafford RS. No beneficial effects of pine bark extract on cardiovascular disease risk factors. *Arch Intern Med.* 2010; 170: 1541-7.
157. Datau EA, Wardhana, Surachmanto EE, Pandelaki K, Langi JA, Fias. Efficacy of *Nigella sativa* on serum free testosterone and metabolic disturbances in central obese male. *Acta Med Indones.* 2010; 42: 130-4.
158. Silveira JQ, Dourado GK, Cesar TB. Red-fleshed sweet orange juice improves the risk factors for metabolic syndrome. *Int J Food Sci Nutr.* 2015; 66: 830-6.
159. Hosseini B, Saedisomeolia A, Wood LG, Yaseri M, Tavasoli S. Effects of pomegranate extract supplementation on inflammation in overweight and obese individuals: A randomized controlled clinical trial. *Complement Ther Clin Pract.* 2016; 22: 44-50.
160. Persson T, Popescu BO, Cedazo-Minguez A. Oxidative stress in Alzheimer's disease: why did antioxidant therapy fail? *Oxid Med Cell Longev.* 2014; 2014: 427318.
161. Nash SH, Till C, Song X, Lucia MS, Parnes HL, Thompson IM, Jr., *et al.* Serum Retinol and Carotenoid Concentrations and Prostate Cancer Risk: Results from the Prostate Cancer Prevention Trial. *Cancer Epidemiol Biomarkers Prev.* 2015; 24: 1507-15.

162. Pantavos A, Ruiter R, Feskens EF, de Keyser CE, Hofman A, Stricker BH, *et al.* Total dietary antioxidant capacity, individual antioxidant intake and breast cancer risk: the Rotterdam Study. *Int J Cancer.* 2015; 136: 2178-86.
163. Freitas Bde J, Lloret GR, Visacri MB, Tuan BT, Amaral LS, Baldini D, *et al.* High 15-F<sub>2t</sub>-Isoprostane Levels in Patients with a Previous History of Nonmelanoma Skin Cancer: The Effects of Supplementary Antioxidant Therapy. *Biomed Res Int.* 2015; 2015: 963569.
164. Speakman J, Hambly C, Mitchell S, Krol E. Animal models of obesity. *Obes Rev.* 2007; 8 Suppl 1: 55-61.
165. Speakman J, Hambly C, Mitchell S, Krol E. The contribution of animal models to the study of obesity. *Lab Anim.* 2008; 42: 413-32.
166. Lutz TA, Woods SC. Overview of animal models of obesity. *Curr Protoc Pharmacol.* 2012; Chapter 5: Unit5 61.
167. Wong SK, Chin KY, Suhaimi FH, Fairus A, Ima-Nirwana S. Animal models of metabolic syndrome: a review. *Nutr Metab (Lond).* 2016; 13: 65.
168. Fernandes MR, Lima NV, Rezende KS, Santos IC, Silva IS, Guimaraes RC. Animal models of obesity in rodents. An integrative review. *Acta Cir Bras.* 2016; 31: 840-4.
169. Nilsson C, Raun K, Yan FF, Larsen MO, Tang-Christensen M. Laboratory animals as surrogate models of human obesity. *Acta Pharmacol Sin.* 2012; 33: 173-81.

170. Johnson W, Li L, Kuh D, Hardy R. How Has the Age-Related Process of Overweight or Obesity Development Changed over Time? Co-ordinated Analyses of Individual Participant Data from Five United Kingdom Birth Cohorts. *PLoS Med.* 2015; 12: e1001828; discussion e.
171. Kelsey MM, Zaepfel A, Bjornstad P, Nadeau KJ. Age-related consequences of childhood obesity. *Gerontology.* 2014; 60: 222-8.
172. de Oliveira MR. Phloretin-induced cytoprotective effects on mammalian cells: A mechanistic view and future directions. *Biofactors.* 2016; 42: 13-40.
173. Ehrenkranz JR, Lewis NG, Kahn CR, Roth J. Phlorizin: a review. *Diabetes/metabolism research and reviews.* 2005; 21: 31-8.
174. Crespy V, Aprikian O, Morand C, Besson C, Manach C, Demigne C, *et al.* Bioavailability of phloretin and phloridzin in rats. *J Nutr.* 2001; 131: 3227-30.
175. Chang WT, Huang WC, Liou CJ. Evaluation of the anti-inflammatory effects of phloretin and phlorizin in lipopolysaccharide-stimulated mouse macrophages. *Food chemistry.* 2012; 134: 972-9.
176. Min J, Huang K, Tang H, Ding X, Qi C, Qin X, *et al.* Phloretin induces apoptosis of non-small cell lung carcinoma A549 cells via JNK1/2 and p38 MAPK pathways. *Oncol Rep.* 2015; 34: 2871-9.

177. Kim JL, Kang MK, Gong JH, Park SH, Han SY, Kang YH. Novel antiosteoclastogenic activity of phloretin antagonizing RANKL-induced osteoclast differentiation of murine macrophages. *Mol Nutr Food Res*. 2012; 56: 1223-33.
178. Pinnell SR, Zielinski J, Hansenne I. Anti-aging composition containing phloretin. Google Patents; 2009.
179. Schulze C, Bangert A, Kottra G, Geillinger KE, Schwanck B, Vollert H, *et al*. Inhibition of the intestinal sodium-coupled glucose transporter 1 (SGLT1) by extracts and polyphenols from apple reduces postprandial blood glucose levels in mice and humans. *Mol Nutr Food Res*. 2014; 58: 1795-808.
180. Najafian M, Jahromi MZ, Nowroznejhada MJ, Khajeaiaian P, Kargar MM, Sadeghi M, *et al*. Phloridzin reduces blood glucose levels and improves lipids metabolism in streptozotocin-induced diabetic rats. *Molecular biology reports*. 2012; 39: 5299-306.
181. Behzad S, Sureda A, Barreca D, Nabavi SF, Rastrelli L, Nabavi SM. Health effects of phloretin: from chemistry to medicine. *Phytochemistry Reviews*. 2017: 1-7.
182. Gao M, Zhang C, Ma Y, Bu L, Yan L, Liu D. Hydrodynamic delivery of mIL10 gene protects mice from high-fat diet-induced obesity and glucose intolerance. *Mol Ther*. 2013; 21: 1852-61.
183. Darkhal P, Gao M, Ma Y, Liu D. Blocking high-fat diet-induced obesity, insulin resistance and fatty liver by overexpression of Il-13 gene in mice. *Int J Obes (Lond)*. 2015; 39: 1292-9.

184. Yan L, Song K, Gao M, Qu S, Liu D. Modulation of Cell-Mediated Immunity to Suppress High Fat Diet-Induced Obesity and Insulin Resistance. *Pharm Res.* 2016; 33: 395-403.
185. Huang WC, Chang WT, Wu SJ, Xu PY, Ting NC, Liou CJ. Phloretin and phlorizin promote lipolysis and inhibit inflammation in mouse 3T3-L1 cells and in macrophage-adipocyte co-cultures. *Mol Nutr Food Res.* 2013; 57: 1803-13.
186. Higdon JV, Delage B, Williams DE, Dashwood RH. Cruciferous vegetables and human cancer risk: epidemiologic evidence and mechanistic basis. *Pharmacol Res.* 2007; 55: 224-36.
187. Sofrata A, Santangelo EM, Azeem M, Borg-Karlson AK, Gustafsson A, Putsep K. Benzyl isothiocyanate, a major component from the roots of *Salvadora persica* is highly active against Gram-negative bacteria. *PLoS One.* 2011; 6: e23045.
188. Hecht SS. Inhibition of carcinogenesis by isothiocyanates. *Drug Metab Rev.* 2000; 32: 395-411.
189. Wu X, Zhou QH, Xu K. Are isothiocyanates potential anti-cancer drugs? *Acta Pharmacol Sin.* 2009; 30: 501-12.
190. Zhang R, Loganathan S, Humphreys I, Srivastava SK. Benzyl isothiocyanate-induced DNA damage causes G2/M cell cycle arrest and apoptosis in human pancreatic cancer cells. *J Nutr.* 2006; 136: 2728-34.

191. Miyoshi N, Takabayashi S, Osawa T, Nakamura Y. Benzyl isothiocyanate inhibits excessive superoxide generation in inflammatory leukocytes: implication for prevention against inflammation-related carcinogenesis. *Carcinogenesis*. 2004; 25: 567-75.
192. Batra S, Sahu RP, Kandala PK, Srivastava SK. Benzyl isothiocyanate-mediated inhibition of histone deacetylase leads to NF-kappaB turnoff in human pancreatic carcinoma cells. *Mol Cancer Ther*. 2010; 9: 1596-608.
193. Cheng J, Chiao J, Jin H, Zhong C. The use of isothiocyanates compounds in treating prostatic diseases and skin cancer. *Google Patents*; 2012.
194. Choi KM, Lee YS, Kim W, Kim SJ, Shin KO, Yu JY, *et al*. Sulforaphane attenuates obesity by inhibiting adipogenesis and activating the AMPK pathway in obese mice. *J Nutr Biochem*. 2014; 25: 201-7.
195. de Carvalho CC, da Fonseca MMR. Carvone: Why and how should one bother to produce this terpene. *Food chemistry*. 2006; 95: 413-22.
196. de Cassia da Silveira e Sa R, Andrade LN, de Sousa DP. A review on anti-inflammatory activity of monoterpenes. *Molecules*. 2013; 18: 1227-54.
197. Wagner KH, Elmadfa I. Biological relevance of terpenoids. Overview focusing on mono-, di- and tetraterpenes. *Ann Nutr Metab*. 2003; 47: 95-106.
198. Committee ES. Scientific opinion on the safety assessment of carvone, considering all sources of exposure. *EFSA Journal*. 2014; 12.

199. Coppens C. Viral inhibitor compositions for in vivo therapeutic use comprising a combination of (-) -carvone, geraniol and a further essential oil component. Google Patents; 2014.
200. Sabir S, Singh D, Rocha J. In Vitro Antioxidant Activity of S-Carvone Isolated from *Zanthoxylum alatum*. *Pharmaceutical Chemistry Journal*. 2015; 49: 187-91.
201. Rajeshwari T, Raja B. Antioxidant and free radical scavenging effect of D-carvone in hypertensive rats. In vivo and in vitro study. *International Letters of Natural Sciences*. 2015; 35: 6-12.
202. Elmastaş M, Dermirtas I, Isildak O, Aboul - Enein HY. Antioxidant Activity of S - Carvone Isolated from Spearmint (*Mentha Spicata* L. Fam Lamiaceae). *Journal of liquid chromatography & related technologies*. 2006; 29: 1465-75.
203. Kazemipoor M, Radzi CW, Hajifaraji M, Haerian BS, Mosaddegh MH, Cordell GA. Antiobesity effect of caraway extract on overweight and obese women: a randomized, triple-blind, placebo-controlled clinical trial. *Evid Based Complement Alternat Med*. 2013; 2013: 928582.
204. Gadkari PV, Balaraman M. Catechins: sources, extraction and encapsulation: a review. *Food and Bioproducts Processing*. 2015; 93: 122-38.
205. Saijo R, Katoh M. A history of catechin chemistry with special reference to tea leaves. *Tea Research Journal (Japan)*. 2009.

206. Actis GL, Viton F, Barron D, Dionisi F. Synthesis of catechin and epicatechin conjugates. Google Patents; 2013.
207. Sutherland BA, Rahman RM, Appleton I. Mechanisms of action of green tea catechins, with a focus on ischemia-induced neurodegeneration. *J Nutr Biochem.* 2006; 17: 291-306.
208. Khalesi S, Sun J, Buys N, Jamshidi A, Nikbakht-Nasrabadi E, Khosravi-Boroujeni H. Green tea catechins and blood pressure: a systematic review and meta-analysis of randomised controlled trials. *Eur J Nutr.* 2014; 53: 1299-311.
209. Inanami O, Watanabe Y, Syuto B, Nakano M, Tsuji M, Kuwabara M. Oral administration of (-)catechin protects against ischemia-reperfusion-induced neuronal death in the gerbil. *Free Radic Res.* 1998; 29: 359-65.
210. Kao YH, Hiipakka RA, Liao S. Modulation of obesity by a green tea catechin. *Am J Clin Nutr.* 2000; 72: 1232-4.
211. Wang H, Wen Y, Du Y, Yan X, Guo H, Rycroft JA, *et al.* Effects of catechin enriched green tea on body composition. *Obesity (Silver Spring).* 2010; 18: 773-9.
212. Murase T, Nagasawa A, Suzuki J, Hase T, Tokimitsu I. Beneficial effects of tea catechins on diet-induced obesity: stimulation of lipid catabolism in the liver. *Int J Obes Relat Metab Disord.* 2002; 26: 1459-64.
213. Rains TM, Agarwal S, Maki KC. Antiobesity effects of green tea catechins: a mechanistic review. *J Nutr Biochem.* 2011; 22: 1-7.



214. Graziose R, Lila MA, Raskin I. Merging traditional Chinese medicine with modern drug discovery technologies to find novel drugs and functional foods. *Curr Drug Discov Technol.* 2010; 7: 2-12.
215. Mereles D, Hunstein W. Epigallocatechin-3-gallate (EGCG) for clinical trials: more pitfalls than promises? *Int J Mol Sci.* 2011; 12: 5592-603.
216. Momose Y, Maeda-Yamamoto M, Nabetani H. Systematic review of green tea epigallocatechin gallate in reducing low-density lipoprotein cholesterol levels of humans. *Int J Food Sci Nutr.* 2016; 67: 606-13.
217. Pereira S, Garvey S, Edens N. Methods for enhancing the effect of EGCg on mitigating skeletal muscle loss. *Google Patents;* 2017.
218. Gan RY, Li HB, Sui ZQ, Corke H. Absorption, metabolism, anti-cancer effect and molecular targets of epigallocatechin gallate (EGCG): An updated review. *Crit Rev Food Sci Nutr.* 2016: 1-18.
219. Klaus S, Pultz S, Thone-Reineke C, Wolfram S. Epigallocatechin gallate attenuates diet-induced obesity in mice by decreasing energy absorption and increasing fat oxidation. *Int J Obes (Lond).* 2005; 29: 615-23.
220. Bose M, Lambert JD, Ju J, Reuhl KR, Shapses SA, Yang CS. The major green tea polyphenol, (-)-epigallocatechin-3-gallate, inhibits obesity, metabolic syndrome, and fatty liver disease in high-fat-fed mice. *J Nutr.* 2008; 138: 1677-83.

221. Zhan W, Liu Y, Li D, Liu Y. Advancing insights on the anti-obesity biochemical mechanism of (-)-epigallocatechin gallate (EGCG) by inhibiting  $\alpha$ -amylase activity. *RSC Advances*. 2016; 6: 96918-27.
222. Byun JK, Yoon BY, Jhun JY, Oh HJ, Kim EK, Min JK, *et al.* Epigallocatechin-3-gallate ameliorates both obesity and autoinflammatory arthritis aggravated by obesity by altering the balance among CD4<sup>+</sup> T-cell subsets. *Immunol Lett*. 2014; 157: 51-9.
223. Remely M, Ferk F, Sterneder S, Setayesh T, Roth S, Kepcija T, *et al.* EGCG Prevents High Fat Diet-Induced Changes in Gut Microbiota, Decreases of DNA Strand Breaks, and Changes in Expression and DNA Methylation of Dnmt1 and MLH1 in C57BL/6J Male Mice. *Oxid Med Cell Longev*. 2017; 2017: 3079148.
224. Bruno RS, Dugan CE, Smyth JA, DiNatale DA, Koo SI. Green tea extract protects leptin-deficient, spontaneously obese mice from hepatic steatosis and injury. *J Nutr*. 2008; 138: 323-31.
225. Halliwell B, Gutteridge JMC. Redox chemistry: the essentials. Free radicals in biology and medicine. Fifth edition. ed. Oxford, United Kingdom: Oxford University Press; 2015. p. 30-76.
226. Onyango AN. Endogenous Generation of Singlet Oxygen and Ozone in Human and Animal Tissues: Mechanisms, Biological Significance, and Influence of Dietary Components. *Oxid Med Cell Longev*. 2016; 2016: 2398573.

227. Flora SJ. Structural, chemical and biological aspects of antioxidants for strategies against metal and metalloid exposure. *Oxid Med Cell Longev*. 2009; 2: 191-206.

CHAPTER 2

PHLORETIN PREVENTS HIGH-FAT DIET-INDUCED OBESITY AND IMPROVES  
METABOLIC HOMEOSTASIS\*

---

\* Sary Alsanea, Mingming Gao, and Dexi Liu (2017). Phloretin Prevents High-Fat Diet-Induced Obesity and Improves Metabolic Homeostasis. *The AAPS Journal*. 2017; 19: 797-805. Reprinted here with publisher permission (License # 4050340644982).

## **Abstract**

Reactive oxygen species generated as a byproduct in metabolism play a central role in the development of obesity and obesity-related metabolic complications. The objective of the current study is to explore the possibility to block obesity and improve metabolic homeostasis via phloretin, a natural antioxidant product from apple tree leaves and Manchurian apricot. Both preventive and therapeutic activities of phloretin were assessed using a high-fat diet-induced obesity mouse model. Phloretin was injected intraperitoneally twice weekly into regular and obese mice fed a high-fat diet. The effects of phloretin treatment on body weight and composition, fat content in the liver, glucose and lipid metabolism, and insulin resistance were monitored and compared to the control animals. Phloretin treatment significantly blocks high-fat diet-induced weight gain but did not induce weight loss in obese animals. Phloretin improved glucose homeostasis and insulin sensitivity and alleviated hepatic lipid accumulation. RT-PCR analysis showed that phloretin treatment suppresses expression of macrophage marker (*F4/80* and *Cd68*) and pro-inflammatory genes (*Mcp-1* and *Ccr2*) and enhances adiponectin gene expression in white adipose tissue. In addition, phloretin treatment elevated the expression of fatty acid oxidation genes such as carnitine palmitoyltransferase 1a and 1b (*Cpt1a* and *Cpt1b*), and reduced expression of monocyte chemoattractant protein-1 (*Mcp-1*), *de novo* lipogenesis transcriptional factor peroxisome proliferator-activated receptor- $\gamma$  2 (*Ppar $\gamma$ 2*), and its target monoacylglycerol *O*-acyltransferase (*Mgat-1*) genes. These results provide direct evidence to support a possible use of phloretin for mitigation of obesity and maintenance of metabolic homeostasis.

**Key words:** Obesity; insulin resistance; inflammation; phloretin; antioxidant

## **Introduction**

Obesity is generally considered a lifestyle-associated medical condition with a high risk of developing cardiovascular disease, cancer, type 2 diabetes, fatty liver, or other metabolic disorders (1, 2). The prevalence of obesity has increased in the past 30 years; Statistical data shows the population in the United States of America with a BMI greater than 25 (overweight) has reached almost 70%, and the age-adjusted population with a BMI over 30 (obese) is currently at 36.3% (3).

Obesity has a multifactorial origin and is associated with an increase in adipose tissue, especially white adipose tissue (WAT). In diet-induced obesity, expansion of adipose tissue is accompanied by adipose hypertrophy, infiltration of macrophages, generation of reactive oxygen species (ROS), and release of pro-inflammation cytokines (4, 5). More recent studies have shown that oxidative stress plays a critical role in diet-induced obesity (6-8). Using a high-fat diet-induced obesity model, we have previously shown that overexpression of Sod3 gene coding for extracellular superoxide dismutase using a method of hydrodynamic gene delivery was effective in blocking high-fat diet (HFD)-induced obesity and improving metabolic homeostasis (9). Similar beneficial effects were also reported in mice after direct administration of antioxidants (10-13).

In addition to its role in obesity, oxidative stress has been well studied as a risk factor for atherosclerosis (14), cancer (15), neurodegenerative (16) and cardiovascular diseases (17), and aging (18). Antioxidant therapies employing primarily vitamins have been assessed in suppressing atherosclerosis, with contradictory conclusions reported. Some studies demonstrated acute benefits of antioxidants in the aspects of pathophysiology (19), epidemiology (20), and mechanism (21), and others reported

negative results in the setting of chronic prevention therapy (22). One of the possible explanations for these conflicting observations, put forward by Steinhubl SR, is that the lack of benefits seen in clinical trials may be caused by the use of easily available antioxidants but not the ones that are most appropriate in dealing with a particular pathological condition (23). In addition, it is believed that many clinical trials ended prematurely (24) or employed an insufficient dose for treatment (25).

In contrast to a significant number of preclinical and clinical studies focusing on a particular disease, the use of antioxidants for preventing and treating obesity is a new area of research because obesity is normally considered a precursor or intermediate step toward disease development. In the current study, we examined the activity of phloretin (**Fig. 2.1a**), a phenol-based antioxidant enriched in apple tree leaves, pears and strawberries, against diet-induced obesity and obesity-associated metabolic disorders. In addition to its antioxidant activities, phloretin has been shown to activate endogenous antioxidant pathways involving Nrf2, SOD, and glutathione (26-30). It has been previously shown that oral administration of phloretin significantly reduces blood glucose levels and improves dyslipidemia in diabetic rats (31). Hassan *et al.* (32) have shown that phloretin regulates the expression of diverse genes involved in lipogenesis and triglyceride storage, including *Pepck1*, *Acs11*, *Glut4*, *Lipin1* and *Perilipin*. Using porcine primary adipocytes, Shu and colleagues reported a strong activity of phloretin in increasing glucose utilization and non-esterified fatty acids while decreasing the lactate output (33). A microarray analysis reveals that phloretin enhances expression of CAAT enhancer binding protein- $\alpha$  (*C/ebp $\alpha$* ), proliferator-activated receptor- $\gamma$  (*Ppar- $\gamma$* ), and adipose-related genes such as fatty acid translocase and fatty acid synthase (*Fas*) (32).

Phloretin is also found to suppress the activation of mouse dendritic cells by disturbing the multiple intracellular signal pathways mediated by ROS, MAPKs (ERK, JNK, p38 MAPK) and NF- $\kappa$ B and reduces production of inflammatory cytokines and chemokines (34). Phloretin has also been shown to inhibit LPS-induced activation of macrophages (35).

The activities of phloretin in regulating glucose and lipid metabolism and inflammation suggest that phloretin may be an anti-hyperglycemic and anti-hyperlipidemic substance in maintaining metabolic homeostasis. The objective of the experiments described here is to evaluate whether phloretin is capable of blocking HFD-induced obesity and improving metabolic homeostasis. Our results show that phloretin is highly effective in preventing diet-induced adiposity and in attenuating diet-induced insulin resistance and hepatic steatosis in HFD-fed mice.

## **Materials and Methods**

*Materials.* Phloretin was purchased from Sigma-Aldrich (St. Louis, MO). HFD (60% kJ/fat, 20% kJ/protein, and 20% kJ/carbohydrate) was from Bio-serv (Frenchtown, NJ). The insulin kit was obtained from Mercodia AB (Winston-Salem, NC) and glucometer and blood test strips were obtained from NIPRO Diagnostics™ (Fort Lauderdale, FL). The Humulin® insulin was purchased from Eli Lilly (Indianapolis, IN). The Infinity™ kit for triacylglycerol was from Fisher Diagnostics (Middletown, VA). The total cholesterol kit was purchased from Genzyme Diagnostics (Charlottetown, PE Canada). The non-esterified fatty acids (NEFA)-HR kits were obtained from Wako Bioproducts (Richmond, VA). The RNeasy® Lipid Tissue Mini Kit was from QIAGEN® (Hilden, Germany). The



First-stand cDNA Synthesis was obtained from Origene<sup>®</sup> (Rockville, MD). The PerfeCta<sup>®</sup> SYBR<sup>®</sup> Green FastMix<sup>®</sup> ROX<sup>™</sup> was purchased from Quanta Biosciences (Gaithersburg, MD). Oil-Red O solution was purchased from Electron Microscopy Sciences (Hatfield, PA). Nile-red was purchased from Sigma-Aldrich (St. Louis, MO).

*Animals and Animal Treatments.* C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). All procedures and protocols performed on mice were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia, Athens, Georgia. To assess phloretin activity in blocking HFD-induced obesity and obesity-associated metabolic disorders, ten-week-old male C57BL/6 mice were randomly grouped (4 in each group) and fed an HFD and received twice weekly intraperitoneal (*i.p.*) injections of either phloretin (10 mg/kg) or equivalent vehicle volume (20  $\mu$ l) of dimethyl sulfoxide (DMSO) for 12 weeks. For the obesity treatment study, mice were fed an HFD for 6 weeks and then treated with phloretin (10 mg/kg, *i.p.*) or DMSO for 6 weeks while kept on HFD (5 in each group). Mouse body weight was monitored weekly and the body composition was determined using EchoMRI-100<sup>™</sup> from the Echo Medical System (Houston, TX). Mice were euthanized at the end of the experiments by carbon dioxide inhalation followed by cervical dislocation.

*Glucose Tolerance Test (GTT), Insulin Tolerance Test (ITT), and Determination of Insulin Resistance.* Mice were fasted for 6 h and received *i.p* injection of glucose (1 g/kg) dissolved in saline. The blood samples were collected from the tail vein and serum

glucose level was determined at time 0, 30, 60, and 120 min using a glucometer. For ITT, mice were fasted for 4 h and injected *i.p.* with insulin (0.75 U/kg). The blood samples were collected and glucose concentrations determined using the same schedule as GTT. Insulin resistance was determined using the formula: [fasting insulin (ng/ml) x fasting blood glucose (mg/dl)/405] (36).

*Histochemical Analysis by Hematoxylin and Eosin (H&E) Staining.* Liver and epididymal white adipose tissues were collected from animals and fixed in 10% formalin at the end of the experiment. Fixed tissues were dehydrated, embedded in paraffin, sectioned at 6  $\mu$ m in thickness, and stained with H&E following the manufacturer's instructions. Tissue sections were examined and photo images were taken under an optical microscope using the NIS-Elements imaging software from Nikon Instruments Inc. (Melville, NY).

*Oil-Red O and Nile-red Staining.* Fresh liver samples were collected and frozen at -80°C immediately after mice were euthanized. Frozen sections were made using a Leica Cryostat at a thickness of 8  $\mu$ m and fixed with 10% formalin for 30 min. The fixed sections were rinsed with 60% isopropanol, stained with freshly prepared Oil-Red O solution for 15 min, and dipped 5 times in hematoxylin. The Nile-red working solution was prepared and tissue sections stained following the manufacturer's instructions. The stained sections were examined under a fluorescence microscope and images were taken.

*Determination of Serum Insulin and Lipid Level.* Mice were fasted for 6 h and blood samples were collected from the hearts of mice soon after euthanasia at the end of the

experiments. Insulin levels were measured using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Serum concentrations of triglycerides, FFA, and total cholesterol were determined using the procedure provided by the supplier of the lipid analysis kits.

*Gene Expression Analysis.* Total RNAs from mouse adipose tissue were isolated using RNeasy<sup>®</sup> Lipid Tissue Mini Kit. The complementary DNA (cDNA) was synthesized using First-stand cDNA synthesis kit. Quantitative real time polymerase chain reaction (RT-PCR) was performed using a SYBR Green as a detector with a StepOnePlus system (Applied Biosystems). Data was analyzed by the  $\Delta\Delta C_T$  method using *Gapdh* mRNA as the internal control. All primer sequences used are listed in **Table 2.1**.

*Statistical Analysis.* Statistical analysis was performed by unpaired *t*-test and a  $P < 0.05$  was considered significantly different. Each data point represents the mean  $\pm$  SD or SEM.

## **Results**

*Phloretin Protected Mice from HFD-induced Obesity and Low-grade Inflammation in WAT.*

To evaluate the protective activity of phloretin against HFD-induced weight gain, we fed C57BL/6 mice with HFD and performed biweekly injection (*i.p.*) of phloretin (10 mg/kg). Results in **Figure 2.1** show a much slower weight gain for animals with phloretin injections. At the end of 12-week treatment, phloretin-treated animals showed an average body weight of  $32.1 \pm 6.1$  g, about 10 g less than that of control animals (**Fig. 2.1c**). The

apparent difference in size between treated animals and the controls can be seen visually (**Fig. 2.1b**). There is no difference in lean mass between treated and control animals, but phloretin-treated mice have much less fat mass (**Fig. 2.1d**). Results in **Figures 2.1e** and **2.1f** show that phloretin treatment did not affect food intake compared to the untreated control.

The fat mass difference between treated and control mice was confirmed by measurements of WAT collected from different locations, including epididymal WAT (eWAT), perirenal WAT (pWAT), and inguinal WAT (iWAT). Results in **Figure 2.2a** show a smaller size of WATs in phloretin-treated mice, which is in agreement with the results of weight measurements (**Fig. 2.2b**). Photo images from H&E staining of adipose tissue (**Fig. 2.2c**) showed that the average diameter of adipocytes is 42-50% smaller in phloretin-treated animals than that of control mice (**Fig. 2.2d-f**). H&E staining of brown adipose tissue (BAT) revealed a decreased density of vacuole-type structures (**Fig. 2.2c**). These results suggest that phloretin suppresses lipid accumulation in adipose tissues.

#### *Phloretin Treatment Blocks Macrophage Infiltration into WAT.*

Macrophage infiltration in adipose tissue is a characteristic of HFD-induced obesity (37, 38). The effects of phloretin treatment on macrophage infiltration were examined by RT-PCR using macrophage-specific gene markers, including antigen *F4/80*, cluster differentiation protein 68 (*Cd68*), pro-inflammatory monocyte chemoattractant protein-1 (*Mcp-1*) and its receptor C-C chemokine 2 (*Ccr2*). Results in **Figure 2.3** show that mRNA levels of the macrophage-specific genes were much lower in the WAT of animals receiving phloretin treatment. In contrast, phloretin treatment significantly

increased expression of the adiponectin gene, an adipokine whose expression is suppressed in obese animals. These results demonstrate that phloretin blocks HFD-induced macrophage infiltration in adipose tissue.

*Phloretin Blocks HFD-induced Fatty Liver without Significant Effect on Serum Lipid Level.*

Development of fatty liver is commonly associated with obesity (39). To examine the phloretin effects on fat accumulation in the liver, hepatic lipid level was examined and compared between phloretin treated and control animals. Oil-Red O and Nile-red staining of liver sections showed a significant level of fat content in the livers of control animals in contrast to a below-detection level in phloretin-treated mice (**Fig. 2.4a**). High lipid content in the liver is also accompanied by large liver size in control animals (**Fig. 2.4b**). To study the underlying mechanisms of phloretin effect on fat content in the liver, we determined the mRNA levels of selected genes responsible for hepatic lipid metabolism. Data in **Figure 2.4c** show a reduction in expression of *Mcp-1*, *de novo* lipogenesis transcriptional factor *Ppar $\gamma$ 2*, and its target monoacylglycerol *O*-acyltransferase (*Mgat-1*) in phloretin-treated mice while the expression of genes involved in fatty acid oxidation carnitine palmitoyltransferase 1a and 1b (*Cpt1a* and *Cpt1b*) were significantly increased (**Fig. 2.4c**). Serum biochemistry analysis shows no difference between treated and control animals regarding serum concentrations of total cholesterol, FFA, and triglycerides (**Fig. 2.4d**). Taken together, these results suggest that phloretin blocks hepatic lipid accumulation without changing the circulatory lipid level.

*Phloretin Prevented Diet-induced Insulin Resistance and Hyperinsulinemia.*

Feeding mice with HFD can lead to hyperglycemia and hyperinsulinemia (40). Glucose and insulin tolerance tests were performed to assess the effects of phloretin on glucose homeostasis and insulin resistance. Results in **Figures 2.5a-b** show that phloretin-treated mice had better glucose profiles. Compared to control animals, phloretin-treated animals showed a faster decrease of blood glucose concentration upon glucose injection. Results from insulin tolerance tests (ITT) (**Figs. 2.5c, 2.5d**) showed better insulin sensitivity in phloretin-treated animals with a lower value of homeostasis model assessment-estimated insulin resistance (HOMA-IR) (**Fig. 2.5e**) compared to that of control mice ( $1.7\pm 0.8$  vs.  $5.4\pm 2.2$ ). Moreover, phloretin blocked HFD-induced hyperinsulinemia ( $3.4\pm 0.8$  ng/ml vs.  $8.7\pm 3.2$  ng/ml) (**Fig. 2.5f**). These data suggest that phloretin prevents the development of HFD-induced glucose intolerance, insulin resistance, and hyperinsulinemia.

*Phloretin Treatment did not Induce Weight Loss in Obese Mice, but Alleviated Hepatic Lipid Accumulation, Hyperinsulinemia, and Insulin Resistance.*

The results shown above demonstrate that phloretin is effective in preventing HFD-induced obesity, insulin resistance, and hepatic steatosis. We then examined whether phloretin treatment could offer therapeutic benefits to obese animals. Two groups of animals were fed an HFD for 6 weeks to induce obesity and then treated with phloretin at the same dose and frequency. The data in **Figures 2.6a, 2.6b** show that continuous treatment for 6 weeks did not induce weight loss. There was no difference between treated and control mice in body weight, fat mass, and lean mass (**Figs. 2.6b,**

**2.6c**). However, images from H&E staining of eWAT showed a slight reduction in average diameter of adipocytes ( $97.1 \pm 24.6 \mu\text{m}$  vs.  $114.8 \pm 22.6 \mu\text{m}$ ) (**Figs. 2.6d, 2.6e**) without a difference seen in BAT (**Fig. 2.6d**). Interestingly, phloretin alleviated hepatic lipid accumulation (**Fig. 2.6f**, upper panel), in agreement with the results of Oil-Red O staining (**Fig. 2.6f**, lower panel). Phloretin did not alter fasting blood glucose level (**Fig. 2.6g**) but did show activity in reducing insulin level ( $4.5 \pm 1.1 \text{ ng/ml}$  vs.  $9.8 \pm 3.2 \text{ ng/ml}$ ) and alleviating insulin resistance (HOMA-IR  $1.9 \pm 0.3$  vs.  $5.2 \pm 2$ ) (**Figs. 2.6h, 2.6i**). These results suggest that phloretin treatment resulted in improvement in fatty liver and insulin resistance of obese mice.

## Discussion

In this study, we systematically studied the preventive and therapeutic activities of phloretin on diet-induced obesity and its associated complications. We show that phloretin protected C57BL/6 mice from HFD-induced obesity (**Fig. 2.1**), fatty liver (**Figs. 2.4a-c**), and insulin resistance (**Figs. 2.5a-f**). These beneficial effects were associated with suppression of adipocyte differentiation and macrophage infiltration into adipose tissues (**Figs. 2.2a, 2.2c**). In obese mice, phloretin treatment did not induce weight loss (**Figs. 2.6a, 2.6b**) but alleviated hepatic lipid accumulation (**Fig. 2.6f**) and improved insulin resistance (**Figs. 2.6h, 2.6i**).

Earlier studies have shown that antioxidant and anti-inflammation activities of phloretin are mediated by inhibition of signaling pathways mediated by nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) and mitogen-activated protein kinase (MAPK) (26, 41, 42). At the molecular level, multiple hydroxyl groups with an aromatic phenol ring structure of phloretin (**Fig.**

**2.1a**) are capable of scavenging the ROS generated by increased food consumption and minimizing the ROS-induced tissue damage, therefore inhibiting macrophage infiltration and macrophage-mediated inflammation. Reduction in the number of crown-like structures seen in WAT of phloretin-treated mice (**Fig. 2.2a**) and suppressed expression of macrophage-specific marker genes including *F4/80*, *Cd68*, *Mcp1*, and *Ccr2* in eWAT (**Fig. 2.3**) appear to support such an explanation. In other words, twice weekly injection of phloretin at a dose of 10 mg/kg provided animals with sufficient antioxidants to neutralize ROS, resulting in blockage of body weight and fat mass gain (**Figs. 2.1b, 2.1c, 2.2**). The fact that phloretin treatment did not induce weight loss suggests a different mechanism between inflammation-based adipocyte differentiation and reduction of fat mass that involves lipolysis, *i.e.*, blockade of inflammation is not sufficient to promote lipolysis of pre-existing fats in WAT.

Fatty liver is caused by excessive intracellular fat accumulation in hepatocytes. Hepatic fat accumulation could result from an increase in fatty acid synthesis accompanied by a decrease in lipolysis and biochemical conversion of free fatty acids to triglycerides. Overexpression of *CD36* gene coding for fatty acid transporters could be another reason for the elevation of hepatic fat content (43). Results shown in **Figures 4a, 4b** suggest that phloretin inhibited hepatic fat accumulation in the liver by suppressing the expression of *de novo* lipogenesis genes (*Ppar $\gamma$ 2* and *Mgat-1*) and enhancing the expression of genes responsible for fatty acid oxidation (*Cpt1a* and *Cpt1b*) (**Fig. 2.4c**). Our observation is in agreement with the study demonstrating the hepatoprotective effect of phloretin (44).



The activity of phloretin in improving metabolic homeostasis is likely related to its activity in blocking inflammation, which is known to be involved in obesity-associated hyperglycemia, hyperinsulinemia, and insulin resistance. Using the streptozotocin-induced diabetic model in rats, Najafian *et al.* (31) have previously shown that phloridzin, a phloretin precursor, improves glucose hemostasis and insulin sensitivity. A similar observation has also been reported in a genetically modified BKS diabetic mouse model (33). Consistent with these previous studies, we show that phloretin improved fasting blood glucose level (**Fig. 2.4a**) and insulin sensitivity in both prevention and treatment studies (**Figs. 2.5a-f**). It remains unclear, however, whether the function of phloretin in inhibiting sodium/glucose co-transporter 1 and 2 previously shown (45) plays an important role in improving glucose homeostasis. This is an important issue and requires additional study because it has been previously shown that phloretin inhibits intestinal glucose absorption and glucose reabsorption in the kidneys by inhibiting these transporters (46, 47).

Similar to activities of phloretin observed in the current study, other antioxidants have also been examined in animals (10-12). Cho *et al.* demonstrated that a continuous treatment of animals with resveratrol for 10 weeks was effective in blocking HFD-induced obesity, fatty liver, and hyperlipidemia (10). Different from the effect of phloretin seen in our study, resveratrol treatment resulted in a decrease in serum lipid level. The differences seen between the current and the study by Cho *et al.* could be caused by the difference in chemical structure of the two antioxidants. Alternatively, the difference could also be due to the inclusion of 1% cholesterol in the HFD used in their study to induce hyperlipidemia, which is not included in our study. Similarly, quercetin, a

flavonol type of flavonoid found in plants, has demonstrated an ability to block HFD-induced obesity and insulin resistance (11). The authors attributed quercetin's effects to its activity in increasing the level of glucose transporter 4 and reducing inflammation in epididymal WAT through the AMPK $\alpha$ 1/SIRT1-mediated pathway, which was in full agreement with the function of phloretin. Similar effects and conclusions have also been obtained in mice treated with bardoxolone methyl (12). Judging by the data currently available and generated from preclinical studies, we believe that all antioxidants are capable of suppressing diet-induced obesity and improving metabolic homeostasis as long as appropriate doses and frequency of administration are used without causing toxicity.

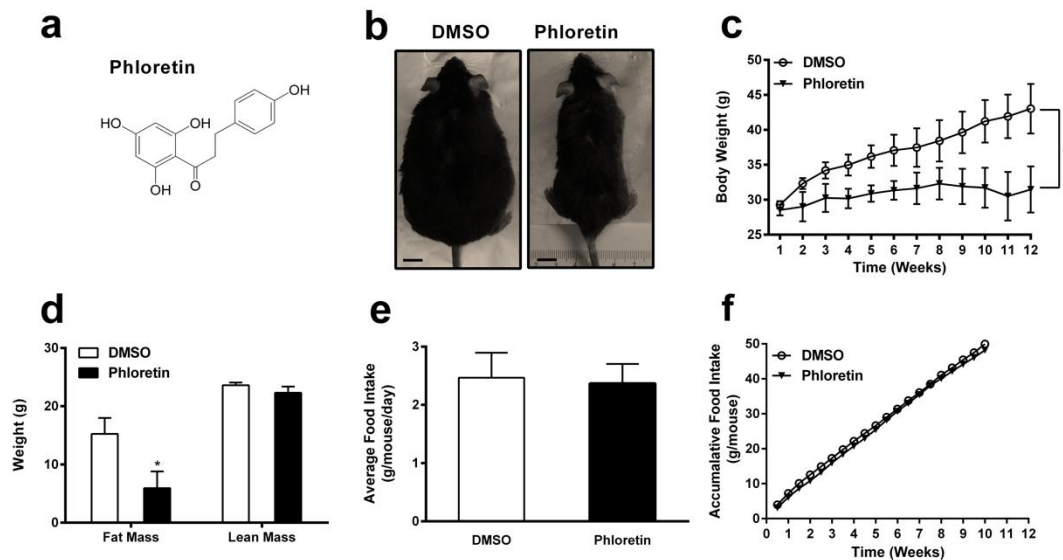
In conclusion, the data presented in this report clearly demonstrate the suppressive effects of phloretin on HFD-induced obesity and obesity-associated metabolic complications. Phloretin's effects are correlated with suppression of HFD-induced inflammation in adipose tissues. In obese mice, phloretin treatment improves hyperinsulinemia and alleviates fatty liver, but does not induce weight loss. These results suggest that phloretin can be considered as a substance to mitigate diet-induced obesity and improve metabolic homeostasis.

**Acknowledgments:** This work was supported in part by NIH grant RO1HL098295. Sary Alsanea is supported by a scholarship from the King Saud University (Riyadh, Saudi Arabia). We thank Dr. Megan Morgan for proofreading and editing the manuscript.

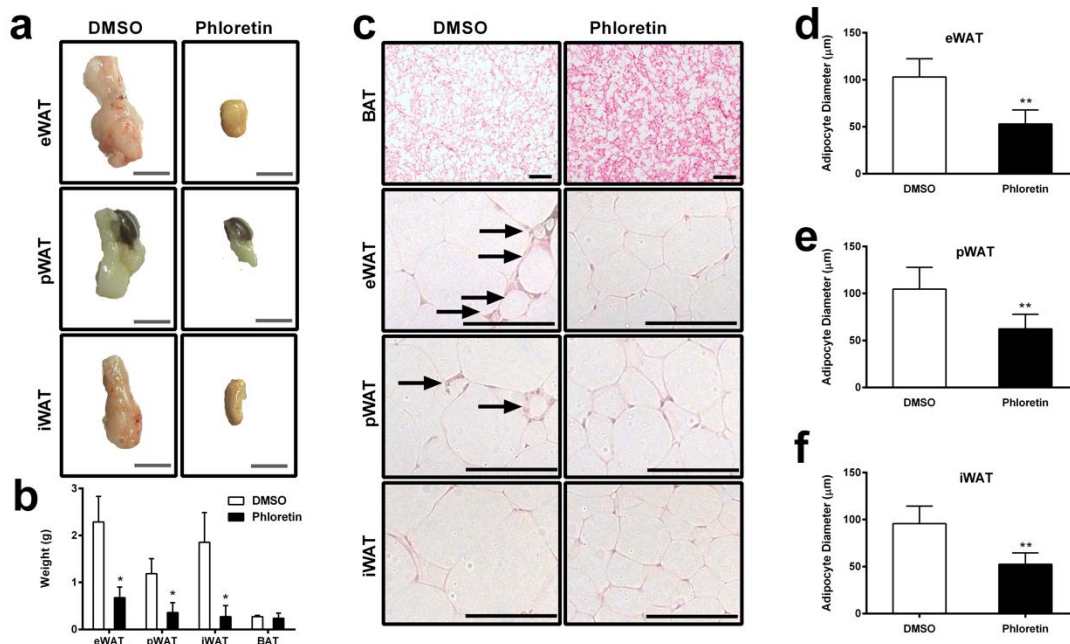
**Conflict of Interest:** The authors declare no conflict of interest.

**Table 2.1: Primers used for quantitative real time PCR analysis of gene expression.**

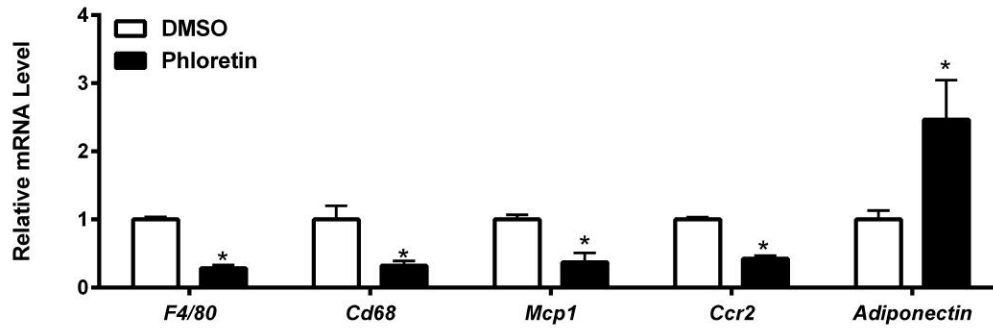
<b>Gene</b>	<b>Forward Primers</b>	<b>Reverse Primers</b>
<i>F4/80</i>	5' CCCAGTGTCTTACAGAGTG 3'	5' GTGCCAGAGTGGATGTCT 3'
<i>Cd68</i>	5' CCATCCTTACGATGACACCT 3'	5' GGCAGGGTTATGAGTGACAGTT 3'
<i>Mcp1</i>	5' ACTGAAGCCAGCTCTCTTCCTC 3'	5' TTCCTTCTGGGGTCAGCACAGAC 3'
<i>Ccr2</i>	5' ATCCACGGCATACTATCAACATC 3'	5' CAAGGCTCACCATCATCGTAG 3'
<i>Adiponectin</i>	5' TGTTCCTCTTAATCCTGCCCA 3'	5' CCAACCTGCACAAGTTCCCTT 3'
<i>Cpt1a</i>	5' CTCCGCCTGAGCCATGAAG 3'	5' CACCAGTGATGATGCCATTCT 3'
<i>Cpt1b</i>	5' GGTCTTCTTCAAGGTCTG 3'	5' CGAGGATTCTTGGAAGTGC 3'
<i>Ppar<math>\gamma</math>1</i>	5' GGAAGACCACTCGCATTCTT 3'	5' GTAATCAGCAACCATTGGGTCA 3'
<i>Ppar<math>\gamma</math>2</i>	5' TCGCTGATGCACTGCCTATG 3'	5' GAGAGTCCACAGAGCTGATT 3'
<i>Mgat1</i>	5' TGGTGCCAGTTTGGTTCCAG 3'	5' TGCTCTGAGGTCGGGTCA 3'
<i>Gapdh</i>	5' AGGTCGGTGTGAACGGATTTG 3'	5' TGTAGACCATGTAGTTGAGGTCA 3'



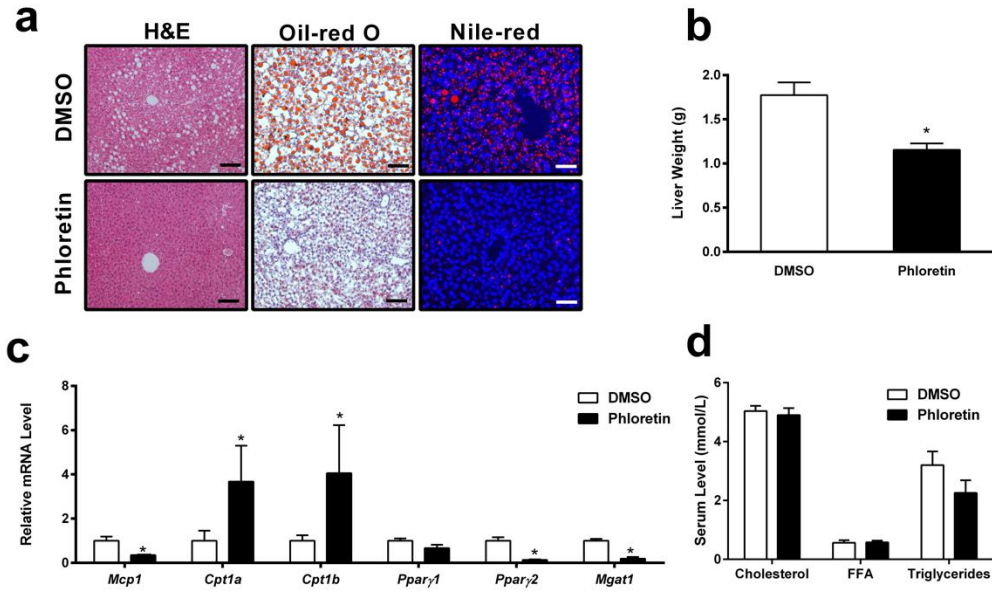
**Figure 2.1. Phloretin inhibits body weight gain in mice fed an HFD.** Ten-week-old C57BL/6 male mice were fed HFD and injected *i.p.* with phloretin (10 mg/kg, twice weekly) or vehicle (DMSO) for 12 weeks. **(a)** chemical structure of phloretin; **(b)** representative pictures of the mice at end of the experiment, scale bar: 1 cm; **(c)** growth curve; **(d)** body composition at week 12; **(e)** average food intake; and **(f)** accumulative food intake. Values represent mean  $\pm$  SEM, \*  $p < 0.05$  (n=4).



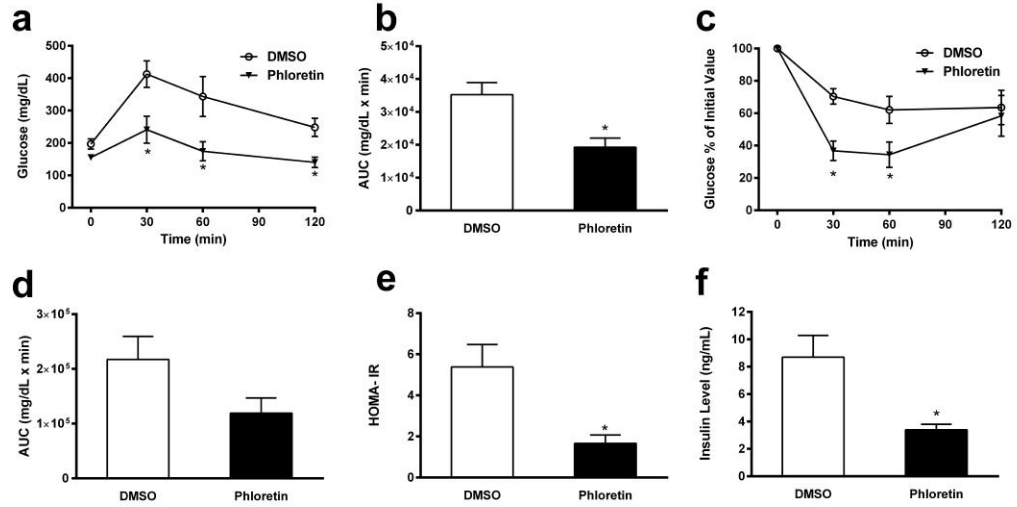
**Figure 2.2. Impacts of phloretin treatment on adipose tissues of animals.** Mice were fed an HFD continuously for 12 weeks, with one group of animals injected *i.p.* with phloretin (10 mg/kg) and the other with vehicle DMSO. Animals were sacrificed at the end of experiments and adipose tissues were collected (n=4). **(a)** Photo images of eWAT, pWAT, and iWAT from treated and control animals, gray scale bar: 1 cm; **(b)** weights of eWAT, pWAT, iWAT, and BAT; **(c)** H&E staining of eWAT, pWAT, and iWAT and BAT, black arrows indicate crown-like structure (CLS), scale bar: 100 μm; **(d-f)** average diameter of adipocytes in different WATs (n= 300). Values are mean ± SD. \* $p < 0.05$ , \*\* $p < 0.01$ .



**Figure 2.3. Phloretin blocked HFD-induced inflammation.** At the end of 12 weeks of HFD feeding and twice weekly *i.p.* injections of phloretin (10 mg/kg) or DMSO, mice were sacrificed. Total RNAs were extracted from eWAT and mRNA levels of macrophage marker genes and adiponectin gene were determined by qPCR. All values represent mean  $\pm$  SEM, \* $p < 0.05$  (n=4).

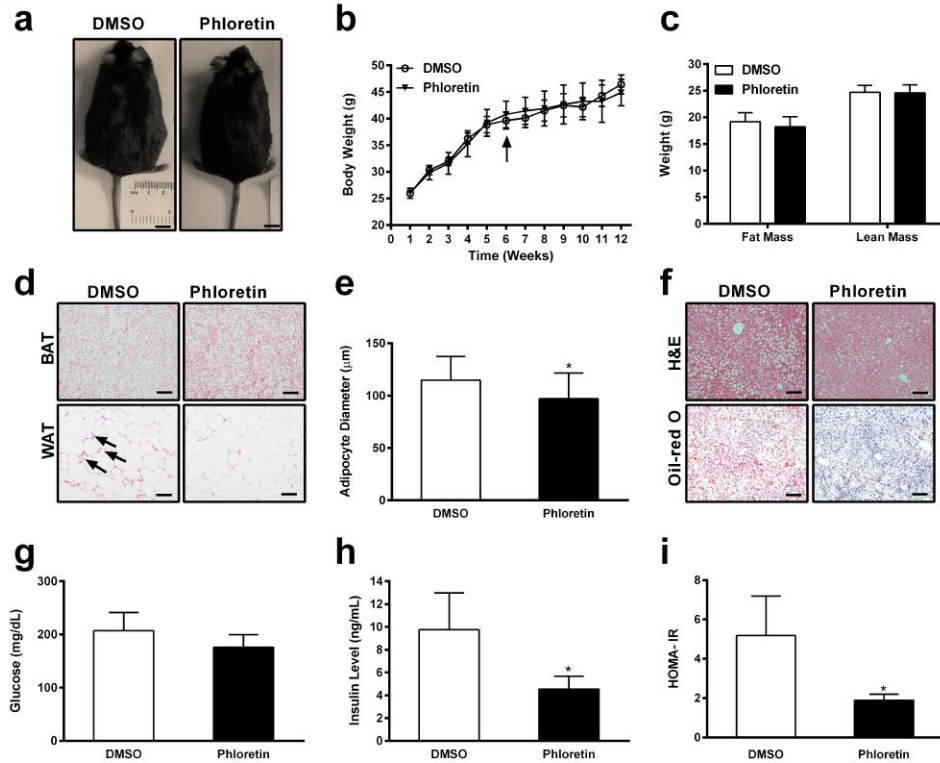


**Figure 2.4. Phloretin prevented HFD-induced fatty liver.** Mice were sacrificed at the end of week 12. **(a)** Photo images of H&E (the left panel), Oil-Red O (the middle panel), and Nile-red (the right panel) staining of liver sections were photographed at x100 magnification, scale bars: 100  $\mu$ m; **(b)** liver weight; **(c)** relative hepatic mRNA expression of *Mcp1*, *Cpt1a*, *Cpt1b*, *Pparγ1*, *Pparγ2*, and *Mgat1*; and **(d)** serum levels of cholesterol, FFA, and triglycerides. Each data point represents mean  $\pm$  SEM, \* $p < 0.05$  (n=4).



**Figure 2.5. Phloretin improved glucose homeostasis and hyperinsulinemia in HFD-fed mice.** (a) Profile of serum glucose concentration after *i.p.* glucose injection (1 g/kg); (b) area under the curve of glucose tolerance test; (c) time-dependent glucose concentration in blood after *i.p.* insulin injection (0.75 U/kg); (d) area under the curve of insulin tolerance test; (e) homeostasis model assessment-estimated insulin resistance (HOMA-IR); and (f) fasting serum insulin concentration. Each data point represents mean  $\pm$  SEM, \*  $p < 0.05$  (n=4).





**Figure 2.6. Effects of phloretin treatment on obese mice fed an HFD.** Mice were fed an HFD for 6 weeks and developed obesity, followed by 6 weeks of twice weekly *i.p.* injection of phloretin when continuing on an HFD. **(a)** Representative pictures of the mice at the end of experiment, scale bar: 1 cm; **(b)** growth curve, arrow indicates starting of phloretin treatment (n=5); **(c)** body composition at the end of the experiment (n=5); **(d)** H&E staining were performed on BAT (the upper panel) and eWAT (the lower panel), arrow indicates crown-like structure (CLS); **(e)** average diameter of epididymal adipocytes (n≈500 from sections of 3 mice); **(f)** Liver images of H&E staining (the upper panel) and Oil-Red O staining (the lower panel), bar scale equals 100 μm; **(g)** fasting blood glucose level (n=5); **(h)** fasting serum insulin concentration (n=4); **(i)** homeostasis model assessment-estimated insulin resistance (HOMA-IR) (n=4). All values represent mean ± SD. \**p* < 0.05.

## References

1. Pi-Sunyer X. The medical risks of obesity. *Postgraduate medicine*. 2009; 121: 21-33.
2. Daniels SR. Complications of obesity in children and adolescents. *International journal of obesity*. 2009; 33 Suppl 1: S60-5.
3. Ogden CL, Carroll MD, Fryar CD, Flegal KM. Prevalence of Obesity Among Adults and Youth: United States, 2011-2014. *NCHS data brief*. 2015: 1-8.
4. Lee YH, Pratley RE. The evolving role of inflammation in obesity and the metabolic syndrome. *Current diabetes reports*. 2005; 5: 70-5.
5. Sun S, Ji Y, Kersten S, Qi L. Mechanisms of inflammatory responses in obese adipose tissue. *Annual review of nutrition*. 2012; 32: 261-86.
6. Vincent HK, Taylor AG. Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans. *International journal of obesity*. 2006; 30: 400-18.
7. Matsuzawa-Nagata N, Takamura T, Ando H, Nakamura S, Kurita S, Misu H, *et al*. Increased oxidative stress precedes the onset of high-fat diet-induced insulin resistance and obesity. *Metabolism: clinical and experimental*. 2008; 57: 1071-7.
8. Paglialunga S, Ludzki A, Root-McCaig J, Holloway GP. In adipose tissue, increased mitochondrial emission of reactive oxygen species is important for short-term high-fat diet-induced insulin resistance in mice. *Diabetologia*. 2015; 58: 1071-80.

9. Cui R, Gao M, Qu S, Liu D. Overexpression of superoxide dismutase 3 gene blocks high-fat diet-induced obesity, fatty liver and insulin resistance. *Gene therapy*. 2014; 21: 840-8.
10. Cho SJ, Jung UJ, Choi MS. Differential effects of low-dose resveratrol on adiposity and hepatic steatosis in diet-induced obese mice. *Br J Nutr*. 2012; 108: 2166-75.
11. Dong J, Zhang X, Zhang L, Bian HX, Xu N, Bao B, *et al*. Quercetin reduces obesity-associated ATM infiltration and inflammation in mice: a mechanism including AMPKalpha1/SIRT1. *J Lipid Res*. 2014; 55: 363-74.
12. Dinh CH, Szabo A, Camer D, Yu Y, Wang H, Huang XF. Bardoxolone methyl prevents fat deposition and inflammation in the visceral fat of mice fed a high-fat diet. *Chem Biol Interact*. 2015; 229: 1-8.
13. Ma Y, Gao M, Liu D. Chlorogenic acid improves high fat diet-induced hepatic steatosis and insulin resistance in mice. *Pharmaceutical research*. 2015; 32: 1200-9.
14. Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. *Am J Cardiol*. 2003; 91: 7A-11A.
15. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med*. 2010; 49: 1603-16.
16. Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr Neuropharmacol*. 2009; 7: 65-74.

17. Fearon IM, Faux SP. Oxidative stress and cardiovascular disease: novel tools give (free) radical insight. *J Mol Cell Cardiol.* 2009; 47: 372-81.
18. Romano AD, Serviddio G, de Matthaes A, Bellanti F, Vendemiale G. Oxidative stress and aging. *J Nephrol.* 2010; 23 Suppl 15: S29-36.
19. Basu A, Sanchez K, Leyva MJ, Wu M, Betts NM, Aston CE, *et al.* Green tea supplementation affects body weight, lipids, and lipid peroxidation in obese subjects with metabolic syndrome. *J Am Coll Nutr.* 2010; 29: 31-40.
20. Graffouillere L, Deschasaux M, Mariotti F, Neufcourt L, Shivappa N, Hebert JR, *et al.* Prospective association between the Dietary Inflammatory Index and mortality: modulation by antioxidant supplementation in the SU.VI.MAX randomized controlled trial. *The American journal of clinical nutrition.* 2016; 103: 878-85.
21. Suzuki-Sugihara N, Kishimoto Y, Saita E, Taguchi C, Kobayashi M, Ichitani M, *et al.* Green tea catechins prevent low-density lipoprotein oxidation via their accumulation in low-density lipoprotein particles in humans. *Nutr Res.* 2016; 36: 16-23.
22. de Gaetano G, Collaborative Group of the Primary Prevention P. Low-dose aspirin and vitamin E in people at cardiovascular risk: a randomised trial in general practice. Collaborative Group of the Primary Prevention Project. *Lancet.* 2001; 357: 89-95.
23. Steinhubl SR. Why have antioxidants failed in clinical trials? *Am J Cardiol.* 2008; 101: 14D-9D.

24. Alves NE, Enes BN, Martino HS, Alfenas Rde C, Ribeiro SM. Meal replacement based on Human Ration modulates metabolic risk factors during body weight loss: a randomized controlled trial. *Eur J Nutr.* 2014; 53: 939-50.
25. Mielgo-Ayuso J, Barrenechea L, Alcorta P, Larrarte E, Margareto J, Labayen I. Effects of dietary supplementation with epigallocatechin-3-gallate on weight loss, energy homeostasis, cardiometabolic risk factors and liver function in obese women: randomised, double-blind, placebo-controlled clinical trial. *Br J Nutr.* 2014; 111: 1263-71.
26. Rezk BM, Haenen GR, van der Vijgh WJ, Bast A. The antioxidant activity of phloretin: the disclosure of a new antioxidant pharmacophore in flavonoids. *Biochemical and biophysical research communications.* 2002; 295: 9-13.
27. Yang YC, Lii CK, Lin AH, Yeh YW, Yao HT, Li CC, *et al.* Induction of glutathione synthesis and heme oxygenase 1 by the flavonoids butein and phloretin is mediated through the ERK/Nrf2 pathway and protects against oxidative stress. *Free Radic Biol Med.* 2011; 51: 2073-81.
28. Ullen A, Fauler G, Bernhart E, Nussold C, Reicher H, Leis HJ, *et al.* Phloretin ameliorates 2-chlorohexadecanal-mediated brain microvascular endothelial cell dysfunction in vitro. *Free Radic Biol Med.* 2012; 53: 1770-81.
29. Vineetha VP, Soumya RS, Raghu KG. Phloretin ameliorates arsenic trioxide induced mitochondrial dysfunction in H9c2 cardiomyoblasts mediated via alterations in membrane permeability and ETC complexes. *Eur J Pharmacol.* 2015; 754: 162-72.

30. de Oliveira MR. Phloretin-induced cytoprotective effects on mammalian cells: A mechanistic view and future directions. *Biofactors*. 2016; 42: 13-40.
31. Najafian M, Jahromi MZ, Nowroznejad MJ, Khajeaian P, Kargar MM, Sadeghi M, *et al*. Phloridzin reduces blood glucose levels and improves lipids metabolism in streptozotocin-induced diabetic rats. *Molecular biology reports*. 2012; 39: 5299-306.
32. Hassan M, El Yazidi C, Malezet-Desmoulin C, Amiot MJ, Margotat A. Gene expression profiling of 3T3-L1 adipocytes exposed to phloretin. *The Journal of nutritional biochemistry*. 2010; 21: 645-52.
33. Shu G, Lu NS, Zhu XT, Xu Y, Du MQ, Xie QP, *et al*. Phloretin promotes adipocyte differentiation in vitro and improves glucose homeostasis in vivo. *The Journal of nutritional biochemistry*. 2014; 25: 1296-308.
34. Lin CC, Chu CL, Ng CS, Lin CY, Chen DY, Pan IH, *et al*. Immunomodulation of phloretin by impairing dendritic cell activation and function. *Food & function*. 2014; 5: 997-1006.
35. Chang WT, Huang WC, Liou CJ. Evaluation of the anti-inflammatory effects of phloretin and phlorizin in lipopolysaccharide-stimulated mouse macrophages. *Food chemistry*. 2012; 134: 972-9.
36. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985; 28: 412-9.

37. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. *The Journal of clinical investigation*. 2003; 112: 1796-808.
38. Bu L, Gao M, Qu S, Liu D. Intraperitoneal injection of clodronate liposomes eliminates visceral adipose macrophages and blocks high-fat diet-induced weight gain and development of insulin resistance. *AAPS J*. 2013; 15: 1001-11.
39. Anstee QM, Goldin RD. Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. *International journal of experimental pathology*. 2006; 87: 1-16.
40. Winzell MS, Ahren B. The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. *Diabetes*. 2004; 53 Suppl 3: S215-9.
41. Huang WC, Wu SJ, Tu RS, Lai YR, Liou CJ. Phloretin inhibits interleukin-1beta-induced COX-2 and ICAM-1 expression through inhibition of MAPK, Akt, and NF-kappaB signaling in human lung epithelial cells. *Food & function*. 2015.
42. Huang WC, Chang WT, Wu SJ, Xu PY, Ting NC, Liou CJ. Phloretin and phlorizin promote lipolysis and inhibit inflammation in mouse 3T3-L1 cells and in macrophage-adipocyte co-cultures. *Molecular nutrition & food research*. 2013; 57: 1803-13.
43. Kawano Y, Cohen DE. Mechanisms of hepatic triglyceride accumulation in non-alcoholic fatty liver disease. *J Gastroenterol*. 2013; 48: 434-41.

44. Zuo AR, Yu YY, Shu QL, Zheng LX, Wang XM, Peng SH, *et al.* Hepatoprotective effects and antioxidant, antityrosinase activities of phloretin and phloretin isonicotinyl hydrazone. *Journal of the Chinese Medical Association : JCMA.* 2014; 77: 290-301.
45. Schulze C, Bangert A, Kottra G, Geillinger KE, Schwanck B, Vollert H, *et al.* Inhibition of the intestinal sodium-coupled glucose transporter 1 (SGLT1) by extracts and polyphenols from apple reduces postprandial blood glucose levels in mice and humans. *Molecular nutrition & food research.* 2014; 58: 1795-808.
46. Skopec MM, Green AK, Karasov WH. Flavonoids have differential effects on glucose absorption in rats (*Rattus norvegicus*) and American robins (*Turdus migratorius*). *J Chem Ecol.* 2010; 36: 236-43.
47. Ehrenkranz JR, Lewis NG, Kahn CR, Roth J. Phlorizin: a review. *Diabetes/metabolism research and reviews.* 2005; 21: 31-8.



## CHAPTER 3

### BITC AND S-CARVONE RESTRAIN HIGH-FAT DIET-INDUCED OBESITY AND AMELIORATE HEPATIC STEATOSIS AND INSULIN RESISTANCE<sup>#</sup>

---

<sup>#</sup>Sary Alsanea and Dexi Liu (2017). BITC and S-Carvone Restrain High-fat Diet-induced Obesity and Ameliorate Hepatic Steatosis and Insulin Resistance. *Pharmaceutical Research*. 2017; 34: 2241-9. Reprinted here with publisher permission (License # 4154270258007)

## **Abstract**

**Purpose:** To investigate the preventative activity of benzyl isothiocyanate and *S*-carvone against high-fat diet-induced obesity and metabolic complications.

**Methods:** Ten-week-old C57BL/6 male mice were fed a high-fat diet and injected intraperitoneally twice per week with benzyl isothiocyanate, *S*-carvone, or vehicle for 8 weeks. The body weight, food intake, and body composition were monitored, and glucose tolerance and insulin tolerance tests were performed at the end of the experiment. Serum and tissue samples were studied using serum biochemistry, histological, and gene expression analysis to define the effects of benzyl isothiocyanate and *S*-carvone treatments on lipid and glucose metabolism and inflammatory responses.

**Results:** Benzyl isothiocyanate and *S*-carvone blocked high-fat diet-induced weight gain, fat accumulation in the liver, and insulin resistance. The beneficial effects were found to be associated with an improvement of expression of macrophage marker genes in white adipose tissue including *F4/80*, *Cd11b*, *Cd11c*, *Cd206*, and *Tnf- $\alpha$* , and reduced expression of genes (*Ppar $\gamma$ 2*, *Scd1*, *Cd36*) responsible for lipid synthesis and transport in the liver.

**Conclusion:** Benzyl isothiocyanate and *S*-carvone block high-fat diet-induced obesity and metabolism disorders and can be considered for management of obesity epidemics that affects about 36% adults and 17% children in the USA.

**Key words:** Obesity; inflammation; BITC; *S*-carvone; antioxidant

## **Abbreviations**

ACC: Acetyl-CoA carboxylase

AUC: Area under the curve

BAT: Brown adipose tissue

BITC: Benzyl isothiocyanate

DMSO: Dimethyl sulfoxide

EWAT: Epididymal white adipose tissues

FAS: Fatty acid synthase

FFA: Free fatty acid

GTT: Glucose tolerance test

HFD: High-fat diet

HOMA-IR: Homeostatic model assessment of insulin resistance

ITT: Insulin tolerance test

IWAT: Inguinal white adipose tissues

PPAR $\gamma$ : Peroxisome proliferator-activated receptor- $\gamma$

PWAT: Perirenal white adipose tissues

SCD: Stearoyl-CoA desaturase

SREBP: Sterol regulatory element-binding protein

TG: Triglyceride

TNF- $\alpha$ : Tumor necrosis factor  $\alpha$

## **Introduction**

The obesity prevalence in the United States has been significantly increasing in the past five years reaching 36% for adults and 17% for youth (1). Obesity is a risk factor for several common diseases including cardiovascular diseases, type 2 diabetes, hypertension, hyperlipidemia, nonalcoholic fatty liver disease (NAFLD, or hepatic steatosis), kidney diseases, osteoarthritis, depression, and some types of cancer (2). A 2008 estimate of medical costs for obesity in the United States is \$147 billion. The annual productive costs of obesity-related absenteeism range between \$3.38 billion and \$6.38 billion (3). Thus, an indispensable strategy is needed to prevent and control obesity epidemics and its associated complications.

In obesity pathogenesis, low-grade chronic inflammation plays an important role in adipose tissue remodeling and expansion (4). This includes macrophage infiltration into white adipose tissue, pro-inflammatory cytokine release, and dysregulation of adipokine release (5). Previous studies have shown that reactive oxygen species (ROS), an indicator of oxidative stress, serve as a trigger for inflammation in adipose tissue (6, 7). We have previously reported that suppression of ROS activity by overexpression of endogenous antioxidant enzyme or by use of antioxidants was able to block high-fat diet (HFD)-induced weight gain and its metabolic complications (8, 9). These earlier studies have provided a strong indication that antioxidants could be an effective agent to prevent obesity and obesity-associated metabolic disorders. In the current study, we selected two different antioxidants, benzyl isothiocyanate (BITC) and *S*-carvone, and explored their activities in blocking HFD-induced obesity in a mouse model.

BITC (**Fig. 3.1a**) is a natural product found in Cruciferous vegetables such as cabbage, broccoli, turnip, rapeseeds, radish and others in the cabbage family. It is produced by hydrolysis of glucotropaeolin under the influence of myrosinase (10). In addition to its antioxidant activity, BITC is also known for its anti-cancer activity shown via inhibition of P450 mediated-carcinogen activation *in vivo* and induction of cell cycle arrest *in vitro* (10-12). BITC has been shown to inhibit lipopolysaccharide-induced inflammatory response in Raw 264.7 murine macrophage cell line and suppressed 12-O-tetradecanoylphorbol 13-acetate-induced ear edema formation in mice (13). The antioxidant activity of BITC involves inhibition of NADPH oxidase to reduce superoxide generation and activation of P450 phase II enzymes (11, 14).

*S*-carvone (**Fig. 3.1a**), also called D-carvone or (+)-carvone, belongs to terpenoids family of natural components of essential oils such as caraway, spearmint, and dill. Carvone has *S* and *R* isomers, and the *S* form is the main isomer in caraway seed and used in food and flavor industry (15). The antioxidant activity of *S*-carvone has been demonstrated *in vitro* systems (16-18). Studies have shown that *S*-carvone has antibacterial, antifungal, anticancer, immunomodulatory and anti-inflammatory activities (15, 19-21). Mechanistically, *S*-carvone induces glutathione *S*-transferase (22) which is downregulated by HFD (23).

We demonstrate here that both BITC and *S*-carvone are effective in blocking HFD-induced obesity, insulin resistance and fatty liver via a mechanism that involves inhibition of HFD-induced inflammation and down-regulation of mRNA level of genes responsible for lipogenesis.

## **Materials and Methods**

*Materials.* BITC and *S*-carvone were purchased from Sigma-Aldrich (St. Louis, MO). HFD (60% kJ/fat, 20% kJ/protein, and 20% kJ/carbohydrate) was from Bio-serv (Frenchtown, NJ). The insulin assay kits were obtained from Mercodia AB (Winston-Salem, NC) and glucometer and blood test strips were obtained from NIPRO Diagnostics™ (Fort Lauderdale, FL). The Humulin® insulin was purchased from Eli Lilly (Indianapolis, IN). The Infinity™ kit for triglycerides (TG) was from Fisher Diagnostics (Middletown, VA). The non-esterified fatty acids kits were obtained from Wako Bioproducts (Richmond, VA). The RNeasy® Lipid Tissue Mini Kit was purchased from QIAGEN® (Hilden, Germany). The First-stand cDNA Synthesis kit was obtained from Origene® (Rockville, MD). The PerfeCta® SYBR® Green FastMix® ROX™ was purchased from Quanta Biosciences (Gaithersburg, MD).

*Animals and Animal Treatments.* C57BL/6 mice (male, ten weeks old) were from Charles River Laboratories (Wilmington, MA). All procedures performed on mice were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia, Athens, Georgia. To assess BITC and *S*-carvone activity in blocking HFD-induced obesity and obesity-associated metabolic disorders, mice were grouped randomly (n=5) and fed a HFD, and received twice weekly injection (*i.p.*) of BITC (12.5 mg/kg), *S*-carvone (175 mg/kg), or carrier solution of dimethyl sulfoxide (DMSO) for 8 weeks. The volume of each injection was fixed at 20 µl per mouse. Mouse body weight was monitored weekly and the body composition was determined using EchoMRI-100™

from the Echo Medical System (Houston, TX). All mice were euthanized using the method of carbon dioxide inhalation followed by cervical dislocation.

*Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT).* At the beginning of 8<sup>th</sup> week, mice were fasted for six hours and received *i.p* injection of glucose (1.5 g/kg) dissolved in saline. About 5  $\mu$ l of blood from each mouse was collected from the tail and glucose level was determined at time 0, 30, 60, and 120 min using a glucometer. ITT was performed at the end of the 8<sup>th</sup> week. Mice were fasted for four hours and injected *i.p.* with insulin (0.75 U/kg). The blood glucose level was determined applying the same schedule as GTT. Animals were put back on HFD after each procedure.

*Histological Examination by Hematoxylin and Eosin (H&E) Staining.* Liver, epididymal white adipose tissue (eWAT), perirenal white adipose tissues (pWAT), inguinal white adipose tissues (iWAT), and brown adipose tissue (BAT) were collected from animals and fixed in 10% formalin at the end of the experiment. The fixed tissues were dehydrated, embedded in paraffin, sectioned at 6  $\mu$ m in thickness, and stained with H&E following the manufacturer's instructions. Tissue sections were examined, and photo images were taken under an optical microscope using the Nikon's NIS-Elements imaging software (Melville, NY).

*Determination of Hepatic Triglyceride Level.* For lipid extraction, liver samples (100 mg) were homogenized in 1 ml of PBS. Tissue homogenate (300  $\mu$ l) was mixed with 5 ml of chloroform/methanol mixture (3:2, v/v ratio) and incubated overnight at 4°C. The

mixture was centrifuged at 2,000 rpm for 10 min and supernatant (organic phase) was transferred to a new tube, dried, and dissolved in 2% Triton-X100. Triglyceride concentration was determined using the Infinity™ kit following manufacturer's instructions.

*Determination of Insulin Resistance and Serum Lipid Level.* Three days after ITT, mice were fasted for six hours and euthanized by CO<sub>2</sub> inhalation. Whole blood was collected from each animal and serum concentrations of insulin were determined by an ELISA following the procedure provided by the supplier of the insulin analysis kit. Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was determined using the formula: [fasting insulin (ng/ml) x fasting blood glucose (mg/dl)/405] (24). Serum concentrations of triglyceride and free fatty acids were determined using the procedure provided by the supplier of the lipid analysis kits.

*Gene Expression Analysis.* Total RNAs from mouse adipose tissue and liver were isolated using RNeasy® Lipid Tissue Mini Kit. The complementary DNA (cDNA) was synthesized using First-stand cDNA synthesis kit. Real-time quantitative polymerase chain reaction (qPCR) was performed using a SYBR Green as a detector with a StepOnePlus system (Applied Biosystems). Data was analyzed by the  $\Delta\Delta C_T$  method using *Gapdh* mRNA as the internal control. All primer sequences used are listed in **Table 3.1**.



*Statistical Analysis.* Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparisons test using the plotting software (Prism) from GraphPad Inc. (La Jolla, CA). A value of  $P < 0.05$  was considered statistically significant, and each data point represents the mean  $\pm$  standard deviation (SD) or standard error of the mean (SEM).

## Results

### *BITC and S-Carvone Blocked HFD-induced Weight Gain.*

C57BL/6 mice were fed a HFD and injected twice weekly with BITC (12.5 mg/kg), *S*-carvone (175 mg/kg) (*i.p.*), or the vehicle for 8 weeks. Results in **Figure 3.1b** show smaller size of mice with BITC and *S*-carvone treatment compared to the DMSO treated control. At the end of week 8, the average body weight of BITC-treated and *S*-carvone-treated mice were  $29.3 \pm 3.5$  g and  $30.9 \pm 1.1$  g, respectively, compared to control mice at  $40.2 \pm 3.1$  g (**Fig. 3.1c**). The recognized difference in body weight of treated mice was due to the difference in fat mass and no difference was seen in lean mass (**Fig. 3.1d**). The average food intake was the same (**Fig. 3.1e**) between BITC and *S*-carvone treated and control mice.

The apparent size (**Fig. 3.2a**) and average weight (**Fig. 3.2b**) of eWAT, pWAT, iWAT and BAT were examined and compared between the treated and control mice. With the exception of BAT, the WATs of control animals are significantly larger than those of BITC and *S*-carvone treated mice. The images of H&E staining show miniature adipocytes and lack of crown-like structure in BITC and *S*-carvone treated mice compared to that of DMSO treated control mice (**Fig. 3.2c**). Lower lipid content in BAT

compared to control (**Fig. 3.2c**, the right panel) was also seen. The average diameter of adipocytes in BITC and *S*-carvone treated animals was at 61.7-74%, 70.6-86.7%, 58.1-69.2% of the size of control animals in eWAT, pWAT, and iWAT, respectively (**Fig. 3.2d**). These results demonstrate that BITC and *S*-carvone treatment suppressed lipid accumulation in adipose tissues.

#### *BITC and S-Carvone Treatment Reduces Inflammation in WAT.*

Diet-induced obesity is associated with inflammation in adipose tissue with characteristics of accumulation of type-1 macrophages and crown-like structure (**Figs 3.2c**). To extend the study of the BITC and *S*-carvone effects on diet-induced inflammation in adipose tissue at the molecular level, we extracted total RNA from WAT and determined the mRNA levels of macrophage-specific marker genes including *F4/80*, *Cd11b*, *Cd11c*, and proinflammatory cytokine *Tnf- $\alpha$* . In addition, mRNA levels of the anti-inflammation gene (*Cd206*) and that of leptin gene were also examined. Results in **Figure 3a** show decreased mRNA levels of *F4/80*, *Cd11b* and *Cd11c* gene in eWAT of *S*-carvone treated mice. An enhanced expression of anti-inflammatory *Cd206* gene was seen in eWAT of BITC treated mice compared to that of control. *Tnf- $\alpha$*  and *leptin* gene expression was lower in iWAT of *S*-carvone and BITC treated mice, respectively. In addition to lack of crown-like structure in adipose tissue, a landmark of macrophage infiltration, in BITC and *S*-carvone treated animals shown in **Figure 3.2c**, these results confirm that BITC and *S*-carvone suppress HFD-induced macrophage infiltration and inflammation in WAT.

*BITC and S-Carvone Prevent HFD-induced Hepatic Steatosis.*

Hepatic steatosis or fatty liver is commonly associated with obesity (25). To evaluate the BITC and *S*-carvone impact on hepatic fat accumulation, lipid level in the liver was examined. The images of H&E staining showed a high level of hepatic lipid level in control mice, but not in those treated with BITC and *S*-carvone (**Fig. 3.4a**). This was consistent with results of lipid measurements. Results in **Figure 4b** show that hepatic triglyceride is at  $21.5 \pm 4$  mg/g level in control mice compared to that  $15.8 \pm 0.7$  and  $16.2 \pm 1.3$  mg/g of BITC and *S*-carvone treated mice, respectively. Livers of control animals weigh about  $1.5 \pm 0.3$  g compared to  $1.1 \pm 0.1$  g of treated animals (**Fig. 3.4c**). There was no significant difference observed in serum levels of triglycerides and free fatty acids (**Fig. 3.4d-e**).

To understand the protective mechanism of BITC and *S*-carvone against HFD-induced fatty liver, mRNA levels of genes responsible for hepatic lipid metabolism were examined. Both BITC and *S*-carvone treatment resulted in expression reduction of peroxisome proliferator-activated receptor- $\gamma$  2 gene (*Ppar- $\gamma$ 2*) coding for *de novo* lipogenesis transcriptional factor, fatty acid transporter gene (*Cd36*), and stearoyl-CoA desaturase 1 gene (*Scd1*) (**Fig. 3.5**). Moreover, *S*-carvone down regulated mRNA levels of genes encoding *de novo* lipogenesis transcriptional factor sterol regulatory element-binding protein 1 c (*Srebp1c*) and genes responsible for fatty acids synthesis including acetyl-CoA carboxylase 1 (*Acc1*) and fatty acid synthase (*Fas*) (**Fig. 3.5**). These data suggest that BITC and *S*-carvone prevented hepatic lipid accumulation through suppression of lipid synthesis and accumulation.

### *BITC and S-Carvone Restrained Diet-induced Hyperglycemia and Insulin Resistance.*

Obesity is strongly associated with insulin resistance and hyperglycemia (26). To evaluate the impacts of BITC and *S*-carvone on glucose homeostasis, non-fasting blood glucose level was determined at the end of 8-week treatments. The blood glucose concentration in BITC-treated mice showed  $167.6 \pm 21.1$  mg/dL and  $161.8 \pm 5.4$  mg/dL in *S*-carvone treated mice, compared to control mice at  $223.8 \pm 22.1$  mg/dL (**Fig. 3.6a**). In addition, BITC and *S*-carvone treated mice were more tolerant to glucose as revealed by GTT (**Fig. 3.6b**) and a plot of the area under the curve (**Fig. 3.6c**). Results from ITT showed that BITC and *S*-carvone treated mice had a significantly higher response to insulin injection (**Fig. 3.6d**). The area under the curve of ITT confirmed the improvement of insulin sensitivity due to BITC and *S*-carvone treatment (**Fig. 3.6e**). HFD-induced hyperinsulinemia and insulin resistance were blocked by BITC and *S*-carvone (**Figs. 3.6f-g**). These data demonstrate the protective activity of BITC and *S*-carvone against HFD-induced hyperglycemia, hyperinsulinemia, and insulin resistance.

### **Discussion**

Inflammation is an essential step toward the development of obesity and obesity-associated metabolic diseases. We have previously shown that blockade of HFD-induced inflammation is a successful strategy to repress the weight gain and improve obesity-associated complications (27-30). In this study, we examined the protective activity of BITC and *S*-carvone against HFD-induced obesity, fatty liver, and insulin resistance. Our results demonstrate that twice-weekly injection intraperitoneally of BITC and *S*-carvone restrains HFD-induced weight gain (**Figs. 3.1-2**), hepatic lipid accumulation (**Fig. 3.4**),

and improve glucose homeostasis (**Fig. 3.6**). These beneficial effects were directly linked to lower expression of macrophage marker genes in adipose tissue (**Fig. 3.3**) as well as those responsible for hepatic *de novo* lipogenesis (**Fig. 3.5**).

Our conclusion is in agreement with previous studies that showed a potent effect of BITC and *S*-carvone against inflammatory diseases (11, 13, 21). BITC has been shown to reduce lipopolysaccharide-induced secretion of inflammatory cytokines (e.g. IL-1 $\beta$ , TNF- $\alpha$ , and IL-6) and their mRNA levels in Raw264.7 macrophage cell line in a dose-dependent manner involving inhibition of NF $\kappa$ B, Akt, and ERK1/2 signaling pathways (13). In the same study, the authors also demonstrated that BITC inhibits ear edema inflammation and increases iNOS and COX-2 protein levels in mice. Waterman *et al.* (31) recently showed that *Moringa oleifera* extract, which is rich in isothiocyanates (BITC precursor), suppressed body weight gain and food intake in a diet-induced obesity animal model. The conclusion derived from the study serves as additional evidence in support the activity of BITC in blocking HFD-induced obesity as shown here in the current study.

*S*-carvone has been shown *in vitro* for its antioxidant activity in several studies and reduced pro-oxidants induced stress (16, 18). In addition, an *in vivo* study has shown that daily *S*-carvone treatment for 30 days protected rats from developing hypertension and restored vitamin-C, vitamin-E, and glutathione levels (17). Moreover, *S*-carvone had the highest activity in inducing glutathione *S*-transferase compared with other carvone derivatives or isomers (22).

Hepatic steatosis is associated with obesity where excess fat is accumulated in the liver due to induction of *de novo* lipogenesis and enhanced expression of fatty acid

transporters in hepatocytes. Feeding mice with HFD for 2 weeks has been reported to induce fatty liver coinciding with elevation of mRNA levels of hepatic *Ppar $\gamma$*  and *Cd36* in mice (32). In agreement, our data showed that both BITC and *S*-carvone significantly blocked HFD-induced up-regulation of *Ppar $\gamma$*  and *Cd36* (**Fig. 3.5**) and *Scd-1*, a gene encoding stearoyl-CoA desaturase-1 that controls the rate-limiting step in monounsaturated fatty acid formation. The enzyme activity of stearoyl-CoA desaturase-1 has been shown to be elevated in humans with fatty liver disease (33). *S*-carvone but not BITC reduced the expression of the genes involving lipid synthesis including *Srebp1c*, *Acc1*, and *Fas* (**Fig. 3.5**). A previous study using *Moringa oleifera* extract has also demonstrated suppression of lipid accumulation in mouse liver with no change of serum concentrations of triacylglycerol (31), in agreement with our results (**Fig. 3.4**).

Inflammation of the adipose tissue is known to cause insulin resistance and glucose intolerance (34, 35). Insulin resistance increases gluconeogenesis and decrease blood glucose absorption, leading to glucose intolerance. Many studies have shown that antioxidants are effective in blocking inflammation, increasing insulin sensitivity and improving glucose homeostasis (9, 30, 31). Consistent with these studies, our results show that BITC and *S*-carvone treatment alleviated insulin resistance and improved glucose intolerance in HFD-fed mice (**Fig. 3.6**) by suppressing TNF $\alpha$  gene expression and macrophage activation in adipose tissue (**Fig. 3.3**).

In conclusion, the current study demonstrates the preventative activity of BITC and *S*-carvone against HFD-induced obesity and obesity-associated metabolic complications. BITC and *S*-carvone effects are achieved by the alteration in inflammatory and lipogenesis gene expression in adipose and liver tissues, respectively. These results

suggest that BITC and *S*-carvone could be considered protecting agents against diet-induced obesity and metabolic disorders.

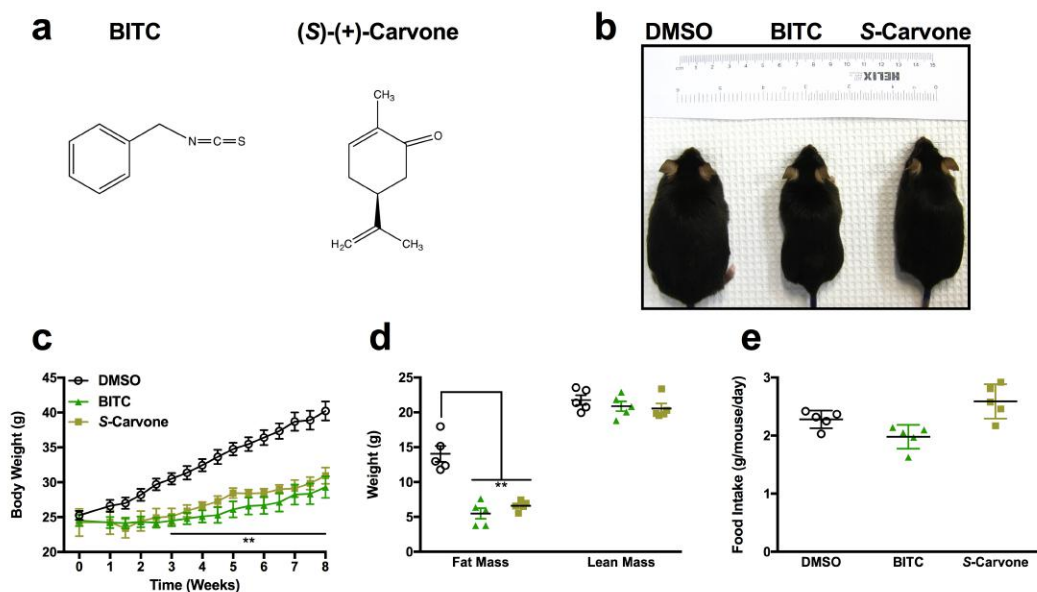
**Acknowledgments:** This work was supported in part by NIH grant RO1HL098295. Mr. Sary Alsanea is supported by a scholarship from the King Saud University, Riyadh, Saudi Arabia. We thank Dr. Megan Morgan for proofreading and editing the manuscript.

**Conflict of Interest:** The authors declare no conflict of interest.

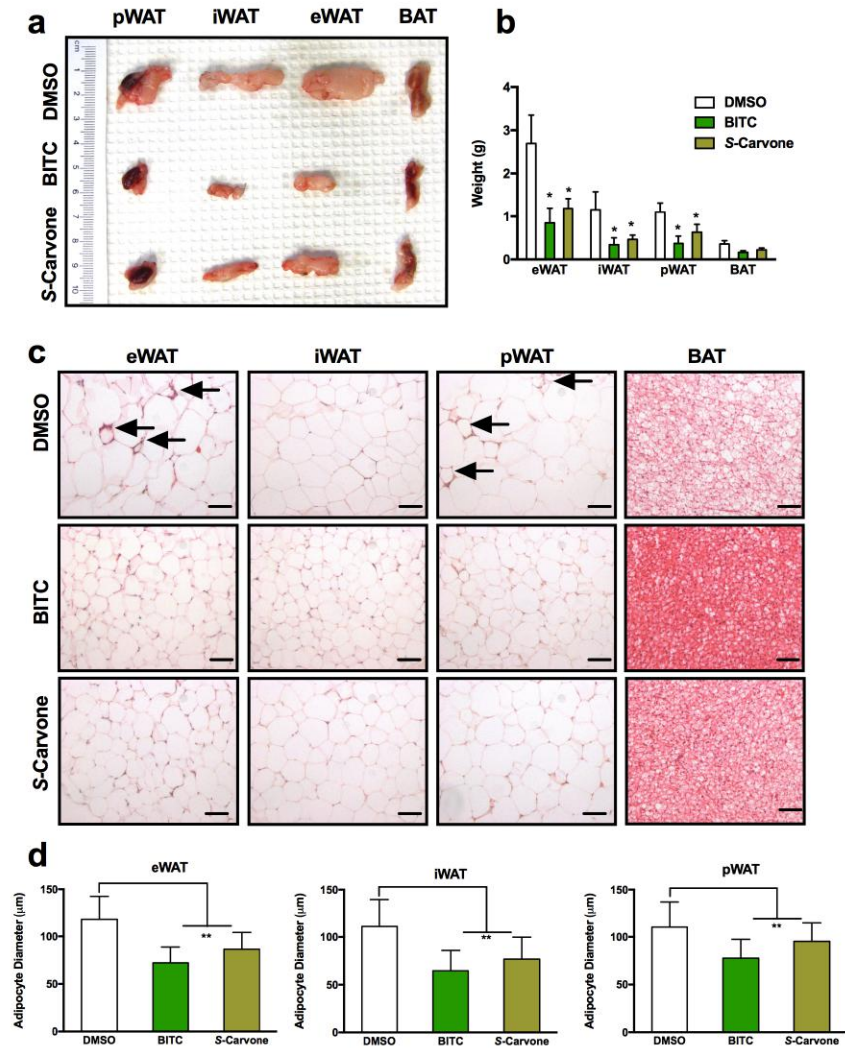
**Table 3.1: Primers used for gene expression analysis by real time PCR**

<b>Gene</b>	<b>Forward Primers</b>	<b>Reverse Primers</b>
<i>F4/80</i>	5' CCCAGTGCCTTACAGAGTG 3'	5' GTGCCAGAGTGGATGTCT 3'
<i>Cd68</i>	5' CCATCCTTCACGATGACACCT 3'	5' GGCAGGGTTATGAGTGACAGTT 3'
<i>Cd11b</i>	5' ATGGACGCTGATGGCAATACC 3'	5' TCCCATTACAGTCTCCCA 3'
<i>Cd11c</i>	5' CTGGATAGCCTTTCTTCTGCTG 3'	5' GCACACTGTGTCCGAACTCA 3'
<i>Cd206</i>	5' CTCTGTTTCAGCTATTGGACGC 3'	5' CGGAATTTCTGGGATTCAGCTTC 3'
<i>Tnf- α</i>	5' CCCTCACACTCAGATCATCTTCT 3'	5' GCTACGACGTGGGCTACAG 3'
<i>Leptin</i>	5' GAGACCCCTGTGTTCGGTTC 3'	5' CTGCGTGTGTGAAATGTCATTG 3'
<i>Pparγ2</i>	5' TCGCTGATGCACTGCCTATG 3'	5' GAGAGGTCCACAGAGCTGATT 3'
<i>Cd36</i>	5' CCTTAAAGGAATCCCCGTGT 3'	5' TGCATTTGCCAATGTCTAGC 3'
<i>Srebp1c</i>	5' CCCTGTGTGTACTGGCCTTT 3'	5' TTGCGATGTCTCCAGAAGTG 3'
<i>Acc1</i>	5' GCCTCTTCTGACAAACGAG 3'	5' TGA CTGCCGAAACATCTCTG 3'
<i>Fas</i>	5' GGAGGTGGTG ATAGCCGGTAT 3'	5' TGGGTAATCCATAGAGCCCAG 3'
<i>Scd1</i>	5' TTCTTACACGACCACCACCA 3'	5' CCGAAGAGGCAGGTGTAGAG 3'
<i>Gapdh</i>	5' AGGTCGGTGTGAACGGATTTG 3'	5' TG TAGACCATGTAGTTGAGGTCA 3'



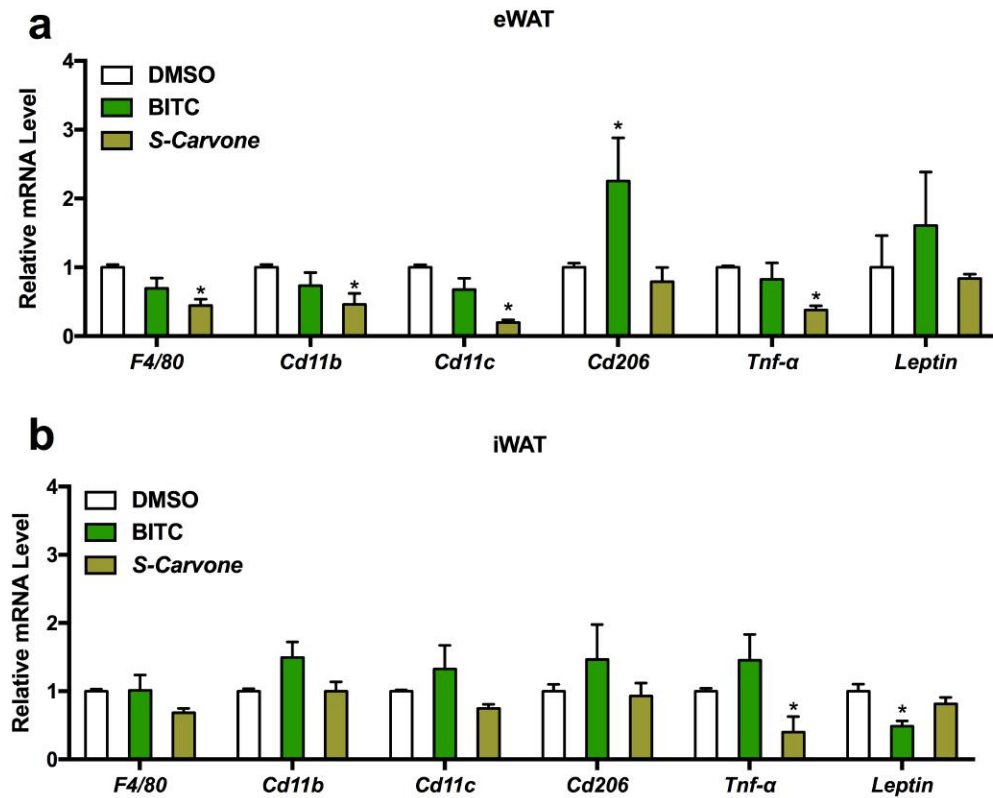


**Figure 3.1. BITC and S-carvone inhibit body weight gain in mice fed an HFD.** Ten-week-old C57BL/6 male mice were fed an HFD and injected *i.p.* twice weekly with benzyl isothiocyanate (12.5 mg/kg), *S*-carvone (175 mg/kg), or vehicle (DMSO) for 8 weeks. **(a)** Chemical structures of benzyl isothiocyanate (left) and *S*-(+)-carvone (right); **(b)** representative picture of the mice at the end of the experiment; **(c)** growth curve; **(d)** body composition at week 8; and **(e)** average food intake. Values represent mean  $\pm$  SEM, \*  $p < 0.05$  (n=5).

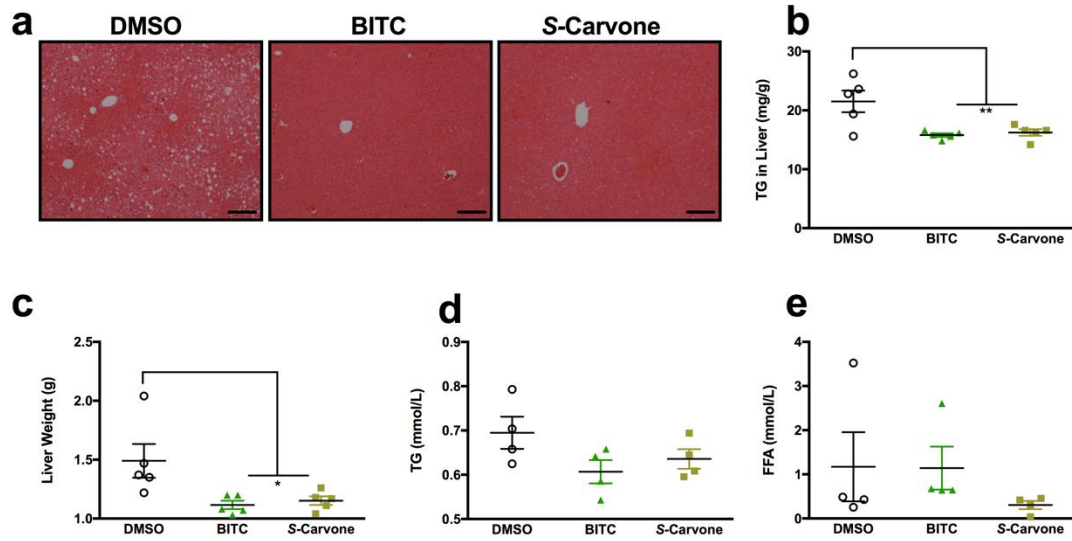


**Figure 3.2. Impacts of BITC and S-carvone treatment on adipose tissues of animals.**

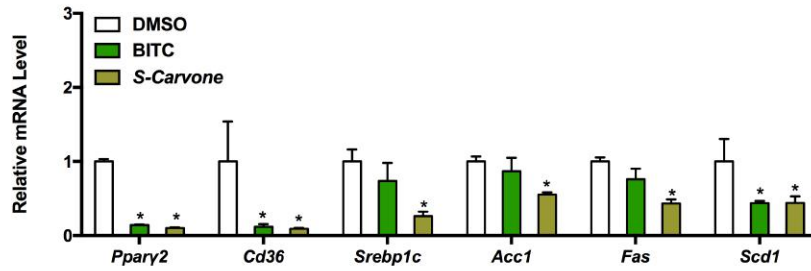
Mice were fed an HFD continuously for 8 weeks, and injected *i.p.* twice weekly with benzyl isothiocyanate (12.5 mg/kg), S-carvone (175 mg/kg), or vehicle DMSO for 8 weeks. Animals were sacrificed at the end of experiment and adipose tissues were collected and examined (n=5). **(a)** Photo images of pWAT, iWAT, eWAT, and BAT from treated and control animals; **(b)** weights of eWAT, iWAT, pWAT, and BAT; **(c)** photo-images of H&E staining of eWAT, iWAT, pWAT and BAT, black arrows indicate crown-like structure (CLS), scale bar: 100 µm; **(d)** average diameter of adipocytes in different WATs (n= 300). Values are mean ± SD. \* $p < 0.05$ , \*\*  $p < 0.01$ .



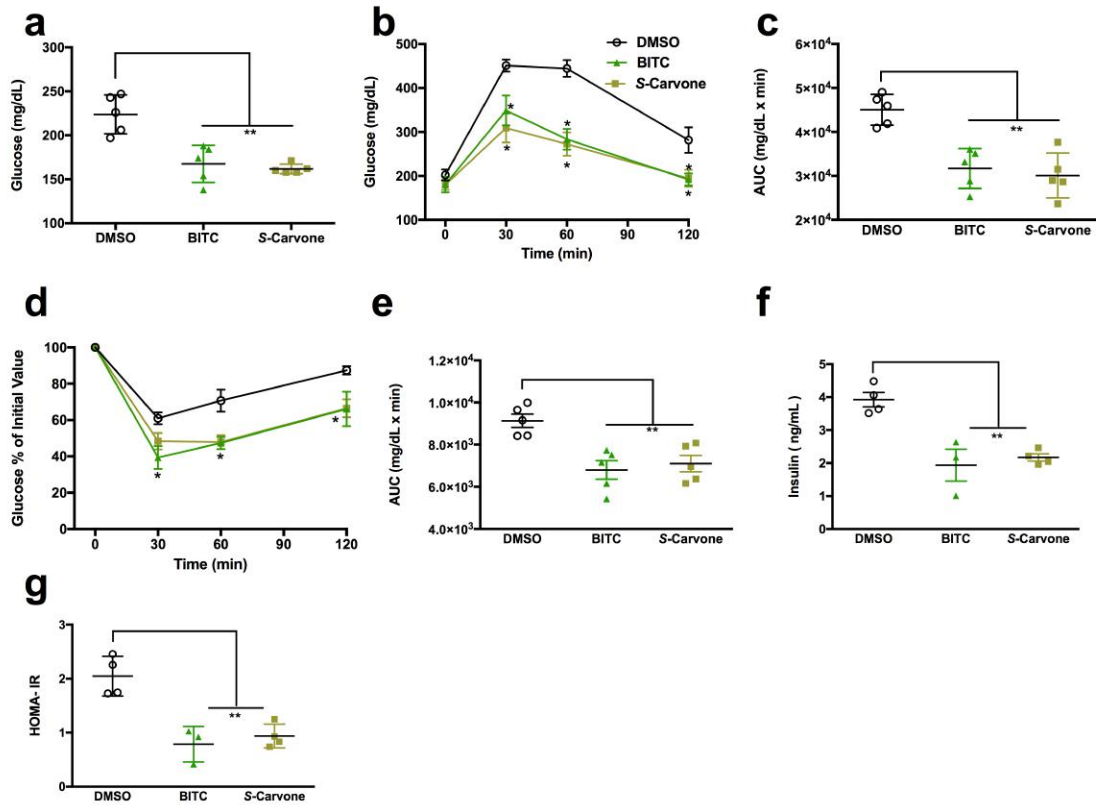
**Figure 3.3. BITC and S-carvone suppressed HFD-induced inflammation.** At the end of experiments, mice were sacrificed and total RNAs were extracted from eWAT or iWAT and gene expression was determined by real-time quantitative PCR. (a) mRNA levels of macrophage marker genes, *Tnf- $\alpha$* , and *leptin* genes in eWAT; and (b) mRNA levels of selected genes in iWAT. All values represent mean  $\pm$  SEM, \* $p < 0.05$  (n=4).



**Figure 3.4. BITC and S-carvone blocked HFD-induced fatty liver.** Mice were fed HFD during the entire study, and received twice weekly *i.p.* injections of benzyl isothiocyanate (12.5 mg/kg), *S*-carvone (175 mg/kg), or DMSO. Mice were sacrificed at the end of the experiments and liver samples were collected. (a) Photo images of H&E staining of liver sections photographed at x100 magnification, scale bars: 100  $\mu$ m; (b) triacylglycerol level in the liver (n=5); (c) liver weight (n=5); (d) serum levels of triacylglycerol (n=4); and (e) serum levels of free fatty acids (n=4). Each data point represents mean  $\pm$  SEM, \* $p$  < 0.05.



**Figure 3.5. Impacts of BITC and S-carvone treatment on mRNA levels of genes responsible for hepatic lipid metabolism.** Total RNA was isolated from liver tissue, and relative mRNA level of selected genes were determined by qPCR. All values represent mean  $\pm$  SEM, \* $p < 0.05$  (n=4).



**Figure 3.6. BITC and S-carvone improved glucose homeostasis and hyperinsulinemia in HFD-fed mice.** (a) Non-fasting blood glucose level (n=5); (b) profile of serum glucose concentration after *i.p.* glucose injection (1.5 g/kg) (n=5); (c) area under the curve of glucose tolerance test; (d) time-dependent percentage of initial glucose concentration in blood after *i.p.* insulin injection (0.75 U/kg) (n=5); (e) area under the curve of insulin tolerance test; (f) fasting serum insulin concentration (n=4); (g) homeostasis model assessment-estimated insulin resistance (HOMA-IR) (n=4). Each data point represents mean  $\pm$  SEM, \*  $p < 0.05$ , \*\*  $p < 0.01$ .

## References

1. Ogden CL, Carroll MD, Fryar CD, Flegal KM. Prevalence of Obesity Among Adults and Youth: United States, 2011-2014. NCHS data brief. 2015: 1-8.
2. Pi-Sunyer X. The medical risks of obesity. *Postgraduate medicine*. 2009; 121: 21-33.
3. Hammond RA, Levine R. The economic impact of obesity in the United States. *Diabetes Metab Syndr Obes*. 2010; 3: 285-95.
4. Monteiro R, Azevedo I. Chronic inflammation in obesity and the metabolic syndrome. *Mediators Inflamm*. 2010; 2010.
5. Choe SS, Huh JY, Hwang IJ, Kim JI, Kim JB. Adipose Tissue Remodeling: Its Role in Energy Metabolism and Metabolic Disorders. *Front Endocrinol (Lausanne)*. 2016; 7: 30.
6. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, *et al*. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest*. 2004; 114: 1752-61.
7. Le Lay S, Simard G, Martinez MC, Andriantsitohaina R. Oxidative stress and metabolic pathologies: from an adipocentric point of view. *Oxid Med Cell Longev*. 2014; 2014: 908539.

8. Cui R, Gao M, Qu S, Liu D. Overexpression of superoxide dismutase 3 gene blocks high-fat diet-induced obesity, fatty liver and insulin resistance. *Gene Ther.* 2014; 21: 840-8.
9. Ma Y, Gao M, Liu D. Chlorogenic acid improves high fat diet-induced hepatic steatosis and insulin resistance in mice. *Pharmaceutical research.* 2015; 32: 1200-9.
10. Higdon JV, Delage B, Williams DE, Dashwood RH. Cruciferous vegetables and human cancer risk: epidemiologic evidence and mechanistic basis. *Pharmacol Res.* 2007; 55: 224-36.
11. Miyoshi N, Takabayashi S, Osawa T, Nakamura Y. Benzyl isothiocyanate inhibits excessive superoxide generation in inflammatory leukocytes: implication for prevention against inflammation-related carcinogenesis. *Carcinogenesis.* 2004; 25: 567-75.
12. Hecht SS. Inhibition of carcinogenesis by isothiocyanates. *Drug Metab Rev.* 2000; 32: 395-411.
13. Lee YM, Seon MR, Cho HJ, Kim JS, Park JH. Benzyl isothiocyanate exhibits anti-inflammatory effects in murine macrophages and in mouse skin. *J Mol Med (Berl).* 2009; 87: 1251-61.
14. Wu X, Zhou QH, Xu K. Are isothiocyanates potential anti-cancer drugs? *Acta Pharmacol Sin.* 2009; 30: 501-12.



15. de Carvalho CC, da Fonseca MMR. Carvone: Why and how should one bother to produce this terpene. *Food Chemistry*. 2006; 95: 413-22.
16. Elmastaş M, Dermirtas I, Isildak O, Aboul - Enein HY. Antioxidant Activity of S - Carvone Isolated from Spearmint (*Mentha Spicata* L. Fam Lamiaceae). *Journal of liquid chromatography & related technologies*. 2006; 29: 1465-75.
17. Rajeshwari T, Raja B. Antioxidant and free radical scavenging effect of D-carvone in hypertensive rats. In vivo and in vitro study. *International Letters of Natural Sciences*. 2015; 35: 6-12).
18. Sabir S, Singh D, Rocha J. In Vitro Antioxidant Activity of S-Carvone Isolated from *Zanthoxylum alatum*. *Pharmaceutical Chemistry Journal*. 2015; 49: 187-91.
19. Vinothkumar R, Sudha M, Viswanathan P, Kabalimoorthy J, Balasubramanian T, Nalini N. Modulating effect of d-carvone on 1,2-dimethylhydrazine-induced pre-neoplastic lesions, oxidative stress and biotransforming enzymes, in an experimental model of rat colon carcinogenesis. *Cell Prolif*. 2013; 46: 705-20.
20. Raphael T, Kuttan\* G. Immunomodulatory activity of naturally occurring monoterpenes carvone, limonene, and perillic acid. *Immunopharmacology and immunotoxicology*. 2003; 25: 285-94.
21. de Cassia da Silveira e Sa R, Andrade LN, de Sousa DP. A review on anti-inflammatory activity of monoterpenes. *Molecules*. 2013; 18: 1227-54.

22. Zheng G-q, Kenney PM, Lam LK. Effects of carvone compounds on glutathione S-transferase activity in A/J mice. *Journal of agricultural and food chemistry*. 1992; 40: 751-5.
23. Noeman SA, Hamooda HE, Baalash AA. Biochemical study of oxidative stress markers in the liver, kidney and heart of high fat diet induced obesity in rats. *Diabetol Metab Syndr*. 2011; 3: 17.
24. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985; 28: 412-9.
25. Festi D, Colecchia A, Sacco T, Bondi M, Roda E, Marchesini G. Hepatic steatosis in obese patients: clinical aspects and prognostic significance. *Obes Rev*. 2004; 5: 27-42.
26. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature*. 2006; 444: 840-6.
27. Bu L, Gao M, Qu S, Liu D. Intraperitoneal injection of clodronate liposomes eliminates visceral adipose macrophages and blocks high-fat diet-induced weight gain and development of insulin resistance. *AAPS J*. 2013; 15: 1001-11.
28. Darkhal P, Gao M, Ma Y, Liu D. Blocking high-fat diet-induced obesity, insulin resistance and fatty liver by overexpression of Il-13 gene in mice. *Int J Obes (Lond)*. 2015; 39: 1292-9.

29. Yan L, Song K, Gao M, Qu S, Liu D. Modulation of Cell-Mediated Immunity to Suppress High Fat Diet-Induced Obesity and Insulin Resistance. *Pharmaceutical research*. 2016; 33: 395-403.
30. Alsanea S, Gao M, Liu D. Phloretin Prevents High-Fat Diet-Induced Obesity and Improves Metabolic Homeostasis. *AAPS J*. 2017; 19: 797-805.
31. Waterman C, Rojas-Silva P, Tumer TB, Kuhn P, Richard AJ, Wicks S, *et al*. Isothiocyanate-rich *Moringa oleifera* extract reduces weight gain, insulin resistance, and hepatic gluconeogenesis in mice. *Mol Nutr Food Res*. 2015; 59: 1013-24.
32. Inoue M, Ohtake T, Motomura W, Takahashi N, Hosoki Y, Miyoshi S, *et al*. Increased expression of PPARgamma in high fat diet-induced liver steatosis in mice. *Biochem Biophys Res Commun*. 2005; 336: 215-22.
33. Kotronen A, Seppanen-Laakso T, Westerbacka J, Kiviluoto T, Arola J, Ruskeepaa AL, *et al*. Hepatic stearoyl-CoA desaturase (SCD)-1 activity and diacylglycerol but not ceramide concentrations are increased in the nonalcoholic human fatty liver. *Diabetes*. 2009; 58: 203-8.
34. Samuel VT, Petersen KF, Shulman GI. Lipid-induced insulin resistance: unravelling the mechanism. *Lancet*. 2010; 375: 2267-77.
35. Chen L, Chen R, Wang H, Liang F. Mechanisms Linking Inflammation to Insulin Resistance. *Int J Endocrinol*. 2015; 2015: 508409.

## CHAPTER 4

### ANTIOXIDANTS REVERSE OBESITY-ASSOCIATED INSULIN RESISTANCE WITHOUT REDUCING OBESE MOUSE WEIGHT

#### **Introduction**

Obesity prevalence has been increased among the adult American population, reaching 35% of men and 40.4% of women in 2014 (1). According to the State of Obesity Organization's statistics for 2015 (2), Louisiana had the highest obesity prevalence (36.2%), whereas Colorado had the lowest obesity rate (20.2%), and Georgia was ranked as the 19<sup>th</sup> state, with a 30.7% obesity rate. Umashanker *et al.* (3) recently reviewed the economic burden associated with obesity. The estimated medical cost of an obese individual was 42% higher than a lean individual in 2006 (4). The number of obesity-associated diseases (e.g., type 2 diabetes, cardiovascular diseases, non-alcoholic fatty liver disease, etc.) explains the increase in obesity-related medical costs.

The United States Food and Drug Administration has approved few anti-obesity drugs that inhibit fat absorption or suppress appetite (5). However, these anti-obesity drugs have limited capacity in reducing body weight ( $\approx 5.8 - 9.3$  kg reduction in body weight) and are associated with considerable side effects, such as dizziness, dry mouth, irritability, oily spotting, insomnia, and back pain (3, 5). For more severe obesity cases, bariatric surgeries work as an alternative solution. Although these surgeries have great results in reducing body weight and mortality (6, 7), they result in foreign instruments in the body (adjustable gastric band surgery) or permanent removal of part of the stomach

(sleeve gastrectomy surgery). In addition, regained weight after surgery might lead to additional reoperative bariatric surgery, which has more risks than the primary surgery (8). Thus, obesity treatment is a challenge and requires multiple approaches, as Dixon has proposed (9).

Inflammation plays a primary role in obesity development and obesity-associated complications (10). We have previously shown, in **Chapters 2 and 3**, the preventative role of antioxidants via inhibition of inflammation. Green tea extract has shown the inhibitory impact on mice weight gain and obesity-associated fatty liver (11, 12). Catechin (**Fig. 4.1a**) and epigallocatechin gallate (EGCG) (**Fig. 4.1b**) are natural antioxidants in green tea extract (13). A study has shown that acute treatment of EGCG for 4-7 days increased energy excretion and reduced hepatic triglycerides and glycogen (14). A similar effect was observed with longer intake of green tea extract (11). These studies provided supportive indication for the antioxidant to serve as an anti-obesity candidate. Here we are testing whether the antioxidant alone (EGCG) or a combination of antioxidants will be able to reverse obesity and its complications in high-fat diet (HFD)-induced obese mice. Our findings show that antioxidants alone can prevent HFD-induced weight gain, where EGCG improves insulin resistance without reducing body weight of obese mice.

## **Materials and Methods**

*Materials.* Catechin, EGCG, and phloretin were purchased from Sigma-Aldrich (St. Louis, MO). HFD (60% kJ/fat, 20% kJ/protein, and 20% kJ/carbohydrate) was from Bioserv (Frenchtown, NJ). The insulin kit was obtained from Merckodia AB (Winston-Salem,

NC), and glucometer and blood test strips were obtained from NIPRO Diagnostics™ (Fort Lauderdale, FL). The Humulin® insulin was purchased from Eli Lilly (Indianapolis, IN). The Infinity™ kit for triacylglycerol was from Fisher Diagnostics (Middletown, VA). The total cholesterol kit was purchased from Genzyme Diagnostics (Charlottetown, PE Canada). The free fatty acids (NEFA)-HR kits were obtained from Wako Bioproducts (Richmond, VA). The RNeasy® Lipid Tissue Mini Kit was from QIAGEN® (Hilden, Germany). The First-stand cDNA Synthesis was obtained from Origene® (Rockville, MD). The PerfeCta® SYBR® Green FastMix® ROX™ was purchased from Quanta Biosciences (Gaithersburg, MD). Oil-Red O solution was purchased from Electron Microscopy Sciences (Hatfield, PA).

*Animals and Animal Treatments.* C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). All procedures and protocols performed on mice were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia, Athens, Georgia. To assess catechin and EGCG activity in blocking HFD-induced obesity and obesity-associated metabolic disorders, seven-week-old male C57BL/6 mice were randomly arranged (n=5) and fed an HFD and received twice weekly intraperitoneal (*i.p.*) injections of catechin (34 mg/kg), EGCG (20 mg/kg), or equivalent vehicle volume of dimethyl sulfoxide (DMSO) for 12 weeks. For the obesity treatment study, mice were fed an HFD for 6 weeks and then treated with EGCG (20 mg/kg, *i.p.*) or DMSO for 6 weeks while kept on HFD (n=5). Antioxidant combination treatment (catechin 50 mg/kg + phloretin 10 mg/kg) was injected *i.p.* twice

weekly for 3 weeks. Mouse body weight was monitored weekly, and body composition was determined using EchoMRI-100™ from the Echo Medical System (Houston, TX).

*Glucose Tolerance Test (GTT), Insulin Tolerance Test (ITT), and Determination of Insulin Resistance.* Mice were fasted for 6 h and received an *i.p.* injection of glucose (1 g/kg) dissolved in saline. The blood glucose level was examined at time 0, 30, 60, and 120 min using a glucometer. For ITT, mice were fasted for 4 h and injected *i.p.* with insulin (0.75 U/kg). The blood glucose level was determined using the same schedule as GTT. Serum concentrations of insulin were determined by ELISA, following the procedure provided by the supplier of the insulin analysis kit. Insulin resistance was determined using the formula: [fasting insulin (ng/ml) x fasting blood glucose (mg/dl)/405] (15).

*Histochemical Analysis by Hematoxylin and Eosin (H&E) Staining.* Liver and epididymal white adipose tissues were collected from sacrificed animals and fixed in 10% neutral buffered formalin. Next, tissues were dehydrated, fixed in paraffin, sectioned at 6 µm in thickness, and stained with H&E, following the manufacturer's instructions. Tissue sections were examined and photo images were taken under an optical microscope using the NIS-Elements imaging software from Nikon Instruments Inc. (Melville, NY).

*Oil-Red O Staining.* Fresh liver samples were collected and frozen at -80°C immediately after mice were euthanized. Frozen sections were made using a Leica Cryostat at a thickness of 8 µm and fixed with 10% formalin for 30 min. The fixed sections were

rinsed with 60% isopropanol, stained with freshly prepared Oil-Red O solution for 15 min, and dipped 5 times in hematoxylin. The stained sections were examined under the optical microscope and images were taken.

*Determination of Serum Insulin and Lipid Level.* Mice were starved for 6 h and blood samples were collected from the heart after euthanasia. Insulin levels were measured using ELISA, according to the manufacturer's instructions. Serum concentrations of triglycerides, FFA, and total cholesterol were determined using the procedure provided by the supplier of the lipid analysis kits.

*Statistical Analysis.* Statistical analysis was conducted by unpaired *t*-test or one-way analysis of variance (ANOVA) followed by multiple comparisons test. A value of  $P < 0.05$  was considered significantly different. Each data point represents the mean  $\pm$  standard deviation (SD) or standard error of the mean (SEM).

## **Results**

*Catechin and EGCG Protected Mice from HFD-induced Weight Gain.*

Results in **Figure 4.1** show that catechin and EGCG block body weight gain of animals fed an HFD for 12 weeks. Compared to the control animals injected with DMSO, the carrier solution, the body weights of catechin- and EGCG-treated animals were  $35.7 \pm 3.5$  g and  $35.8 \pm 5.1$  g, respectively, compared to  $45.2 \pm 3.1$  g for the control (**Fig. 4.1d**). The size difference between treated and control mice can also be detected with the naked eye (**Fig.4.1c**). Body composition analysis showed that catechin and EGCG blocked HFD-



induced increase in fat mass (**Fig. 4.1e**) without affecting lean mass (**Fig. 4.1f**). Catechin and EGCG had no effect on food intake during the 12-week feeding of HFD (data not shown).

*Catechin and EGCG Treatment Reduces Lipid Accumulation in WAT and BAT.*

Histological examinations of white adipose tissue (WAT) and brown adipose tissue (BAT) confirmed lower lipid accumulation in adipocytes of catechin- and EGCG-treated animals (**Fig. 4.2a**). The average diameters of adipocytes in treated animals were  $73.1 \pm 22.3 \mu\text{m}$  and  $81.9 \pm 23.1 \mu\text{m}$ , respectively, around 35% smaller than that of control animals ( $118.3 \pm 24.5 \mu\text{m}$ ) (**Figs. 4.2a, b**). Moreover, EGCG-treated mice had no crown-like structures in WAT, compared to control. However, less fat accumulation in BAT was observed in both catechin- and EGCG-treated animals suggested by the lower density of vacuole structures (**Fig. 4.2a**, lower panel). These results suggest that catechin and EGCG suppress lipid accumulation in adipose tissues.

*Catechin and EGCG Blocks HFD-induced Fatty Liver without Significant Impact on TC and FFA.*

Oil-red O staining was performed on liver samples from treated and control mice. Results in **Figure 4.3** show that catechin and EGCG protected mice from fatty liver after 12 weeks of feeding mice with HFD, whereas significant fatty liver was observed in control mice, shown as the more lipid-soluble red dye in liver cells (**Fig. 4.3a**). However, serum analysis of total cholesterol (TC) and fatty acids (FA) did not show a statistical difference (**Fig. 4.3b-c**), although catechin significantly reduced serum triglycerides

(TG), by 47% ( $1.7 \pm 0.2$  vs.  $3.2 \pm 1$  mmol/L) (**Fig. 4.3d**). Taken together, these results indicate that catechin and EGCG block hepatic lipid accumulation and catechin lowers serum TG.

*Catechin and EGCG Prevented Diet-induced Insulin Resistance and Hyperinsulinemia.*

The effects of catechin and EGCG treatment on glucose and insulin homeostasis were examined, and the results are shown in **Figure 4.4**. The fasting blood glucose level (**Fig. 4.4a**) was significantly lower in EGCG-injected mice than in control ( $192 \pm 45$  mg/dl vs.  $249 \pm 15$  mg/dl). Moreover, catechin and EGCG blocked HFD-induced hyperinsulinemia compared with control mice ( $4 \pm 1.5$  and  $2.9 \pm 0.5$  vs.  $8.7 \pm 3.2$  ng/ml, respectively) (**Fig. 4.4b**). To evaluate the impact of catechin and EGCG on HFD-induced glucose intolerance, the glucose tolerance test showed a little improvement of glucose profile (**Fig. 4.4c**), without a significant difference in the area under the curve (**Fig. 4.4d**). However, the insulin tolerance test (ITT) and the homeostasis model assessment-estimated insulin resistance (HOMA-IR) demonstrated a reduction in insulin resistance (**Fig. 4.4e-f**). Furthermore, catechin- and EGCG-treated mice had significantly lower HOMA-IR (**Fig. 4.4h**). These data suggest that catechin and EGCG prevent diet-induced hyperinsulinemia and insulin resistance.

*EGCG Treatment Did Not Induce Weight Loss in Obese Mice but Slightly Alleviated*

*Insulin Resistance.*

The results collected so far support the notion that antioxidants such as catechin and EGCG can block HFD-induced obesity, obesity-associated fatty liver, and insulin

resistance. These preventive effects are likely due to blockage of inflammation and macrophage infiltration into adipose tissue. We then asked if an individual antioxidant or in combination can reverse obesity. We selected EGCG because it is more potent than catechin and it showed better beneficial results against HFD-induced fatty liver and hyperglycemia. Obese mice were generated by HFD feeding for 6 weeks to develop obesity-related metabolic complications. Mice were treated twice weekly with EGCG (20 mg/kg) or DMSO for 6 weeks, and body weight and fat mass were monitored every week. The results show that there was no difference in body weight and composition (**Figs. 4.5a-d**). There was a reduction in adipocyte size and crown-like structures (CLS) (**Fig. 4.6**), without any significant change in hepatic levels, serum lipids, fasting glucose, and insulin (**Figs. 4.7, 4.8a-b**). In contrast, EGCG-treated mice had better response to insulin, judging by the lower glucose profile in ITT, which was confirmed by measuring the area under the curve. These results demonstrate that EGCG treatment reduced adipocytes and improved diet-induced insulin resistance.

#### *Antioxidant Combination Did Not Reverse Obesity.*

Obesity is a preventable disease, as we have shown earlier, in previous chapters (**2** and **3**) and with catechin and EGCG in this chapter. However, obesity is still a challenge that a lone antioxidant (i.e., EGCG or phloretin) was not able to reverse. Here, we use an antioxidant combination [phloretin (10 mg/kg) / catechin (50 mg/kg)] and treated mice with twice-weekly *i.p.* injections for 3 weeks. The results showed that antioxidant combination did not induce any significant change in body weight, body composition, or the food intake of the obese mice (**Fig. 4.9a-c**).

## Discussion

In the current study, we examined the preventive effect of the antioxidants catechin and EGCG against HFD-induced obesity, fatty liver, and insulin resistance. We also investigated the ability of an antioxidant alone (EGCG) or in combination (catechin plus phloretin) to induce weight loss in obese mice. The results show that catechin or EGCG was able to protect mice from HFD-induced weight gain and alleviate its complications manifesting as fatty liver and insulin resistance (**Figs. 4.1-4**). Moreover, catechin and EGCG improved insulin resistance of obese mice. However, neither antioxidant treatment alone (**Fig. 4.5**) nor in combination (**Fig. 4.9**) induced weight loss of obese mice.

Earlier studies have shown the beneficial effects of catechins (11, 16-18). Murase *et al.* (11) have shown that feeding mice for 11 months with catechin-rich extract 0.5% (w/w) (including catechin and EGCG) suppressed HFD-induced weight gain and lipid accumulation. EGCG content is higher than catechin in green tea extract (18). Feeding animals with EGCG for short terms (4 weeks) or long terms (16 weeks) alleviated diet-induced obesity (16, 17). The proposed anti-obesity mechanisms of EGCG are: increasing energy expenditure, enhancing fat oxidation, decreasing nutrient absorption, suppressing appetite, and reducing inflammation (17, 18). In our preventative and treatment studies, we have focused on antioxidants for inflammation inhibition. Our preventative results were in agreement with the previous studies despite different doses, route of administration, and treatment period. We confirmed the preventative activity of catechin and EGCG before conducting the treatment experiment to induce weight loss with EGCG. Our treatment study shows that EGCG was not able to reduce the body

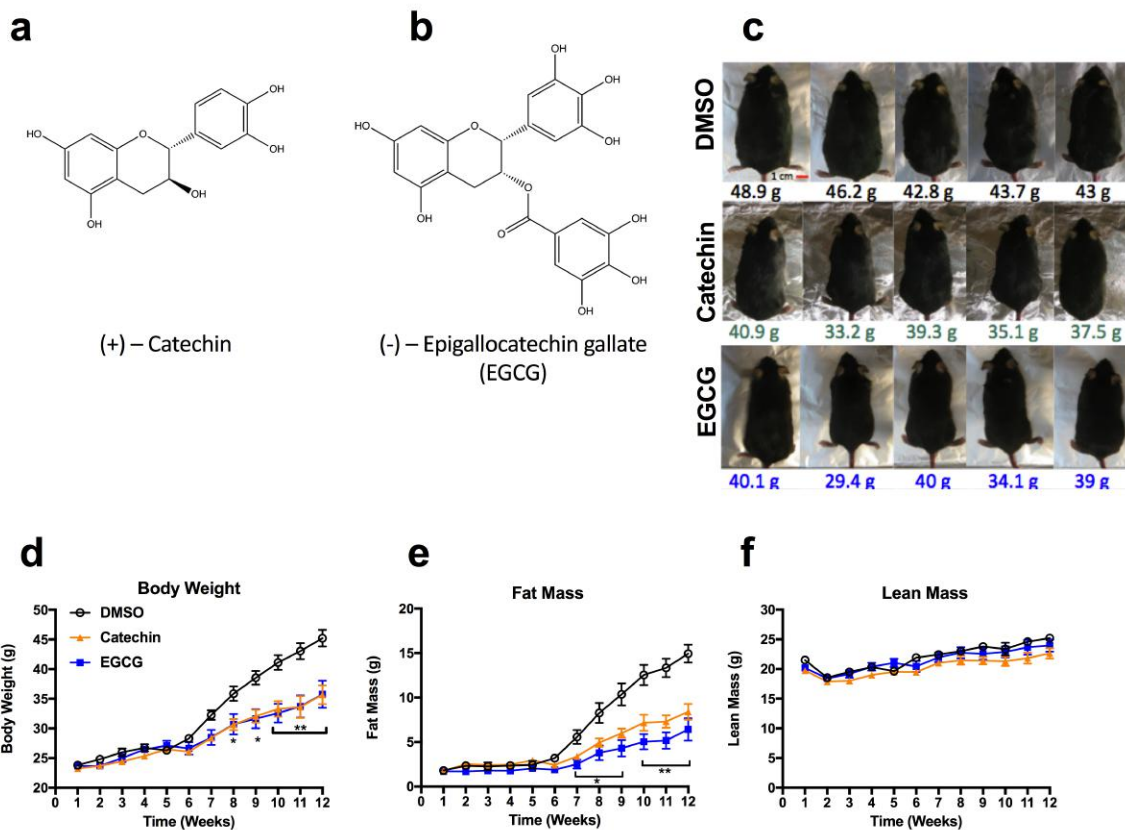
weight of obese mice (**Fig. 4.5**). In contrast, Friedrich and colleagues reported that an acute dose of EGCG (1% w/w) reduced body weight within 4 days of feeding HFD (14), which cannot be seen with the lower dose (0.5% w/w). The same lower dose was effective in another prevention study (16) but not in the treatment, similar to our observation with dosage of EGCG, which suggests a different strategy to treat obesity either by increasing the antioxidant dose or by using an antioxidant combination. We tried the antioxidant combination of catechin and phloretin, and it was not strong enough to induce body weight loss (**Fig. 4.9**). Additional mechanisms, such as suppressing appetite and/or causing energy expenditure, are required.

Fatty liver is tightly associated with obesity. Our lab and others have shown that obesity prevention is related to fatty liver prevention (11, 17, 19, 20) (**Figs. 4.1-3**). However, not all antioxidants are equal in their ability to reverse fatty liver of obese mice. For instance, we have shown (**Chapter 2**) (21) that phloretin was able to alleviate hepatic steatosis due to up-regulation of fatty acid oxidation-responsible genes and down-regulation of *de novo* lipogenesis genes. In the current study, EGCG was not able to reverse fatty liver in the obese mice (**Fig. 4.7**). However, Bruno *et al.* (12) have shown an improvement of fatty liver in leptin-deficient (*ob/ob*) mice. This observation variance could be due to differences in the model or the different doses.

The activity of antioxidants in improving glucose homeostasis and insulin sensitivity has been reported in several animal studies (11, 17, 21). We have presented in this study that catechin and EGCG significantly reduced insulin levels and insulin resistance (HOMA-IR) (**Fig. 4.4**). Further, EGCG was able to reduce fasting blood glucose levels in lean (**Fig. 4.4a**) but not in obese mice (**Fig. 4.8a**). This observation can

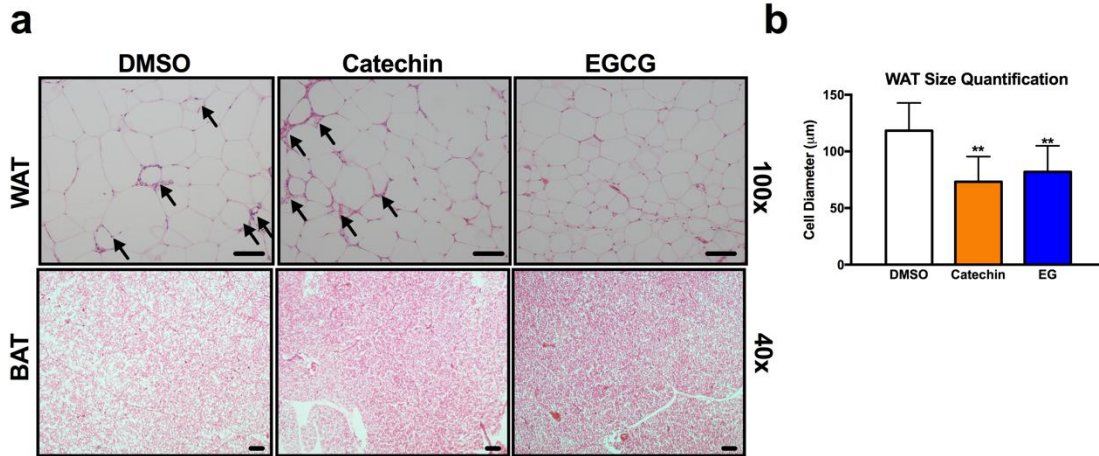
be explained by higher hepatic lipid accumulation (**Fig. 4.7a vs. 4.3a**) and proinflammatory macrophage infiltration in adipose tissue (**Fig. 4.6 vs. 4.2**), which play a crucial role in insulin resistance (10).

In conclusion, the data presented show that antioxidants are effective in obesity prevention and treatment of its complications, while neither one antioxidant alone nor an antioxidant combination reduced body weight. These results suggest that antioxidants are good candidates for protection against obesity and to alleviate obesity-associated insulin resistance.



**Figure 4.1. Catechin and EGCG inhibit body weight gain in mice fed an HFD.**

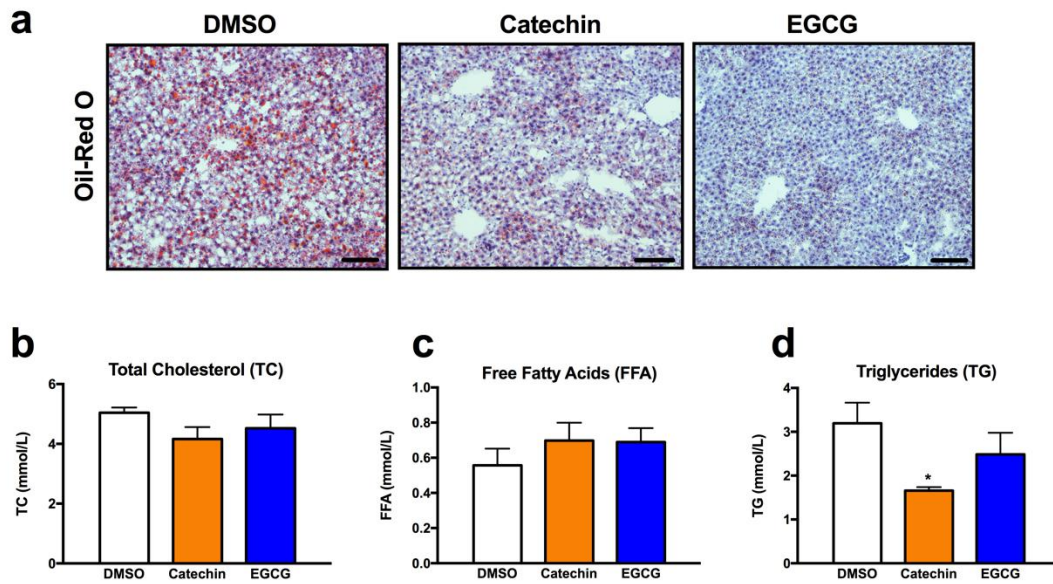
Seven-week-old C57BL/6 male mice were fed an HFD and injected *i.p.* twice weekly with catechin (34 mg/kg), EGCG (20 mg/kg), or vehicle (DMSO) for 12 weeks. (a) chemical structure of catechin; (b) chemical structure of EGCG; (c) mice pictures at the end of the experiment, scale bar: 1 cm; (d) growth curve; (e) fat mass; and (f) lean mass. Values represent mean  $\pm$  SEM, \*  $p < 0.05$  (n=5).



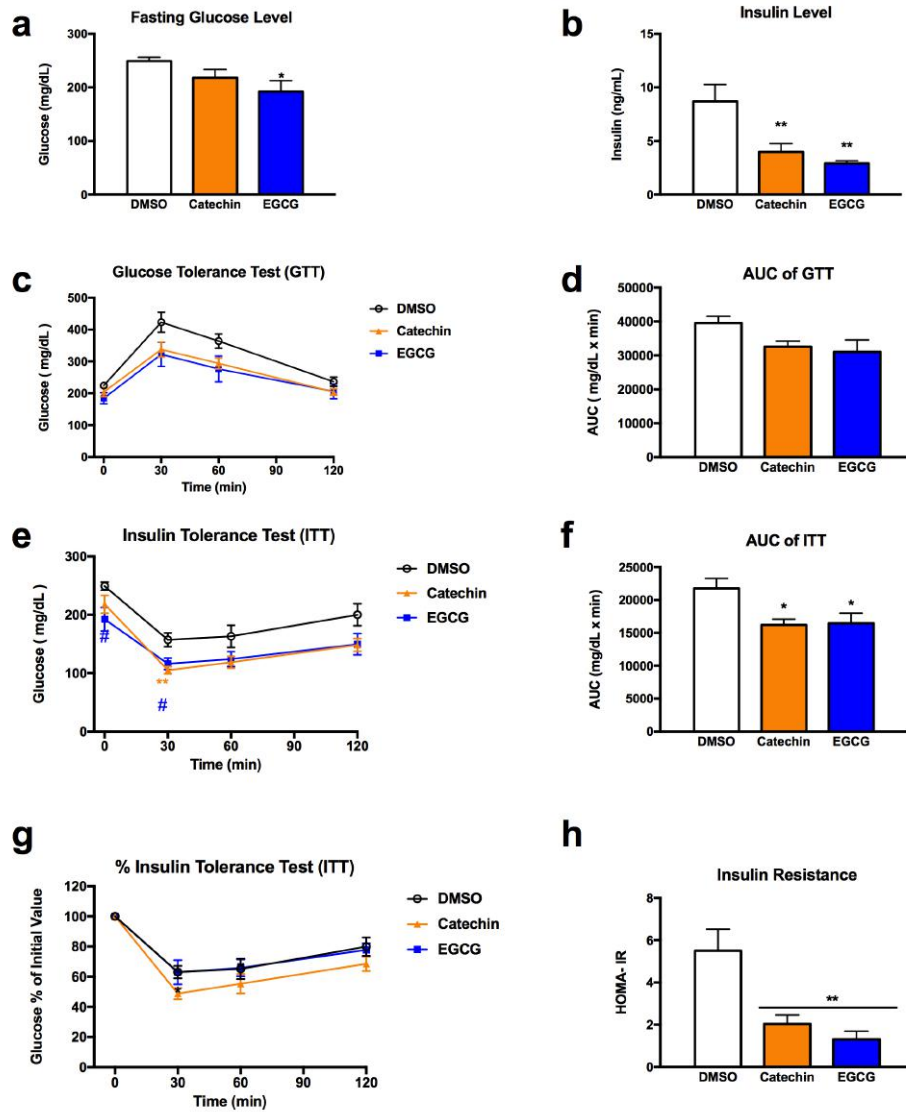
**Figure 4.2. Impacts of catechin and EGCG treatment on adipose tissues of animals.**

Mice were fed an HFD continuously for 12 weeks, where animals were injected *i.p.* twice weekly with catechin (34 mg/kg), EGCG (20 mg/kg), or vehicle (DMSO) for the entire 12 weeks. Adipose tissues were collected after mice were sacrificed at the end of experiments (n=4). (a) H&E staining of epididymal fat pads (WAT) and brown adipose tissue (BAT), black arrows indicate crown-like structures (CLS), scale bar: 100 μm; (b) average diameter of adipocytes in of epididymal WAT; Values are mean ± SD. \* $p < 0.05$ , \*\*  $p < 0.01$ . (n=5)

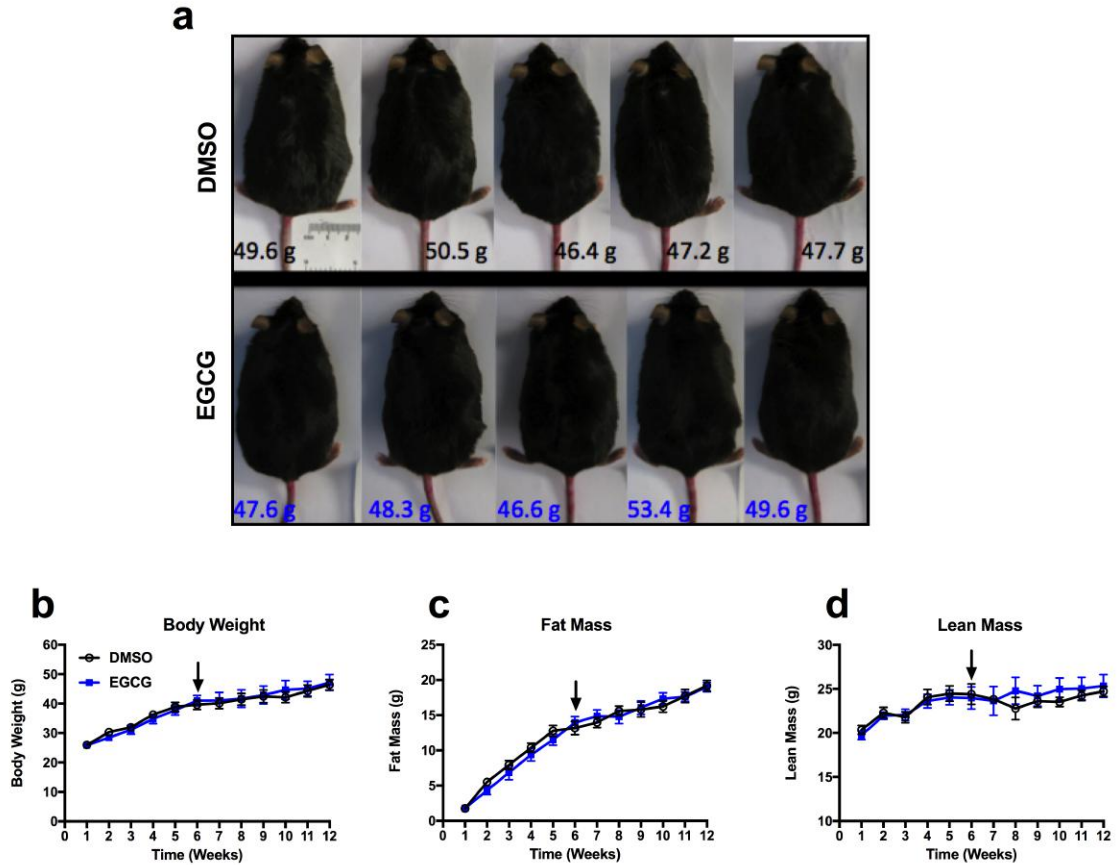




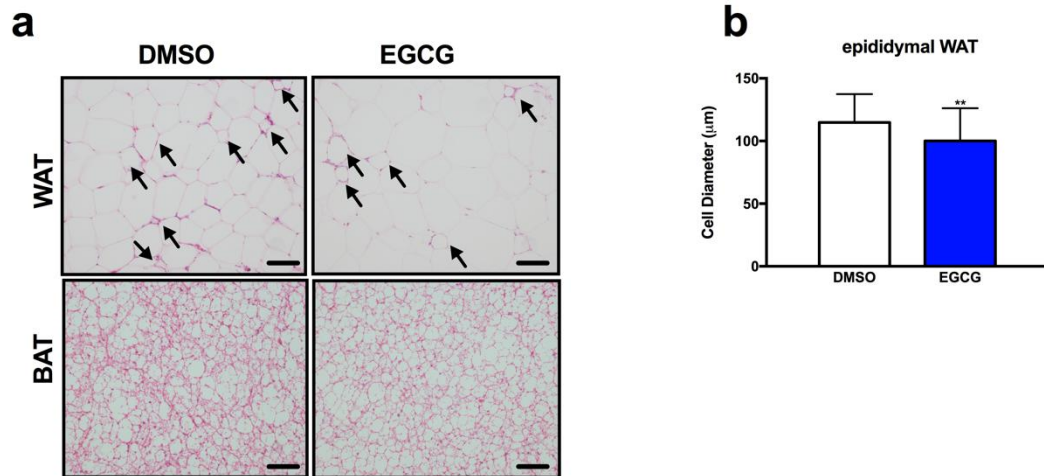
**Figure 4.3. Catechin and EGCG suppressed HFD-induced hepatic lipid accumulation.** Mice were sacrificed at the end of week 12. (a) Photo images of Oil-Red O staining of liver sections were photographed at x100 magnification, scale bars: 100  $\mu$ m; (b) serum level of total cholesterol; (d) FFA; and (e) triglycerides. Each data point represents mean  $\pm$  SEM, \* $p < 0.05$  (n=5).



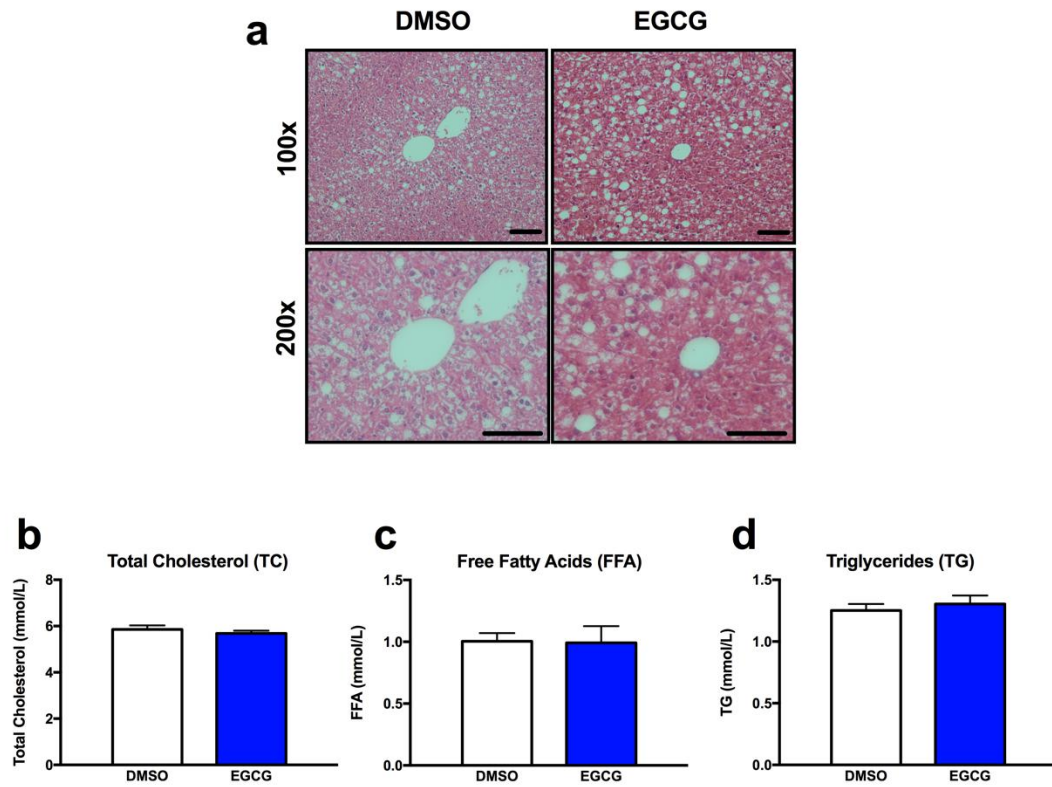
**Figure 4.4. Catechin and EGCG improved hyperinsulinemia and insulin resistance in HFD-fed mice.** (a) Fasting glucose level; (b) fasting serum insulin concentration. (c) Profile of serum glucose concentration after *i.p.* glucose injection (1 g/kg); (d) area under the curve of glucose tolerance test; (e) time-dependent glucose concentration in blood after *i.p.* insulin injection (0.75 U/kg); (f) area under the curve of insulin tolerance test (ITT); (g) ITT represented by percentage decrease of initial glucose value; and (h) homeostasis model assessment-estimated insulin resistance (HOMA-IR). Each data point symbolizes mean  $\pm$  SEM, \*  $p < 0.05$  (n=4-5).



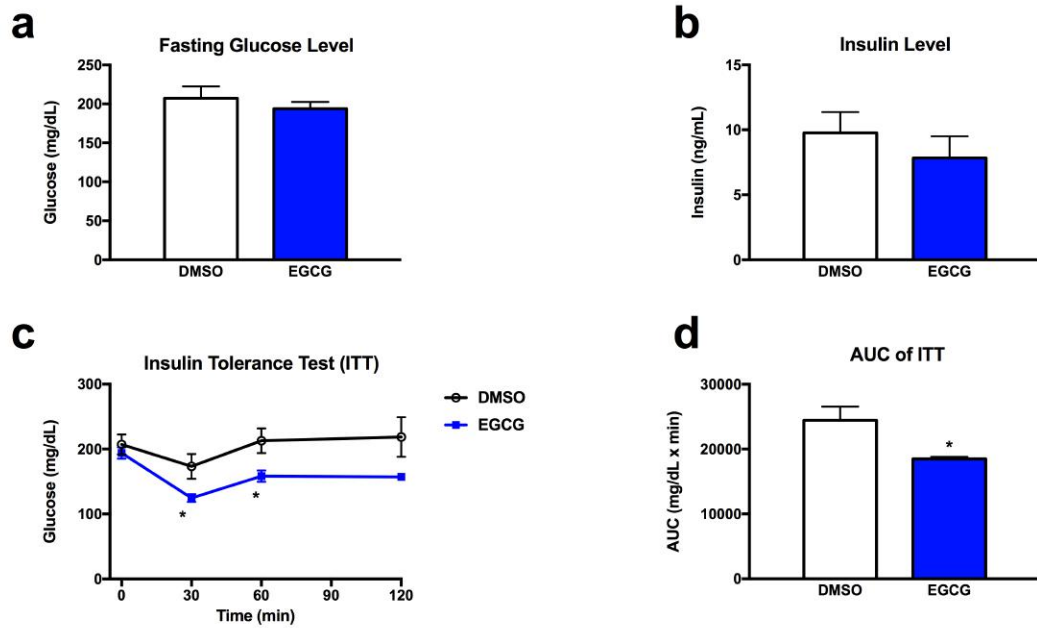
**Figure 4.5. EGCG did not induce weight loss in obese mice.** Mice were fed an HFD for 6 weeks and developed obesity, followed by 6 weeks of twice-weekly *i.p.* injection of EGCG while continuing an HFD. **(a)** Mice pictured at the end of the experiment; **(b)** growth curve, arrow indicates starting of EGCG treatment (n=5); **(c)** fat mass; and **(d)** lean mass. Values represent mean  $\pm$  SEM, \*  $p < 0.05$  (n=5).



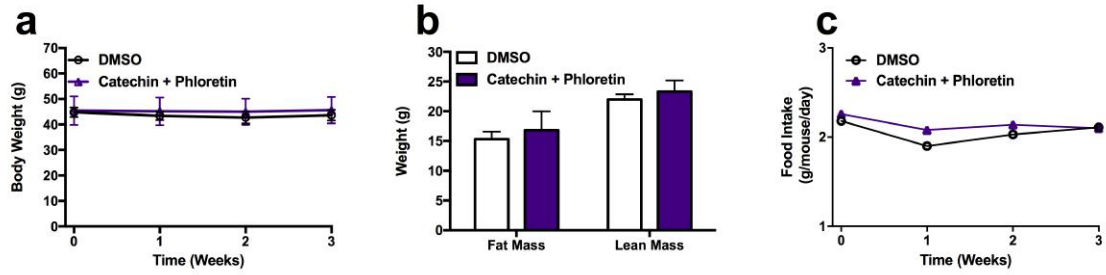
**Figure 4.6. Impacts of EGCG treatment on adipose tissues of animals.** Mice were fed an HFD continuously for 12 weeks, where were animals injected *i.p.* twice weekly with EGCG (20 mg/kg) or vehicle (DMSO) from week 6 until week 12. Mice were euthanized at the end of experiments and adipose tissues were collected. **(a)** H&E staining of epididymal WAT and BAT, black arrows indicate crown-like structures (CLS), scale bar: 100 µm; **(b)** average diameter of adipocytes in of epididymal WAT. Values are mean ± SD. \* $p < 0.05$ , \*\*  $p < 0.01$ . (n=5)



**Figure 4.7. EGCG failed to restore HFD-induced fatty liver in obese mice.** EGCG treatment started at the 6<sup>th</sup> week for 6 weeks, and mice were sacrificed at the end of week 12. (a) Photo images of Oil-Red O staining of liver sections were photographed at x100 magnification, scale bars: 100  $\mu$ m; (b) TC; (d) FFA; and (e) TG. Each data point represents mean  $\pm$  SEM, \* $p$  < 0.05 (n=5).



**Figure 4.8. EGCG slightly improved insulin sensitivity in obese mice.** Mice were fed an HFD for 6 weeks and developed obesity, followed by 6 weeks of twice weekly *i.p.* injection of EGCG when continuing on an HFD. **(a)** Fasting glucose level; **(b)** fasting serum insulin concentration; **(c)** Insulin tolerance test (ITT) after *i.p.* insulin injection (0.75 U/kg); and **(d)** area under the curve of ITT. Each data point represents mean  $\pm$  SEM, \*  $p < 0.05$  (n=4-5).



**Figure 4.9. Antioxidant combination treatment study.** Obese C57BL/6 male mice were fed an HFD and injected with *i.p.* twice weekly with a combined dose of phloretin (10 mg/kg) / catechin (50 mg/kg) or DMSO for 3 weeks. **(a)** Growth curve; **(b)** average food intake; **(c)** body composition at week 3. All values represent mean  $\pm$  SEM \* $p$  < 0.05 (n=3-4).

## References

1. Flegal KM, Kruszon-Moran D, Carroll MD, Fryar CD, Ogden CL. Trends in Obesity Among Adults in the United States, 2005 to 2014. *JAMA*. 2016; 315: 2284-91.
2. The State of Obesity. Adult Obesity in the United States 2015 [updated 9-1-2016. Available from: <http://stateofobesity.org/adult-obesity/>.]
3. Umashanker D, Igel LI, Kumar RB, Aronne LJ. Current and Future Medical Treatment of Obesity. *Gastrointest Endosc Clin N Am*. 2017; 27: 181-90.
4. Finkelstein EA, Trogon JG, Cohen JW, Dietz W. Annual medical spending attributable to obesity: payer-and service-specific estimates. *Health Aff (Millwood)*. 2009; 28: w822-31.
5. Daneschvar HL, Aronson MD, Smetana GW. FDA-Approved Anti-Obesity Drugs in the United States. *Am J Med*. 2016; 129: 879 e1-6.
6. Sjostrom CD, Lissner L, Wedel H, Sjostrom L. Reduction in incidence of diabetes, hypertension and lipid disturbances after intentional weight loss induced by bariatric surgery: the SOS Intervention Study. *Obes Res*. 1999; 7: 477-84.
7. Sjostrom L, Narbro K, Sjostrom CD, Karason K, Larsson B, Wedel H, *et al*. Effects of bariatric surgery on mortality in Swedish obese subjects. *N Engl J Med*. 2007; 357: 741-52.



8. Brethauer SA, Kothari S, Sudan R, Williams B, English WJ, Brengman M, *et al.* Systematic review on reoperative bariatric surgery: American Society for Metabolic and Bariatric Surgery Revision Task Force. *Surg Obes Relat Dis.* 2014; 10: 952-72.
9. Dixon JB. Obesity in 2015: Advances in managing obesity. *Nat Rev Endocrinol.* 2016; 12: 65-6.
10. Chen L, Chen R, Wang H, Liang F. Mechanisms Linking Inflammation to Insulin Resistance. *Int J Endocrinol.* 2015; 2015: 508409.
11. Murase T, Nagasawa A, Suzuki J, Hase T, Tokimitsu I. Beneficial effects of tea catechins on diet-induced obesity: stimulation of lipid catabolism in the liver. *Int J Obes Relat Metab Disord.* 2002; 26: 1459-64.
12. Bruno RS, Dugan CE, Smyth JA, DiNatale DA, Koo SI. Green tea extract protects leptin-deficient, spontaneously obese mice from hepatic steatosis and injury. *J Nutr.* 2008; 138: 323-31.
13. Higdon JV, Frei B. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit Rev Food Sci Nutr.* 2003; 43: 89-143.
14. Friedrich M, Petzke KJ, Raederstorff D, Wolfram S, Klaus S. Acute effects of epigallocatechin gallate from green tea on oxidation and tissue incorporation of dietary lipids in mice fed a high-fat diet. *Int J Obes (Lond).* 2012; 36: 735-43.
15. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from

- fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985; 28: 412-9.
16. Klaus S, Pultz S, Thone-Reineke C, Wolfram S. Epigallocatechin gallate attenuates diet-induced obesity in mice by decreasing energy absorption and increasing fat oxidation. *Int J Obes (Lond)*. 2005; 29: 615-23.
  17. Bose M, Lambert JD, Ju J, Reuhl KR, Shapses SA, Yang CS. The major green tea polyphenol, (-)-epigallocatechin-3-gallate, inhibits obesity, metabolic syndrome, and fatty liver disease in high-fat-fed mice. *J Nutr*. 2008; 138: 1677-83.
  18. Rains TM, Agarwal S, Maki KC. Antiobesity effects of green tea catechins: a mechanistic review. *J Nutr Biochem*. 2011; 22: 1-7.
  19. Cui R, Gao M, Qu S, Liu D. Overexpression of superoxide dismutase 3 gene blocks high-fat diet-induced obesity, fatty liver and insulin resistance. *Gene Ther*. 2014; 21: 840-8.
  20. Gao M, Ma Y, Liu D. Rutin suppresses palmitic acids-triggered inflammation in macrophages and blocks high fat diet-induced obesity and fatty liver in mice. *Pharm Res*. 2013; 30: 2940-50.
  21. Alsanea S, Gao M, Liu D. Phloretin Prevents High-Fat Diet-Induced Obesity and Improves Metabolic Homeostasis. *AAPS J*. 2017; 19: 797-805.

## CHAPTER 5

### CONCLUSION AND FUTURE PERSPECTIVES

While obesity has been heavily researched for decades, the prevalence of obesity continues to increase (1, 2). A newly released health report by the Centers for Disease Control and Prevention (CDC) reveals that obesity prevalence in the US increased from 37.7% in 2013-2014 to 39.6% in 2015-2016 (3). Public health efforts have been made to encourage physical activity in the community and to raise awareness regarding the correlation between obesity and many diseases (4). Recent studies have shown the great need to take action against obesity as similar in urgency to the treatment of human immunodeficiency virus infection and immune deficiency syndrome (5).

Obesity has been linked to oxidative stress and low-grade chronic inflammation (6, 7). Strategies to eliminate oxidative stress and inflammation have been explored as a means to manage obesity. In my dissertation studies, I have systematically examined the effects of antioxidants on animals subjected to developing obesity. These studies focused on preventing HFD-induced obesity, according to a philosophy reflected by a famous quote from Benjamin Franklin: “An ounce of prevention is worth a pound of cure” (8). Obesity prevention is a cost-effective approach with long-term benefits in decreasing obesity prevalence.

Among the 5 compounds tested, phloretin, benzyl isothiocyanate [BITC], and *S*-carvone, which exist in dietary fruits or vegetables, showed activity in suppressing HFD-induced weight gain and other metabolic complications. These results are in full

agreement with previous studies showing that plants or seeds containing these compounds tend to inhibit weight gain. One finding resulting from my work is that these compounds do not affect appetite or food intake. Mechanistic studies showed that these compounds are able to suppress expression of inflammatory genes in adipose tissues such as *F4/80*, *Cd68*, *Cd11b*, *Cd11c*, *Mcp1*, *Ccr2*, and *Tnf- $\alpha$* . Future work is needed to demonstrate the impacts of antioxidants on reducing the actual level of ROS not only in adipose tissues but also in other organs that play an important role in energy metabolism.

Treating obesity with antioxidants such as phloretin or epigallocatechin gallate did not show a body weight reduction in obese mice, though fatty liver and/or insulin resistance were improved (**Chapter 2** and **Chapter 4**). Future work should focus on whether these antioxidants bring about other beneficial effects. The results from my work also indicate that body weight loss may require enhanced lipolysis, increased energy expenditure, and reduction of energy intake. The possibility of using leptin sensitizers to achieve weight loss may represent a new research area (9) and deserves more attention.

ROS are the main cause of low-grade chronic inflammation which, consequently, contributes to ROS once developed. Obesity and associated fatty liver and insulin resistance were blocked in the prevention study (10, 11), whereas only obesity complications were alleviated in the treatment study, without reversal of high-fat diet-induced obesity (10). Future studies need to determine whether ROS levels in various specific organs are different upon antioxidant treatment.

The diet-induced obesity model employed in my dissertation study is commonly used to study obesity and obesity-associated complications (12, 13). While fatty liver, insulin resistance, and hyperglycemia were observed in the study, other obesity-

associated complications such as hyperlipidemia did not develop within 8-12 weeks of HFD feeding. It is possible that a longer period is needed for animals to develop hyperlipidemia, or alternatively, that cholesterol (0.2%) should be included in HFD to regulate lipid metabolism. Another future direction is to establish the relationship between ROS production and the content of the diets employed.

Reduction/oxidation (redox) homeostasis plays a crucial role in maintaining normal physiological functions. ROS overproduction leads to oxidative stress, while antioxidant overdose generates reductive stress. Redox homeostasis is controlled mainly by the 2GSH/GSSG ratio. In reductive stress, GSSG decreases and, consequently, reduces protein content disulfides. These thiol groups are important for many ROS-dependent signaling proteins, such as AP-1 and NF- $\kappa$ B transcriptional factors, protein tyrosine phosphatases, Src kinases, MAP kinases (JNK and p38), and insulin receptor kinase (14). Thus, excessive antioxidant activity can transform oxidative stress into reductive stress and lead to protein dysfunction or proteotoxicity (15). Villanueva and Kross (16) suggested several factors that contribute to stress due to antioxidants, including antioxidant redox potential, antioxidant concentration, the presence of another antioxidant or transition metals, and endogenous antioxidant activity and concentration. Reductive stress can further trigger ROS production in the mitochondrial respiratory chain as negative feedback through dihydroxyacetone phosphate reduction and glycerol-3 phosphate (G3P) production (17, 18). Future studies should pay close attention to the redox balance to avoid any toxicity caused by antioxidants.

Some antioxidants might have “off-target” effects due to their function as independent transcriptional factors or as estrogen-like phytochemicals (19, 20). Estrogen

pathway activation has shown beneficial effects on glucose homeostasis (21), leptin regulation (22), energy balance (23), fatty liver amelioration (24), and energy expenditure (25). Consequently, choosing the right antioxidant is important in order to take advantage of its clearly defined mechanism of action.

The effects of antioxidants have been clearly demonstrated in animal but not in human studies. The major reason for such a discrepancy may lie in the differences of the objectives of the studies and the type of subjects involved. Compared to animal studies, in which antioxidants have commonly been studied for their activities in preventing obesity and improving metabolic homeostasis, clinical studies tend to focus on demonstrating the therapeutic benefits to obese patients or patients with metabolic diseases such as diabetes, cardiovascular diseases, hyperlipidemia or hypertension. With the exception of three studies available in the literature showing a slight reduction in body weight (1.9-4.5 kg, 1.8% - 5.8%) (26-28), no clinical studies have demonstrated the positive influence of antioxidants on reducing body weight for obese patients with or without metabolic diseases. Future human studies should consider the benefits of antioxidants in preventing obesity and obesity-associated diseases. In addition, population, experimental design, carbohydrate/fat ratio in diet, antioxidant dose and frequency, treatment duration, and, more importantly, treatment starting point are all important factors to be considered and paid close attention. While most of the discussed human studies focused on obesity treatment, there is still hope for upcoming studies to show the impact of antioxidants on obesity prevention.

## References

1. Ogden CL, Carroll MD, Fryar CD, Flegal KM. Prevalence of Obesity Among Adults and Youth: United States, 2011-2014. NCHS Data Brief. 2015: 1-8.
2. Collaborators GBDO. Health Effects of Overweight and Obesity in 195 Countries over 25 Years. N Engl J Med. 2017.
3. Hales CM CM, Fryar CD, Ogden CL. Prevalence of Obesity Among Adults and Youth: United States, 2015-2016. NCHS data brief, no 288. Hayysville, MD: National Center for Health Statistics. 2017 [Available from: <https://www.cdc.gov/nchs/data/databriefs/db288.pdf>.]
4. Chan RS, Woo J. Prevention of overweight and obesity: how effective is the current public health approach. Int J Environ Res Public Health. 2010; 7: 765-83.
5. Spoer B, Fullilove R. Obesity: a syndemics perspective. Clin Obes. 2016; 6: 171-4.
6. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, *et al*. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest. 2004; 114: 1752-61.
7. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest. 2003; 112: 1796-808.

8. Woolf SH. The power of prevention and what it requires. *JAMA*. 2008; 299: 2437-9.
9. Liu J, Lee J, Salazar Hernandez MA, Mazitschek R, Ozcan U. Treatment of obesity with celastrol. *Cell*. 2015; 161: 999-1011.
10. Alsanea S, Gao M, Liu D. Phloretin Prevents High-Fat Diet-Induced Obesity and Improves Metabolic Homeostasis. *AAPS J*. 2017; 19: 797-805.
11. Alsanea S, Liu D. BITC and S-Carvone Restrain High-Fat Diet-Induced Obesity and Ameliorate Hepatic Steatosis and Insulin Resistance. *Pharm Res*. 2017; 34: 2241-9.
12. Anstee QM, Goldin RD. Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. *International journal of experimental pathology*. 2006; 87: 1-16.
13. Winzell MS, Ahren B. The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. *Diabetes*. 2004; 53 Suppl 3: S215-9.
14. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol*. 2007; 39: 44-84.



15. Dodson M, Redmann M, Rajasekaran NS, Darley-USmar V, Zhang J. Correction: KEAP1-NRF2 signalling and autophagy in protection against oxidative and reductive proteotoxicity. *Biochem J.* 2015; 471: 431.
16. Villanueva C, Kross RD. Antioxidant-induced stress. *Int J Mol Sci.* 2012; 13: 2091-109.
17. Tilton RG. Diabetic vascular dysfunction: links to glucose-induced reductive stress and VEGF. *Microsc Res Tech.* 2002; 57: 390-407.
18. Mracek T, Drahota Z, Houstek J. The function and the role of the mitochondrial glycerol-3-phosphate dehydrogenase in mammalian tissues. *Biochim Biophys Acta.* 2013; 1827: 401-10.
19. Zand RS, Jenkins DJ, Diamandis EP. Steroid hormone activity of flavonoids and related compounds. *Breast Cancer Res Treat.* 2000; 62: 35-49.
20. Bhathena SJ, Velasquez MT. Beneficial role of dietary phytoestrogens in obesity and diabetes. *Am J Clin Nutr.* 2002; 76: 1191-201.
21. Bryzgalova G, Gao H, Ahren B, Zierath JR, Galuska D, Steiler TL, *et al.* Evidence that oestrogen receptor-alpha plays an important role in the regulation of glucose homeostasis in mice: insulin sensitivity in the liver. *Diabetologia.* 2006; 49: 588-97.
22. Yi KW, Shin JH, Seo HS, Lee JK, Oh MJ, Kim T, *et al.* Role of estrogen receptor-alpha and -beta in regulating leptin expression in 3T3-L1 adipocytes. *Obesity (Silver Spring).* 2008; 16: 2393-9.

23. Park CJ, Zhao Z, Glidewell-Kenney C, Lazic M, Chambon P, Krust A, *et al.* Genetic rescue of nonclassical ERalpha signaling normalizes energy balance in obese Eralpha-null mutant mice. *J Clin Invest.* 2011; 121: 604-12.
24. Han SI, Komatsu Y, Murayama A, Steffensen KR, Nakagawa Y, Nakajima Y, *et al.* Estrogen receptor ligands ameliorate fatty liver through a nonclassical estrogen receptor/Liver X receptor pathway in mice. *Hepatology.* 2014; 59: 1791-802.
25. Dixen K, Basse AL, Murholm M, Isidor MS, Hansen LH, Petersen MC, *et al.* ERRgamma enhances UCP1 expression and fatty acid oxidation in brown adipocytes. *Obesity (Silver Spring).* 2013; 21: 516-24.
26. Basu A, Sanchez K, Leyva MJ, Wu MY, Betts NM, Aston CE, *et al.* Green Tea Supplementation Affects Body Weight, Lipids, and Lipid Peroxidation in Obese Subjects with Metabolic Syndrome. *Journal of the American College of Nutrition.* 2010; 29: 31-40.
27. Datau EA, Wardhana, Surachmanto EE, Pandelaki K, Langi JA, Fias. Efficacy of *Nigella sativa* on serum free testosterone and metabolic disturbances in central obese male. *Acta Med Indones.* 2010; 42: 130-4.
28. Hosseini B, Saedisomeolia A, Wood LG, Yaseri M, Tavasoli S. Effects of pomegranate extract supplementation on inflammation in overweight and obese individuals: A randomized controlled clinical trial. *Complement Ther Clin Pract.* 2016; 22: 44-50.