DIETARY COCOA (*THEOBROMA CACAO*) SUPPLEMENTATION IMPROVES 
OBESITY-RELATED FATTY LIVER DISEASE IN MICE

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

Doctor of Philosophy

December 2016
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ABSTRACT

Nonalcoholic Fatty Liver Disease (NAFLD), defined in 1980s, is a liver disease that histologically mimics alcoholic hepatitis but occurs in individuals who do not abuse alcohol. NAFLD is an increasingly recognized condition that may progress to end-stage liver disease; it can be characterized by fat accumulation in liver exceeding 5–10% by weight. NAFLD is classified into two stages: simple fatty liver (i.e. steatosis) and non-alcoholic Steatohepatitis (NASH). The later stage, NASH, has drawn more cautions because its can evolve to more advanced liver damage, and cannot be treated effectively so far. “Two hit hypothesis” has been proposed to explain the progression from simple steatosis to NASH. According to this hypothesis, steatosis is the “first hit” which increases the vulnerability of the liver to various “second hit” such as chronic inflammation, oxidative damage, and mitochondrial dysfunction, which are the characteristics of NASH. It is expected that food components targeting at “second hit” have therapeutic properties and may help prevent the pathogenesis process of NASH.

Cocoa (or cacao) is a product of Theobroma cacao fruit, which grows in the equatorial tropics including Central and South America, West Africa, and Malaysia. Cocoa has a long history in use, to treat numbers of disorders such as angina and heart pain. Recent studies have confirmed various health-beneficial effects associated with cocoa products including their multifactorial ability to modulate immune response, anti-inflammatory, anti-radical and anti-carcinogenic properties. Considering the pathogenesis of NAFLD and NASH, the multifactorial effects of cocoa may make it a promising candidate in preventing obesity-related fatty liver disease.

To study the protective effect of cocoa, a high-fat-diet-fed (HF) C57BL/6J mouse model is applied. This model is advantageous to investigate metabolic fatty liver disease due to two reasons. Firstly, steatosis developed in healthy livers in this model. Secondly, after steatosis,
inflammatory process is triggered by releasing proinflammatory cytokines and generating oxidative stress that lead to hepatocyte apoptosis. This model allows observing the pathogenesis from simple steatosis to NASH. In our experiments, mice were fed with high fat diet (60% calorie from fat) for 8 weeks to induce steatosis. After 8 weeks, mice were randomly divided into two groups: high fat-high fat cocoa (HF-HFC) and HF group. In HF group, mice were still maintained with high fat diet for additional 10 weeks; in HF-HFC group, mice were treated with 8% cocoa supplemented with high fat diet.

In this research, we hypothesized that cocoa supplementation can ameliorate obesity related NAFLD through modulating lipid metabolism pathway, boosting endogenous antioxidant defense capacity, enhancing mitochondria biogenesis, and reducing chronic inflammation in mice. To test our hypothesis, we proposed three specific aims: **Specific Aim 1:** Evaluation of protective effect of cocoa supplementation on hepatic lipid metabolism pathway in mice model of NAFLD (discussed in chapter 2). **Specific Aim 2:** Evaluation of protective effect of cocoa supplementation on hepatic oxidative stress and mitochondrial function in mice model of NAFLD (discussed in chapter 3). **Specific Aim 3:** Evaluation of protective effect of cocoa supplementation on liver Kupffer cell differentiation in mice model of NAFLD (discussed in chapter 4).

In chapter 2, we demonstrate that cocoa-treated obese mice decrease triglycerides (TAG), an important diagnostic marker of NAFLD, by enhancing fatty acid disposal and reducing lipogenesis in the liver. In chapter 3, we demonstrate that the beneficial effects of cocoa supplementation on NAFLD is through increasing mitochondrial biogenesis and antioxidant response signaling. In chapter 4, we demonstrated that dietary cocoa is able to alternatively activate M2 KCs in HF-fed mice, and this phenotypic switch protects mice from ongoing of liver damage compared with HF treated alone.
In conclusion, in this dissertation, we demonstrated that cocoa supplementation did mitigate obesity-related fatty liver disease, and could be useful in preventing the progression of hepatic steatosis to NASH. These effects are caused by three mechanisms: modulating lipid metabolism pathway, boosting endogenous antioxidant defense capacity, increasing mitochondria biogenesis and reducing chronic inflammation. It is clear that dietary cocoa could be a potential dietary intervention to modulate obesity-related fatty liver disease.
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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Dr. Joshua Lambert, for giving me an opportunity to be his graduate student. I appreciate his constant supervision, support, tolerance and trust during my Ph.D. He always encourages me and lets me keep positive on my study, especially when I feel frustrating on my data. Whenever I have a difficulty or happy news in my life, he is always the person I want to share with. There is no doubt in my mind that I lucked out in having such a great mentor. With his advice and help, I grown as an independent researcher; I have also learnt about persistence in research, inter-disciplinary collaboration, and various aspects of leadership.

I would like to thank my dissertation committee members, Dr. Gregory R. Ziegler, Dr. Gregory Shearer, and Dr. Jairam Vanamala, for their valuable suggestions on my dissertation work. I would also like to thank Dr. Elias for being my candidacy and comprehensive committee, and for the valuable advice on my project. Their thoughts and opinions on my research give me direction and made me think deeper about what I was doing.

I would like to acknowledge Dr. Kamil Borkowski for his kind help with analysis of oxylipins, Dr. Kevin Harvatine and Jackie Yun Ying for their help with the analysis of liver fatty acid profile. I am thankful to my lab mates Shannon Glisan, Karma Jamse, Weslie Khoo, Kuier Zhao, Benjamin Chrisfield, and Qiaoqiao Dai for their support, guidance, input, critiques, and discussions throughout my project. Special thanks to Yeyi Gu and Ling Tao, who helped me when I first started; my best friend Lingzi Xiaozi, who gives me support and encouragement in my study and life.

I would also like to express my gratitude to my parents Mr. Guixiang Sun and Mrs. Xiujuan Li for their love, caring, and constant encouragement that has made this dissertation possible. My special thanks go to my husband Yuliang Xie, my son Andrew Xie, who have
accompanied me and made my life outside the Ph.D study joyful and colorful. My sincerest thanks also go to my friends who have always been there for me.
Chapter 1

Introduction and literature reviews

1.1 Obesity-associated Non-alcoholic fatty liver disease

1.1.1 Definition of Nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease in United States and affects 70 million adults, 30% of the adult population, in the United States\textsuperscript{12}. NAFLD is defined as the accumulation of fatty acid content greater than 5% of liver weight, also known as steatosis\textsuperscript{13}. It includes a spectrum of liver damage ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) which has inflammatory events happening, or even life-threatening form, such as advanced fibrosis, and rarely, progression to cirrhosis\textsuperscript{14}. Once patients with simple steatosis develop NASH, up to 50% of them could develop advanced fibrosis\textsuperscript{15}. Most patients with NAFLD have simple hepatic steatosis without progression to steatohepatitis and fibrosis. However, in 2-3% of patients, NAFLD can progress to NASH that can eventually cause progressive fibrosis and lead to cirrhosis and related complications including hepatocellular carcinoma\textsuperscript{16,17}. The mechanisms underlying disease development and progression are awaiting clarification. Multiple factors including insulin resistance, oxidative stress and subsequent lipid peroxidation, proinflammatory cytokines, adipokines and mitochondrial dysfunction are thought to play key roles in the development and progression of NAFLD\textsuperscript{18-22}. Therefore, the identification of new pharmacological or dietary approaches to effectively prevent, treat, and cure NAFLD is of critical importance.
1.1.2 Hypothesis regarding NAFLD

1.1.2.1 Two Hit hypothesis

Two hypothesis has been proposed to explain the progression of NAFLD to NASH: “two hit model” and “multiple parallel hit model”. The “two-hit” hypothesis was first proposed by Day et al. in 1998\(^3\). According to this hypothesis, steatosis represents the “first hit”, which increases the vulnerability of the liver to various “second hits” such as inflammation, oxidative damage, mitochondrial dysfunction, that in turn lead to the inflammation, fibrosis and cellular death characteristic of NASH\(^4\). Consistent with this hypothesis, administration of variously proposed second hits (e.g. endotoxin and pro-oxidants) results in significantly greater liver damage and lethality in obese mice with fatty liver compared to lean mice with healthy livers\(^{23-25}\).

1.1.2.2 Multiple parallel hit hypothesis

On the contrary of “two hit hypothesis”, it has been suggested that steatosis is not a true ‘first hit’, it interplay with other pathogenetic factors such as insulin resistance, adipokines, adipose tissue inflammation\(^26\). Therefore, the traditional ‘two-hit’ pathophysiological theory has been challenged by “Multiple parallel hit theory”\(^{22,26,27}\). This is so-called multiple parallel hit hypothesis\(^27\). Because simple hepatic steatosis is a benign process in the majority of patients, NASH might be developed in parallel with steatosis with a different pathogenesis. This new model suggests that inflammation may also precede or develop in parallel to hepatic steatosis. Extrahepatic triggers, especially gut-derived and adipose tissue–derived factors may play a central role\(^28\). Inflammation may affect liver function via secreted soluble factors (cytokines and chemokines) leading to the activation of innate immunity, facilitating progression of hepatic steatosis and will in turn exacerbating inflammation.
1.1.3 Pathogenesis of liver steatosis

1.1.3.1 Lipid accumulation/steatosis

NAFLD is characterized by the accumulation of triglycerides, which are formed from the esterification of free fatty acids (FFAs) and glycerol within the hepatocyte. FFAs in the liver come from three distinct sources: lipolysis (the hydrolysis of FFA and glycerol from triglyceride) within adipose tissue, dietary sources, and de novo lipogenesis (DNL)\textsuperscript{29,30}. In contrast, FFA may be utilized either through b-oxidation, re-esterification to triglycerides and storage as lipid droplets, or packaged and exported as very low density lipoprotein (VLDL). Hence fat accumulation within the liver is complex, and can occur as a result of many pathway, such as increased fat synthesis, increased fat delivery, decreased fat export, and/or decreased fat oxidation\textsuperscript{29}.

One major contribution of TAG is esterification of free fatty acids (FFAs) in liver. In healthy individual, FFAs are predominantly stored in adipose tissue due to buffering mechanism. However, in individuals with overweight and obesity, overloading FFAs may exceed storing capacity in adipose tissue, thus prompt translocation via circulating system in a form of circulating FFAs. Therefore, the circulating FFAs is considered as a feature of the insulin resistant, metabolic syndrome\textsuperscript{31}. High level of circulating FFAs in plasma can either increase rate of hepatic FFA uptake, or maintain a normal rate of hepatic FFAs uptake, despite increased hepatic FFAs levels\textsuperscript{32}. In addition, circulating FFAs has been confirmed in contribution of deposition of hepatic triacylglycerol. It was found circulating FFAs, derived by adipose tissue, contributed to 82% and 62% of the total circulating pool in the fasted and fed states, respectively. In turn, they provided 59% and 62% of FFAs in liver and circulating triacylglycerol-rich lipoprotein particles, respectively\textsuperscript{33}. 
In addition to adipose tissue derived FFAs, *de novo* lipogenesis (DNL) is another important source of FFAs. DNL is the synthesis of fatty acid chains from acetyl-CoA subunits produced from a number of different pathways within the cell, most commonly carbohydrate catabolism. The lipogenic pathway plays an important role in NAFLD due to its contribution of FFAs in the liver. Using isotope tracking method, Donnelly et al. (2005) were able to conclude that 59% of TAG in the livers of patients with NAFLD were from serum FFAs, 26% from DNL and 15% from the diet. This study indicated DNL secondary to circulating FFAs contribute to the accumulation of hepatic fat in NAFLD.

1.1.3.2 FFA induced hepatic lipotoxicity

Most circulating FFAs come from adipose tissue, since adipocytes has capacity to store excess FFAs in the form of triglyceride in lipid droplets. But when adipose tissue loses this buffering capacity, FFAs transport to liver in the form of circulating FFAs. It is now recognized that circulating FFAs play a role in promoting liver injury. Circulating FFAs are the primary contributor to the liver triacylglycerol (TG) content, and plasma FFAs levels are correlated with disease severity in NAFLD patients. However, since FFA flow in various hepatocellular compartments and participate in many metabolic pathways, it has been difficult to prove whether FFAs or their metabolites are responsible for the cellular injury seen in lipotoxicity.

FFAs can induce lipotoxicity through multiple pathways. To begin with, saturated fatty acids can serve as activating ligands for toll-like receptor-4, leading to a cascade of events precipitating apoptosis. One study demonstrated that inhibition or loss of function of toll-like receptor-4 can prevent steatohepatitis in mouse models. In addition, FFAs induce c-jun N-terminal kinase (JNK)-dependent activation of apoptotic Bcl-2 proteins Bim and BCL-2-associated X protein, which trigger the mitochondrial apoptotic pathway.
In addition, lipids can activate transcription factors to initiate metabolic, inflammatory, and innate immune response. Several lipid activated transcription factors seem to be crucial for modulating the intersection of these pathways, particularly peroxisome-proliferator activated receptor (PPAR) and liver X receptor (LXR)\textsuperscript{42,43}. Activation of these transcription factors can protect metabolic cell from lipid overload and inhibits the expression of several genes involved in inflammatory response in macrophages and adipocytes\textsuperscript{44}. The PPAR family is composed of three proteins: PPAR-\(\alpha\), PPAR-\(\delta\) (also known as PPAR-\(\beta\)) and PPAR-\(\gamma\). They expressed in different tissues and has different or overlapped functions. This family has been shown to modulate various cellular functions related to metabolic syndrome, including adipocyte and macrophage differentiation, fatty-acid oxidation, glucose metabolism and inflammatory gene expression\textsuperscript{42}.

PPARs are activated by various xenobiotic and endogenous ligands and exert either positive or negative feedback on metabolic and inflammatory genes expression. The ligands for PPARs including unsaturated fatty acids, eicosanoids, components of oxidized low-density lipoproteins (LDLs) and very-low-density lipoproteins (VLDLs) and metabolites of linoleic acid\textsuperscript{42,45}.

PPAR-\(\alpha\) is highly presented in metabolic cells with high requirement of high fatty acids metabolism, such as liver, kidney, heart, muscle, intestinal mucosa\textsuperscript{46}. Many PPAR-\(\alpha\) target genes are involved in mitochondrial and \(\beta\)-oxidation of fatty acids, including those encoding carnitine palmitoyltransferase 1B\textsuperscript{47} and acyl-coenzyme A oxidase\textsuperscript{48}. PPAR-\(\alpha\) -null mice subjected to high fat diet 3 weeks developed steatosis with increased liver-body weight ratio, inflammatory response (TNF-\(\alpha\) and iNOS) and lipid peroxidation compared with the wild type\textsuperscript{49}. In addition, PPAR-\(\alpha\) also controls extend and duration of inflammation via a feedback loop. For example, Leukotriene B4 (LTB4) is a potent lipid mediator of inflammation and natural ligand for PPAR-\(\alpha\). Binding of Leukotriene B4 to PPAR-\(\alpha\) induced the gene expression related to beta or omega oxidation, which in turn inactive Leukotriene B4 through oxidation\textsuperscript{50}.
PPAR-\(\gamma\) is mainly expressed in brown and white adipose tissue, the colon, differentiated myeloid cells and the placenta\(^{51}\). PPAR-\(\gamma\) has a distinct function in regulating adipocyte and is essential for the formation of adipose tissue \textit{in vivo}\(^{52,53}\). For instance, PPAR-\(\gamma\)2 expression activate the adipogenesis in multiple fibroblastic cell lines, including BALB/c 3T3, 3T3-C2, Swiss 3T3, 3T3-F442A and NIH 3T3 cells\(^{52}\). Another function of PPAR-\(\gamma\) is to inhibit inflammatory gene expression by binding to its agonist. For example, in a rat model of polymicrobial sepsis, I.P injection of 15-deoxy-\(\Delta^{12,14}\)-PGJ2 (15d-PGJ2) (1 mg/kg) significantly increase survival rate by 82% and dramatically serum level of decrease IL-6 (50 %), IL-10 (90% ) and TNF-a (45%) compared with vehicle group. This beneficial effect of PPAR-\(\gamma\) is associated with reduced DNA-binding activity of NF-\(\kappa\)B via the degradation of I\(\kappa\)B complex\(^{54}\). Finally, PPAR-\(\gamma\) also plays a role in macrophage differentiation. Deficiency of PPAR-\(\gamma\) in immune cells favors expression of classical macrophage (M1) and impairs alternative macrophage (M2) markers in adipose tissue\(^{55}\).

1.1.3.3 Oxidative stress

Oxidative stress refers to various deleterious processes resulting from an imbalance between the excessive formation of pro-oxidants (ROS and/or reactive nitrogen species (RNS)) and limited antioxidant defenses\(^{56}\). “ROS” is a collective term that broadly describes a variety of molecules and free radicals (chemical species with one unpaired electron) derived from molecular oxygen: singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl. ROS are essential at low levels for molecular signaling but increased levels are toxic since they can damage virtually all biomolecules including lipids, proteins and nucleic acids\(^2\).

Within the liver, there are three potential sites for ROS generation: mitochondria, endoplasmic reticulum and peroxisomes. In addition, various enzymes, including oxidases and
oxygenases, can also generate ROS as part of their enzymatic reaction\textsuperscript{57}. Among those sources, mitochondria is considered to be the major source of ROS production in eukaryotic cells, particularly from the redox state of the respiratory chain\textsuperscript{58}. Mitochondria are eukaryotic organelles involved in various essential functions including energy transduction, iron/sulfur cluster synthesis, lipid metabolism and copper homeostasis biogenesis\textsuperscript{59}. This energy feature made mitochondria the major source of a dangerous superoxide anion radical produced by mitochondrial transportation chain\textsuperscript{60}. The superoxide anion radical can be converted to other types of (ROS)\textsuperscript{61}.

\subsection*{1.1.3.4 Mitochondrial dysfunction}

Considering the closely relation between mitochondrial dysfunction and pathogenesis of NAFLD, NAFLD has been considered as a mitochondrial disease\textsuperscript{62}. Oxidative stress is an important trigger of mitochondrial dysfunction\textsuperscript{2}. Mitochondrial oxygen consumption produces ROS. Under normal conditions, the amount is only about 1\%–2\%. However, NAFLD induce excessive production of ROS, and usually exceeds the cell’s antioxidant capacity. Excess ROS can attack mitochondrial bio-macromolecules (DNA, protein and lipid), and eventually lead to mitochondria dysfunction. Mitochondrial dysfunction contains many aspects, include ultrastructural lesions, depletion of mitochondrial DNA (mtDNA), decreased activity of respiratory chain complexes, and impaired mitochondrial β-oxidation\textsuperscript{62}. Mitochondrial dysfunction affects the pathogenesis of NAFLD through disbalancing hepatic lipid homeostasis, promoting ROS production and lipid peroxidation, cytokine release and cell death\textsuperscript{53}.
Kupffer cell (KCs) differentiation

KCs are suggested to be one of the major immune effectors in the pathogenesis of NASH. KCs can secrete cytokines and chemokines in responding to inflammation and metabolic stresses by orchestrating local immune responses. In addition, KCs can undergo phenotypic switch in response to various local stimuli and express distinct patterns of surface markers. The phenotype switch of KCs depends on the triggering stimuli and genetic background, it can undergo either a “classical” (Th1 dependent; M1) or “alternative” (Th2 dependent; M2) activation pathway. Macrophage M1 response is an essential part of innate immunity and act as the first responders to pathogens, toxins, and tissue damage. M1 macrophage can produce a class of M1 (Th-1) proinflammatory cytokines, such as tumor necrosis factor-a (TNF-α), r-interferon (IFN- γ), and interleukin (IL)-1β. However, if excessive, the inflammatory response becomes detrimental and undesirably result in tissue damage.

To counteracted toxic effects generated by M1 macrophage, an “alternative” (M2) activation pathway is opened. The activation of M2 macrophage results in a protective phenotype. It promotes maturation of alternatively activated macrophages to counteract excessive inflammation, enhance tissue repair and may have a beneficial role in regulating nutrient homeostasis. For example, recent studies demonstrate that blocking the anti-inflammatory or alternative (M2 or Th-2) activation program of KCs exacerbates obesity-induced insulin resistance and decreases hepatocyte fatty acid oxidation. Those study indicate the beneficial role of M2 macrophage activation in the resolution of NAFLD. Therefore, it expect that increase the population of M2 or shape an environment favors M2 macrophage activation would be a pharmacological strategy to reduce hepatic inflammation. Finally, PPAR-γ also plays a role in macrophage differentiation. Deficiency of PPAR-γ in immune cells favors expression of M1 and impairs M2 markers in adipose tissue.
1.1.3.6 Inflammation

Obesity is also characterized by increased production of cytokines and chemokines and immune cells migration to metabolic tissues. For example, elevated TNF-α RNA expression was observed in adipose tissue from four different rodent models of obesity and diabetes. Local and systematic expression of TNF-α was also increased in those models. This observation provided the first clear link between obesity, diabetes and chronic inflammation. In addition to TNF-α, other inflammatory mediators such as IL-6, IL-1β, CCL2, monocyte chemoattractant protein-1 (MCP-1) are produced in adipose tissue with increasing obesity. MCP-1 is produced predominantly by macrophages and endothelial cells and is a potent chemotactic factor for monocytes. Mice engineered to express an MCP-1 transgene in adipose tissue under the control of the aP2 gene promoter exhibited macrophage infiltration into adipose tissue.

1.1.3.7 Endotoxin

Plasma Lipopolysaccharides (LPS) is continuously produced in the gut by Gram-negative intestinal microbiota. The killing of Gram-negative bacteria in vivo lead to the release of LPS, which can cause an bacterial sepsis and septic shock. The mechanism about how LPS is transported from intestine to target tissues has been well studied. It was found that translocation of LPS through impermeant intestinal epithelial barrier occurs after phagocytosis by the enterocytes via TLR4 dependent mechanism. The transportation of LPS from gut lumen to target tissue is facilitated by newly formed chylomicrons, a lipoprotein whose synthesis is sensitive to high fat feeding. In addition, factors (e.g. high fat feeding and alcohol drinking) that increase intestine permeability may contribute to elevated LPS release into blood stream.
Previous study has found that a 4-week high-fat diet chronically increased plasma LPS concentration two to three times, a threshold that they defined as metabolic endotoxemia. This concentration is still far from the values that could be reached during septicemia or other infections. For example, chamber fluid samples from dorso-lumbar region were collected through C57BL/6J mice inoculated with the wild-type P. gingivalis strain. The concentration of LPS in those mice was increased 60 times from day 1 to day 3 when compared with basal level (10 EU/ml on day 1 to 600 EU/ml on day 3). Though low, persistent endotoxemia exposure may cause aggravated systematic infections involve in multi-organs, such as heart, gut, liver, kidney and adipose. Increasing evidence shows that gut microbiota and bacterial products plays a role in the development of hepatic steatosis and its progression to nonalcoholic steatohepatitis. People use the term gut-liver axis to describe the close relationship between these two organs.

In the liver, TLR4 are expressed in many different cell types including Kupffer cells, hepatocytes, and hepatic stellate cells. TLR4 is the specific receptor for the bacterial endotoxin, which is the key inducer of pro-inflammatory cytokines production (such as TNF-α, IL-6, IL-1β). This LPS-triggered cytokine production mainly reply on the activation of the transcription factors NF-kB, AP-1 and LPS-induced TNF-α factor (LITAF) in the liver. In addition, leptin may directly affect hepatic CD14 expression, then lead to hypersensitivity to LPS. In this study, serum leptin levels of HFD-fed WT mice were significantly higher than those in chow-fed WT mice, in parallel with hepatic CD14 expression.

1.1.4 Animal model of NAFLD

To study the pathogenesis of steatosis and steatohepatitis, numerous different rodent models that exhibit histological evidence of hepatic stetaosis are investigated. An idea animal model should resemble hepatic histopathology and pathophysiology of human NAFLD/NASH.
Under this notion, the true feature of steatosis should include accumulation of TAG in the hepatocytes, whereas in steatohepatitis should show inflammation in addition to simple steatosis. Furthermore, the animal model should show metabolic syndrome such as obesity, insulin resistance, fasting hyperglycemia, dyslipidemia, and altered adipokine profile. It is ideal if an animal model can completely fulfill these conditions. Numerous animal models of NAFLD/NASH have been reported to date; however, no animal model is able to completely reflects hepatic histopathology and pathophysiology of human NAFLD/NASH due to natural difference between human and model animals. Thus, researchers need to carefully choose a specific animal models that better meet the aim of study. Research models of NAFLD/NASH may be divided into three broad categories nutritional models, genetic models, and combination models of genetic and nutritional factors.

1.1.4.1 A model of genetically determined leptin deficiency (the ob/ob mouse)

Mutant obese mice (ob/ob) mice possess a spontaneous mutation in the leptin gene (leptin-deficient). Leptin is 16kDa adipokine that produced by white adipose tissue and acts on the hypothalamic ventral median nucleus to generates its notable anorexic effects. ob/ob mice are hyperphagic, inactive, and extremely obese, and show hyperglycemia, insulin resistance, and hyperinsulinemia. This ob/ob mice has several features. Firstly, unlike the human NAFLD population, ob/ob mice develop steatosis spontaneously, but not steatohepatitis. Secondly, it need secondary hit the provoke the transition from steatosis to steatohepatitis, like a methionine- and choline-deficient (MCD) diet, a high fat (HF) diet, or low-dose lipopolysaccharide (endotoxin) in ob/ob mice. Thirdly, fatty liver b-oxidation is compromised, increased PPARα activity in ob/ob mice has not been demonstrated in this model. Finally, leptin as an essential
mediator of hepatic fibrogenesis, and Leptin-deficient mice failed to develop fibrosis during steatohepatitis or in response to chronic toxic liver injury\textsuperscript{84}.

\subsection*{1.1.4.2 A model of genetically determined leptin deficiency (the \textit{db/db} mouse)}

Db/db mice possess a natural mutation in the leptin receptor (Ob-Rb) gene, mapped to mouse chromosome 4\textsuperscript{85,86}. These mice have normal or elevated levels of leptin but are resistant to the effects of leptin\textsuperscript{85}. These mice are obese, insulin resistant, and diabetic, and develop macrovesicular hepatic steatosis under normal conditions and readily develop NASH upon the addition of a second hit, for example, by feeding of an MCD diet\textsuperscript{9,87,88}. MCD diet-fed db/db mice exhibited significantly greater histological inflammation, higher serum alanine aminotransferase levels and marked pericellular fibrosis than ob/ob mice\textsuperscript{88}. Thus, common feature of the ob/ob and db/db mice is that these two phenotypes exhibited human condition of metabolic syndrome in many aspects\textsuperscript{9}.

\subsection*{1.1.4.3 Peroxisome proliferator-activated receptor-\textit{α} knockout mice}

The Peroxisome proliferator-activated receptor (PPARs) are nuclear receptors that bind to fatty acid-derived ligands and activate the transcription of genes that regulate lipid metabolism. Three PPAR isoforms have been described: PPAR\textsubscript{α}, δ (or β), and γ. The primary sites of PPAR\textsubscript{α} includes liver, heart, muscle, and kidney. PPAR\textsubscript{α} activates a program of target gene expression associated with peroxisomal, mitochondrial, and microsomal fatty acid oxidation in the liver\textsuperscript{89}. PPAR\textsubscript{α}-null mice chronically fed a high fat diet showed a massive accumulation of lipid in their livers due to the inhibition of fatty oxidation\textsuperscript{90}. 
1.1.4.4 *Acyl-coenzyme A oxidase null mice*

Acyl-coenzyme A oxidase (AOX) is the key enzyme of peroxisomal β-oxidation of long chain fatty acid (LCFA). AOX null (AOX -/-) mice have defective peroxisomal β-oxidation of LCFA, which accumulate in the liver and exhibit extensive microvesicular steatohepatitis\(^91\). In AOX -/-mice, microvesicular fatty change is able to be observed in liver cells at 7 days. By the end of 2 months of age livers, liver showed extensive steatosis\(^92\). By 4 to 5 months of age, AOX -/-mice has elevated PPARα, cytochrome P450 (Cyp) 4a10, and Cyp4a14 expression, and \( \text{H}_2\text{O}_2 \) levels. However, compensatory mechanism drives hepatocellular regeneration with increase in FA oxidation and reversal of hepatic steatosis by 6 to 7 months of age there is a compensatory. The AOX null mice will finally develop adenomas and carcinomas by 15 month of age\(^93\).

1.1.4.5 *PTEN 10 null mice PTEN*

The phosphatase and tensin homolog deleted on chromosome 10 (PTEN 10) tumor suppressor is a phosphatase that whose major substrate is phosphatidylinositol- 3,4,5-triphosphate (PIP3)\(^9\). PTEN 10 antagonizes the phosphoinositol-3-kinase/AKT signaling pathway and suppresses cell survival as well as cell proliferation\(^94\). Liver-specific Pten knockout mice (AlbCrePTEN flox/flox mice) showed massive hepatomegaly and steatohepatitis with triglyceride accumulation, a phenotype similar to human NASH\(^95\). Mice with global knockout for PTEN are not viable, dying during embryonic development. However, pten knockout mice are hypersensitive to insulin, which is the limitation of these mode\(^96\).
1.1.4.6 SREBP-1c transgenic mice

Sterol regulatory element–binding protein 1c (SREBP-1c) is a transcription factor that is involved in adipocyte differentiation. Transgenic mice overexpressing nuclear SREBP-1c (nSREBP-1c) in adipose tissue spontaneously develop steatohepatitis. By the end of 20 weeks or older, liver featured with mononuclear cell infiltration, pericellular fibrosis, ballooning degeneration, histology similar to NASH. This model can spontaneous develop of NASH without special nutrition. This model is useful in study of lipodystrophy associated steatohepatitis. However, the limitation is obvious with the absence of obesity and low level of leptin.

1.1.4.7 Methionine and choline deficiency (MCD) diet model

The MCD diet contains high sucrose and fat (40% sucrose, 10% fat), but lacks choline and essential amino acids methionine. In addition, choline deficiency impairs hepatic VLDL secretion. Impaired VLDL secretion decrease lipid disposal and lead to fatty acid accumulation in the liver. Compared to other animal nutritional model, MCD model cause more severe form of NASH with more inflammation, oxidative stress, mitochondrial damage, apoptotic cell. However, the severity of this model depends on species, strain and gender of animals. Male C57BL/6 mice developed the most inflammation and necrosis, and best approximated the histological features of NASH. The main disadvantage of the MCD model is MCD diet show significant weight loss (often, more than 20% weight loss after 3 wk, 50% weight loss after 10wk), low plasma TG, no insulin resistance and reduced liver/body weight ratio. To improve these problems, MCD diet are usually added to the diet of genetically obese mice, such as ob/ob and db/db mice, as a second hit to induce NASH. The main advantages is the MCD diet
provide the best response for study the inflammatory and fibrotic elements of the NAFLD spectrum.

1.1.4.8 High fat (HF) diet model

A HF diet mouse model is induced by an HF diet containing 71% of energy from fat, 11% from carbohydrates, and 18% from proteins\(^{101}\). Sprague-Dawley rat fed the high-fat diet ad libitum for 3 wk developed panlobular steatosis. Like human NASH, these rats have abnormal mitochondria dysfunction, oxidative stress and inflammation. For example, these rats develop insulin resistance and exhibited increased hepatic mRNA of tumor necrosis factor \(\alpha\) and cytochrome P450 2E1 and oxidative stress marker 4-hydroxynonenal\(^{101}\). These changes closely resemble human NASH. In the long term study\(^{102}\), chronic exposure to an HF diet (60% of calories from fat) caused steatohepatitis with excess body weight, hyperinsuliemia and hypercholesteremia in male C57BL/6J mice. In addition, this model showed that steatohepatitis was induced after onset of metabolic abnormalities. Thus, the two hit theory can be used to explain the pathogenesis of NASH in this model.

An HF diet is widely used to cause hepatic steatosis and NASH in experimental animals. However, it seems that the degree of severity of this model varies. Factors such as rodent species and strain, the fat content in the diet, the composition of dietary fat, and the duration of treatment have impact on the response\(^9\). For example, Sprague-Dawley rats is more susceptible to steatohepatitis when fed an HF diet due to their susceptibility to diet-induced obesity\(^{103}\).

In summary, many animal models of NASH have been developed to date. These animal models do not replicate the full spectrum of the disease in humans. Those model has two types, either genetically modified model or diet induced model, or the combination of these two. However, it is worthwhile to mention that neither of these models can fully resemble the
pathogenesis of NASH. Researchers need to choose a model to better address the hypothesis and aims of for their studies. It is hopeful that new animal models that closely reflect the histopathology and pathophysiology of human NASH will be developed in the future. And by the aid of these mode, we will collect more information on pathogenesis and treatment of NASH\textsuperscript{9,10}.

1.2 Sirtuins

Sirtuins are members of the silent information regulator 2 (Sir2) family, a group of Class III histone/protein deacetylases. Sirtuins involved in numerous physiological and pathophysiological situations, including metabolic syndrome, oxidative stress, and lipid metabolism through deacetylation of numerous substrates. There are 7 different sirtuins in mammals (SIRT1-7), of which SIRT1 and SIRT3 is the best known and most studied. These two sirtuins are important to the control of mitochondrial function, which are localized to the nucleus and mitochondria, respectively\textsuperscript{104}. SIRT1 is responsible for regulation of protein activation by means of deacetylating a variety of proteins that play important roles in the pathophysiology of metabolic diseases. Sirtuin 3 (SIRT3) mainly regulate mitochondrial antioxidant system by activating forehead transcription factor 3a (Foxo3a) and deacetylating its substrate associated with antioxidant properties. Recent studies have shown that SIRT expression in the liver is significantly decreased in an NAFLD model of rats fed a high-fat diet, and moderate SIRT1 overexpression protects mice from developing NAFLD\textsuperscript{105}. Therefore, pharmacologic activation of SIRT1, will be a potential therapeutic target for treating NAFLD.
1.3 Beneficial effects of cocoa on NAFLD

1.3.1 Cocoa

Cocoa (or cacao) is the product of the fruit of the *Theobroma cacao* tree, grown in the equatorial tropics mainly in the Central and South America, West Africa and Malaysia. Cocoa has a long history in use. In Europe, various historical documents recorded chocolate’s medicinal value. In history, the primarily application of cocoa is treating number of disorders, including angina and heart pain. Today, cocoa is a global commodity associated with pleasure and often luxury in the form of drinking and eating chocolate. Numerous reports have focused on various health-beneficial effects associated with the consumption of cocoa products.

1.3.2 Manufacturing process of Cocoa

Cocoa plant contains 360 known chemicals. In their natural state, cocoa beans are virtually inedible because of their high concentration of polyphenols, which renders them an extremely bitter flavor. Manufacturing process, particularly roasting, fermentation and drying, can alter content of bioactive compounds. For example, in a final cocoa product such as chocolate, polyphenol content might decrease from 100% to 10% throughout the different manufacturing processes. Among the Cocoa products, Cocoa liquor is a dark brown fluid obtained by grinding cocoa nibs, it contains cocoa butter and cocoa powder. The production of cocoa liquor involves in multiple process, such as cleaning of the seeds, fermentation, drying and roasting. The step most affect flavor characteristics and nutrient profile of the final product is the roasting process. The liquor may then be processed with alkali, also known as Dutch processing or Dutching. The aim of Dutching is to increase pH and improve palatability. Furthermore, the alkalizing stage affects the chemical composition of the cocoa liquor. A summary of this
manufacturing steps is shown in Figure 1-1. The term “cocoa component” is intended to refer to a fraction derived from shell-free cocoa nib and includes chocolate liquor, partially- or fully-defatted cocoa solids, cocoa extracts, cocoa butter and cocoa nib.

1.3.3 Bioactive components in cocoa

Many studies have described phenolics compounds as bioactive components in cocoa. These description is based on health-promotion properties of cocoa phenolics, including
antioxidant, anti-oxidant, antiradical, modulating immune response and anticarcinogen\textsuperscript{6,106}. In addition, cocoa contains a higher content of flavonoids per serving than teas or red wine\textsuperscript{107}. The antioxidant effects of the cocoa may have impact on insulin resistance, reduce the risk for diabetes or activate the gene expression of antioxidant defenses\textsuperscript{107}.

Wollgast and Anklam\textsuperscript{108} reviewed the polyphenol content of cocoa depending on its origin and the manufacturing process of the final product. Three groups of polyphenols can be identified in cocoa beans: monomeric catechins, which constitute about 37\% of the polyphenol content in the beans, anthocyanidins (about 4\%), and proanthocyanidins (PAC) (about 58\%)\textsuperscript{8}. Within the catechins, (−)-epicatechin is the most abundant with the percentage up to 35\%. By contrast, (+)-catechin, (+)-gallocatechin, and (−)-epigallocatechin are present in smaller quantities (Figure 1-2). PAC with a degree of polymerization (DP) up to decamer have been identified and quantified by normal-phase high-performance liquid chromatography (HPLC)/mass spectrometry (MS)\textsuperscript{109,110}. In addition, the amount of PAC in cocoa are at significantly higher levels than in other flavanol-rich foods such as red wine or green tea\textsuperscript{111}. In addition to polyphenols, cocoa contains methylxanthine compounds, predominantly theobromine (approximately 2–3\% by weight, Fig. 1-2) and small amounts of caffeine is also present (0.2\%)\textsuperscript{112}. 

In addition to polyphenol, cocoa also contains a high content of dietary fiber (26-40%, mostly insoluble fiber) which could give additional nutritional value to cocoa. Dietary fiber has well documented beneficial effects on human health, a high intake of dietary fiber was associated with the lower risk of western disease, including chronic bowel disorders, obesity, diabetes, cardiovascular disease and cancer\textsuperscript{113-116}. Nutritional properties derived from regular consumption of cocoa fiber has been assessed in several \textit{in vivo} experiment. For example, supplemented hypercholesterolemic diet with 10% cocoa fiber improved lipidemic profile and reduce lipid peroxidation in a rat model of dietary-induced hypercholesterolemia\textsuperscript{117}.

Figure 1-2: Bioactive compounds in cocoa\textsuperscript{112}
1.3.4 Animal model studies of cocoa

There is a growing body of data derived from animal models demonstrating the hepatoprotective effects of cocoa and cocoa polyphenols in prevention of obesity-related fatty liver disease (Table 1.1). Beneficial effects have been observed with regard to lipid metabolism, VLDL transportation, anti-oxidant, inflammatory biomarkers, serum lipid profile and insulin resistance. However, the underlying physiologic and molecular mechanisms are not yet fully understood, and we found studies regarding the beneficial effects of cocoa on NAFLD is still limited.

1.3.4.1 Lipid metabolism

One study has shown that short-term cocoa polyphenol treatment with a high dose could attenuate liver steatosis in part by modulating AMPK signaling pathway. There beneficial effects are likely due to several mechanisms. Firstly, CPs treatment significantly increased AMPK activation at the protein level, as shown in elevation of p-AMPKβ1/2. AMPK phosphorylation/activation switches on fatty acid oxidation and switches off lipogenesis-related genes. Secondly, cocoa increase enhanced fatty acid β-oxidation in the liver of obese rats and down-regulates genes related to the key enzymes of fatty acid synthesis (Fasn and Scd1).

1.3.4.2 Anti-oxidant effects

The endoplasmic reticulum (ER) is an important player in regulating protein synthesis and lipid metabolism. Alteration in endoplasmic reticulum(ER) stress in the liver may disturbs lipid metabolism and promotes fatty acid accumulation. One study found cocoa supplymentation (0.2 and 2%) significantly suppress mRNA expression of ER stress-related...
genes, including activating transcription factor 6 (ATF6), hypoxia upregulated 1 (Hyou1), and x-box-binding protein 1 (XBP1) compared with western diet group in apoE KO mice. In the same experiment, they found cocoa modulate lipid metabolism by upregulating mRNA expression of lipid metabolism related transcription factors and genes, including carbohydrate response element binding protein (chREBP1), sterol regulatory element binding proteins 1(Srebp-1), liver X nuclear receptor alpha variant 1(LXRα), PPARα, fatty acid binding protein 4 (Fabp4), perilipin 2, and LPL. Another study suggested that cocoa could have anti-oxidant effect within the circulating system, especially within RBC’s. 12.5% Cocoa supplementation was associated with partial attenuation of oxidative stress induced by the methionine choline deficient diet in female Sprague Dawley rats. To be specific, red blood cell glutathione was increased by cocoa supplementation, whereas liver glutathione (GSH) was reduced by cocoa compared to methionine choline deficient diet fed animals.

1.3.4.3 Anti-inflammation

Guan et al demonstrated that intake of 0.2% and 2% of cocoa powder improved hypercholesterolemia and inhibited aortic atherosclerosis in apoE KO mice. They found inflammatory reaction genes, tumor necrosis factor alpha and monocyte chemo-attractant protein 1, were significantly down-regulated in the cocoa powder-fed groups.
Table I-I: Animal experiments

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Control</th>
<th>Treatment</th>
<th>Outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Standard diet</td>
<td>HF + CP (600mg/kg bw/day)</td>
<td>↓ Liver weight, total lipid, triglycerides and cholesterol ↓ Scd1 and Fasn gene expression ↑ PPARα, Cpt1, and prkaa1 ↑ p-AMPKβ1 gene expression</td>
<td>118</td>
</tr>
<tr>
<td>7</td>
<td>Standard diet</td>
<td>cocoa+soy+oats+fishoil + (HC+F) (hypercholesterolaemic diet supplemented with 10mL kg⁻¹ BW of 600 g kg⁻¹ fructose solution)</td>
<td>↓ steatosis</td>
<td>124</td>
</tr>
<tr>
<td>9</td>
<td>Standard diet</td>
<td>Standard diet +10% cocoa powder</td>
<td>↑ reduce level of insulin resistance</td>
<td>125</td>
</tr>
<tr>
<td>9</td>
<td>Standard diet</td>
<td>Standard diet +10% cocoa powder</td>
<td>Nrf2 and NF-κB</td>
<td>126</td>
</tr>
<tr>
<td>4</td>
<td>Purine chow</td>
<td>HF+10% cocoa</td>
<td>↑ catalase activity, gene of CYP4a10, PDK4, ACOX1 ↓ liver weight, TAG, ipid droplets, ↓ SCD1 gene and protein level PPARα gene expression slightly ↑</td>
<td>127</td>
</tr>
<tr>
<td>12</td>
<td>Western diet (21% fat)</td>
<td>Western diet+0.2% cocoa Western diet+2% cocoa</td>
<td>↓ lipid ATF 6, Hyou 1, and XBP1 ↑ chREBP1, Srebp-1, LXRα, PPARα, Fabp4, perilipin 2, and LPL.</td>
<td>123</td>
</tr>
<tr>
<td>Sprague–Dawley rats</td>
<td>3 days</td>
<td>R36 Standard diet</td>
<td>cocoa butter- or safflower oil-enriched R36 diets</td>
<td>↓ SCD1 gene expression</td>
</tr>
<tr>
<td>---------------------</td>
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<td>------------------</td>
<td>-----------------------------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Female Sprague Dawley rats</td>
<td>28, 56, 80, and 108 days</td>
<td>MCS diet</td>
<td>MCD diet+12.5% cocoa powder</td>
<td>↑ LFABP mRNA and Protein Expression ↓ RBC GSH, liver weight, circulating TAG</td>
</tr>
</tbody>
</table>
1.3.5 Human intervention study

Human intervention study on Cocoa has received much attention over recent years. Cocoa and cocoa containing product, especially dark chocolate, represents considerably higher concentrations of flavonoids per serving than tea, apple and red wine\textsuperscript{107}. Because of that, dark chocolate has higher antioxidant capacity and provide more contribution to its beneficial health effects\textsuperscript{129}. However, most studies are focus on metabolic syndrome, studies regarding beneficial function of cocoa on NAFLD and NASH is limited. Considering the close relationship between metabolic syndrome and NAFLD, it is reasonable to expect that cocoa may also benefits liver steatosis. Previous studies found the association between daily chocolate consumption and lower BMI. For example, in recent prospective study, with 3-year follow-up among postmenopausal American women aged 50–79 years, found a direct association between chocolate–candy consumption (1oz per day) and weight gain\textsuperscript{130}. 
Chapter 2

Dietary cocoa modulates hepatic fatty acid composition and lipid metabolism in high fat fed mice

2.1 Abstract

The hallmark of Nonalcoholic fatty liver disease (NAFLD) is hepatic neutral lipid accumulation, mainly triacylglycerol, in the absence of significant ethanol consumption. The regulation of triglyceride content in liver is complicated, which results from an imbalance between lipid availability (via circulating lipid uptake or de novo lipogenesis) and lipid disposal (via free fatty acid oxidation or triglyceride-rich lipoprotein secretion). We hypothesize cocoa supplementation is able to modulate this balance and reduce liver steatosis. To determine the protective effects of cocoa on hepatic lipid metabolism, mice were fed with high fat diet for 8 weeks to induce steatosis. After 8 weeks, the animals were fed with 8% cocoa supplemented high fat diet for additional 10 weeks. We found mouse fed with Cocoa significantly decreased the hepatic gene expression of Atgl and Hsl which were associated with lipolysis. By contrast, gene expression of Cpt1α were significantly increased in cocoa fed mouse compared with control group. In addition, dietary cocoa significantly reduced SCD1 activity expressed as product/precursor ratio (C16:1/C16:0 and C18:1/C18:0), were significantly suppressed. These data revealed a novel hepatoprotective effect of dietary cocoa in the context of obesity-related fatty liver disease.
2.2 Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by excessive energy and fat intake, insulin resistance, and/or altered lipid metabolism\textsuperscript{131}. The prevalence of NAFLD is strongly associated with several risk factors of metabolic syndrome (MetS) such as obesity, dyslipidemia, hypertension and type 2 diabetes. NAFLD, a multistage and progressive disease, begins with lipid accumulation in hepatocytes (hepatic steatosis) and hepatic steatosis with a necro-inflammatory component (non-alcoholic steatohepatitis, abbreviated as NASH), and might finally progresses to more advanced liver damage, such liver cirrhosis.

Triglyceride (TAG) is an important diagnostic marker of NAFLD. In early stage of NAFLD, TAG presents in a form of large vacuole within cytosol. TAG level is determined by a balance between fatty acids accumulation and triglyceride disposal. The fatty acids accumulation is through \textit{de novo} lipogenesis in the liver and non-esterified fatty acids from adipose tissue; while the triglyceride disposal is through mechanisms of fatty acid oxidation and secretion of triglyceride-rich lipoproteins. In patient with NAFLD, this balance is usually disturbed, favoring fatty acid accumulation through four major ways: increased uptake of non-esterified fatty acids, increased \textit{de novo} fatty acid synthesis, decreased mitochondrial beta-oxidation or elevated \textit{de novo} lipogenesis. All of these change will accelerate the development of steatosis, an early stage of NAFLD.

One major contribution of TAG is from esterification of free fatty acids (FFAs) in liver. In healthy individual, FFAs are predominantly stored in adipose tissue due to buffering mechanism. However, in individuals with overweight and obesity, overloading FFAs may exceed storing capacity in adipose tissue, thus prompt translocation via circulating system in a form of circulating FFAs. Therefore, the circulating FFAs are considered as a feature of the insulin resistant, metabolic syndrome\textsuperscript{31}. High level of circulating FFAs in plasma can either increase rate
of hepatic FFA uptake, or maintain a normal rate of hepatic FFAs uptake, despite increased hepatic FFAs levels\textsuperscript{32}. In addition, circulating FFAs has been confirmed in contribution of deposition of hepatic triacylglycerol. It was found circulating FFAs, derived by adipose tissue, contributed to 82% and 62% of the total circulating pool in the fasted and fed states, respectively. In turn, they provided 59% and 62% of FFAs in liver and circulating triacylglycerol-rich lipoprotein particles, respectively\textsuperscript{33}.

In addition to esterification of circulating FFAs, liver also synthesis TAG by \textit{de novo} lipogenesis. Several enzymes have been reported to play key roles in this process. For example, Stearoyl-CoA desaturase (SCD) is an endoplasmic reticulum enzyme that catalyzes the critical committed step in the biosynthesis of monounsaturated fatty acids from saturated fatty acids. It catalyzes delta-9-desaturation of long-chain fatty acids, leading to biosynthesis of palmitoleic (C16:1) and oleic (C18:1) acids as major products\textsuperscript{132}. Palmitoleic and oleic acids are major monounsaturated FFAs of triglycerides, cholesteryl esters, and membrane phospholipids. In mouse genome, SCD1 and SCD2 are the most well characterized isoforms of SCD. Most organs of different mouse strains express SCD1 and SCD2, whereas liver expresses mainly the SCD1 isoform. Increased SCD1 gene expression and activity is associated with liver steatosis and apoptosis, thus inhibiting of SCD1 activity might be beneficial for the treatment of NAFLD\textsuperscript{133}.

To curb the increased fat accumulation, liver has its own adaptation mechanisms for TAG disposal. One major way is through increase fatty acid disposal via mitochondrial, an optimal place for long chain fatty acid oxidation due to completed oxidation and lower ROS production. Mitochondrial is able to digest long-chain FFAs inside the mitochondrial matrix with the aid of transporting protein, which is a carnitine-dependent enzyme shuttle such as sequentially carnitine palmitoyltransferase-1 (CPT-1), carnitine translocase, and CPT-2\textsuperscript{134}. Within mitochondria, every FFA undergoes four sequential reactions to generate one acetyl-CoA molecule and a shortened
FA. The cycle is repeated to split FFAs into several acetyl-CoA subunits. Acetyl-CoA can then be completely degraded to CO$_2$ by the tricarboxylic cycle.

Medications/foods that targeting at keys stage of TAG accumulation and disposal can be a good candidate to prevent or delay the onset of NAFLD. Important candidates are dietary components, such as vegetables, fruits and beverage. Cocoa has drawn attentions due to its ability to modulate lipid absorption and metabolism. Our lab and other researchers have demonstrated that cocoa polyphenols have lipid-lowering properties, it inhibites the activity of key digestive enzymes, down-regulates gene expression and modulates obesity relate inflammation$^{112,123,135–137}$. These properties indicate that cocoa may have interesting health protective benefits against the obesity-related NAFLD. However, the mechanism for the preventive activities of cocoa related to lipid metabolism in the liver remains largely unknown. Nevertheless, whether cocoa may modulate liver fatty acid composition has never been addressed. The aim of our study in this chapter is to address those questions.

In this chapter, we hypothesized that cocoa supplementation would prevent the hepatic accumulation of TAGs by stimulating lipid hydrolysis, inhibiting lipogenesis and inducing mitochondria beta-oxidation. We use the high-fat-diet-fed (HF) C57BL/6J mouse model to study effect of cocoa on NAFLD, as this model is widely applied to investigate metabolic diseases$^{9–11}$. We fed mice with high fat diet (60% calorie from fat) for 8 weeks to induce steatosis. After 8 weeks, mice were randomly divided into two groups: high fat-high fat cocoa (HF-HFC) and HF group. HF diet mice still maintain high fat diet for additional 10 weeks, whereas HF-HFC mice were treated with 8% cocoa supplemented high fat diet. Furthermore, we measured messenger RNA (mRNA) levels of *Atgl*, *Hsl* and *Cpt1α*. We also determined lipid profile by Gas Chromatograph (GC). Our results showed the mechanisms for prevention of fatty liver by cocoa are likely multifactorial.
2.3 Material and methods

2.3.1 Animals

The experimental design is shown in Figure 2-1. Wild type (C57BL/6J background) was used in this experiment. Mice were housed in a pathogen-free facility (12/12-hour light/dark cycle) and fed by standard diet (LF, 10% Kcal) or high fat diet (HF, 60% Kcal) for 8 weeks. After 8 weeks, half of the HF mice were randomly selected and treated with a HF-diet supplemented with 8% unsweetened cocoa powder (HF-HFC). The rest of mice were maintained with their original diet. Experimental diet compositions are described in one published paper\textsuperscript{135}. Diets were matched for energy and macronutrient content. By the end of 18 weeks, mice were sacrificed, plasma and liver samples were collected for analysis. All collected samples were stored at −80°C until further analysis.

Figure 2-1: Experimental design
2.3.2 Gene expression

Liver RNA was extracted according to our lab protocol and quantified using NanoDrop 1000 spectrophotometer. cDNA was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen, Germany) using 1000 ng of RNA and then amplified using SYBR Green PCR Master Mix. Applied Biosystems 7900HT Fast Real-Time PCR System and relative quantification software (Applied Biosystems, Foster City, CA) were used for the real-time analyses. Calculations were performed by a comparative method (2-ΔΔCT) using GAPDH as an internal control. Primer sequence were listed in table1. *Atgl* and *Hsl* primers sequence were found from one published paper\(^{138}\).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’ → 3’)</th>
<th>Reverse (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Atgl</em></td>
<td>CAACGCCACTCACATCTACGG</td>
<td>TCACCAGGTTGAAGGGAGGAT</td>
</tr>
<tr>
<td><em>Hsl</em></td>
<td>GGCTCACAGTTACCATCTACC</td>
<td>GAGTACCTTGCTGTCCTGTCC</td>
</tr>
<tr>
<td><em>Cpt1a</em></td>
<td>CATTCCAGGAGAATGCCAGG</td>
<td>CTGGCACTGCTTAGGGATGTC</td>
</tr>
<tr>
<td><em>Gapdh</em></td>
<td>TGAAGCAGGCATCTGAGGG</td>
<td>CGAAGGTGGAAGAGTGGGAG</td>
</tr>
</tbody>
</table>

2.3.3 FFAs detection by GC

FFAs levels were detected in liver. Each liver sample was weighed and kept in liquid nitrogen before lipid extraction and methylation. A one-step lipid extraction and fatty acid methyl ester (FAME) preparation was carried out using a modified method from Garces and Mancha\(^{139}\). Sample was heated with a reagent containing methanol, heptane, toluene, 2,2-dimethoxypropane, and H\(_2\)SO\(_4\). During heating, the simultaneous digestion and lipid transmethylation took place in a
single phase. After cooling two phases were formed with the upper phase containing the FAMEs used for GC analysis. FAMEs were quantified using a GC (Agilent 6890 series, Atlanta, GA) equipped with a silica-fused capillary column and a flame ionization detector. Fatty acid peaks were identified in the GC analysis using pure FAME standards (C13 and C19).

2.3.4 Data analysis

All data are presented as the mean ± standard error (SEM). *P < 0.05, **P < 0.01, ***P < 0.001 vs. obese group according to one-way ANOVA with Dunnett’s post-test (HF as control). Correlation were assessed by GraphPad Prism 7.0 (San Diego, CA, USA).

2.4 Results

2.4.1 Effects of cocoa on fatty acid composition and correlation with liver weight and TAG level

In line with our previous study, where significant elevation of TAG and liver weight were detected in HF compared with LF\textsuperscript{135}, we also observed a significant increase in Fat% of mice fed the HF diet at the end of experiment compared to that of the LF-treated mice (**P < 0.001, Figure 2-2a). Dietary cocoa supplementation (HF-HFC group) for 10 weeks significantly decreased Fat% compared to HF-fed control. In addition, we observe a strong positive relation between Fat% with two fatty liver parameters: TAG level and liver weight, indicating the contributing of Fat% in liver TAG content.
2.4.2 Effects of Cocoa on fatty acid profile and SCD1 activity index

Fatty acid composition in total fat fraction was modulated by cocoa supplementation. The proportions of liver palmitic acid (16:0) was not affected, palmitoleic acid (16:1, n-7) significantly decreased in cocoa diet-fed mice compared with HF controls. SCD1 activity can be reflected by product/precursor ratio: C16:1/C16:0 and C18:1/C18:0. Due to higher oleic acid [18:1(n-9)] and lower Stearic acid (18:0), this results in highest oleic: stearic acid ratio (8.79), a marker for stearoyl-CoA desaturase (SCD D9-desaturase) activity in HF mice. The ratio of [18:1(n-9)]/(18:0) was 3.62 and 6.52 in LF and HF-HFC, respectively. In addition, cocoa also decreased SCD1 activity index expressed as ratio of C16:1 and C16:0. This trend was reversed by cocoa treatment, result in a decrease in SCD1 activity to the level near control group.

Figure 2-2: Effects of cocoa on Fat% (a) and its correlation with TG level (b) and liver weight (c). Values are mean ± SEM (n = 6-12). *P < 0.05, **P < 0.01, ***P < 0.001 vs. obese group according to one-way ANOVA with Dunnett’s post-test (HF as control).
2.4.3 Correlation between SCD1 activity and TAG level, Fat% and liver weight

SCD 1 activity index (C18:1/C18:0) is strong and positively correlation with three liver steatosis parameters: Fat% liver fat content, TAG level and liver weight. However, no correlation was found between SCD1 activity index (C16:1/C16:0) with those three parameters.
2.4.4 Effects of cocoa on PUFA profile

NAFLD is associated with numerous changes in the lipid composition of the liver. Although the levels for linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) remained unaltered, arachidonic acid (20:4n-6), eicosapentanoic acid (20:5n-3) and docosahexanoic acid (22:6n-3) were significantly decreased in total fatty acid pool in HF group compared with LF control. Cocoa supplementation reverses the action by HF feeding, slightly elevated AA level and significantly increase EPA and DHA in the total fatty acid pool. The ratios of 20:4 (n-6): 20:3 (n-6), which is markers for D5-desaturase was significantly increased in HF-HFC compared with HF, whereas 18:3 (n-6):18:2 (n-6), marker for D6-desaturase, did not differ (data not shown).

Figure 2-4: Effects of cocoa supplementation on SCD1 activity with different product/precursor and its correlation between C18:1/C18:0 with TG level (a), Fat% (b) and liver weight (c). Values are mean ± SEM (n = 6-12). *P < 0.05, **P < 0.01, ***P < 0.001 vs. obese group according to one-way ANOVA with Dunnett’s post-test (HF as control).
2.4.5 Effects of cocoa lipid hydrolysis gene expression

The mRNA levels of Hsl and Atgl in the liver tissue were determined. Expression of Atgl and Hsl were unaffected (P < 0.05) in HF-fed obese mice compared to the LF-fed lean mice (Fig. 2-6). Supplementation with dietary cocoa remarkably increased the expression of Hsl by 30% (P < 0.05) compared to HF-fed mice (Fig. 2-6).
2.4.6 Effects of cocoa on Cpt1α gene expression

Cpt1α in the liver mitochondrial outer membrane catalyzes the primary step in overall mitochondrial fatty acid oxidation140. We found Cpt1α gene expression of HF-fed obese mice was significantly suppressed compared to LF-fed mice (P < 0.05, Fig. 2-7). Supplementation with dietary cocoa remarkably reverse this trend by increasing the expression of Cpt1α by 76% compared with HF controls.
2.5 Discussion

The protective role of cocoa in metabolic syndrome in various animal models has been observed to different extents\textsuperscript{112,122,135,141}. These beneficial effects are modulated by multiple pathway, such as anti-inflammation, anti-oxidant, anti-obesity and increasing insulin sensitivity. These multi-factorial effects let us to think cocoa may help prevent NAFLD. However, studies regarding the beneficial effects of cocoa on liver steatosis, especially with the emphasis on lipid metabolism is limited. This may impede the application of cocoa on obesity related fatty liver disease. Under this context, we investigate effects of cocoa supplementation on hepatic lipid metabolism, with focus on gene expression of lipolysis enzyme, SCD1 activity and mitochondria beta-oxidation. To the best of our knowledge, we, for the first time, demonstrated that cocoa-treated obese mice favors TAG and fatty acid disposal vs lipogenesis in the liver. In addition, we found cocoa increased the ratio of ω-3 fatty acid, may also contribute to anti-inflammatory effects.
NAFLD is now considered the main hepatic manifestation of obesity and metabolic syndrome\textsuperscript{14}. In fact, data from previous reports supports high fat feeding increase body weight gain and subsequently formation of liver steatosis, featured with enlarged liver and triglycerides accumulation\textsuperscript{135,142}. This is the first event in the pathogenesis of fatty liver disease. Additional event happen when mitochondrial dysfunction and oxidative stress involve in, accelerate the development of more advanced liver damage\textsuperscript{3,26}. In our previous study, we found cocoa can prevent the development of first event, however, the underlying mechanism remains ambiguous. Answering these questions unveil a new insight into the molecular mechanisms underlying the pharmacological effect of cocoa on steatosis biomarkers in obese mice.

In the early stage of NAFLD, TAG presents in the form large vacuoles within the cytosol. TAG levels are the result of the balance between non-esterified fatty acids from the plasma and by de novo lipogenesis, versus triglyceride disposal by fatty acid oxidation and by the secretion of triglyceride-rich lipoproteins. Therefore, ways to attenuate NAFLD including inhibiting lipogenesis, stimulating lipolysis and beta-oxidation, and increasing external transportation by VLDL. However, in NAFLD and NASH patients, this increase in fat export via VLDL may be impaired or insufficient to prevent fatty liver\textsuperscript{143}. Thus, inhibiting lipogenesis, increasing lipolysis and beta-oxidation are thought to be feasible strategies. In our current study, we found cocoa is able to modulate lipid metabolism by targeting those three strategies.

First of all, we found cocoa suppress SCD1 activity. SCD1 is a delta-9 desaturase that catalyzes the de novo biosynthesis of oleate and palmitoleate, which are major substrates for synthesis of triacylglycerol’s and other lipids\textsuperscript{132}. One way to calculate SCD1 activity is product and precursor ratio, we called this SCD1 activity index. We found both two SCD1 activity index as shown in (C18:1/C18:0) and (C16:1/C16:0) were significantly reduced in cocoa treated group compared with HF group. Interestingly, we found strong positive correlation in SCD1 activity (C18:1/C18:0) with liver TAG, fat% and liver weight, but not in SCD1 activity (C16:1/C16:0).
To explain reason why we only found correlation with \((C_{18:1}/C_{18:0})\), we look at the fatty acid profile in the diet by GC. We found \(C_{18:0}\) and \(C_{18:1}\) are the major fatty acid in HF and HF-HFC group compared with \(C_{16:0}\) and \(C_{16:1}\)(as shown in table 2.3). Thus, liver will prefer to use \(C_{18:0}\) and \(C_{18:1}\) as a source for the synthesis of various lipid.

Table 2-2: Fatty acid profile of mice diet

<table>
<thead>
<tr>
<th>Fat acid profile%</th>
<th>LF</th>
<th>HF</th>
<th>HFC</th>
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<tr>
<td>C14:0</td>
<td>0.8660**</td>
<td>1.107</td>
<td>1.095</td>
</tr>
<tr>
<td>C16:0</td>
<td>14.91***</td>
<td>18.88</td>
<td>19.27</td>
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<tr>
<td>C16:1</td>
<td>0.7959***</td>
<td>1.421</td>
<td>1.398</td>
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<tr>
<td>C18:0</td>
<td>6.662***</td>
<td>10.42</td>
<td>11.25</td>
</tr>
<tr>
<td>C18:1 cis9</td>
<td>24.38***</td>
<td>32.87</td>
<td>32.40</td>
</tr>
<tr>
<td>C 18:1 trans11</td>
<td>1.605***</td>
<td>1.934</td>
<td>1.868</td>
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<tr>
<td>C18:2</td>
<td>34.55***</td>
<td>24.21</td>
<td>23.73</td>
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<td>C18:3</td>
<td>4.116***</td>
<td>1.626</td>
<td>1.574</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.2447**</td>
<td>0.1989</td>
<td>0.2182*</td>
</tr>
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<td>C20:1</td>
<td>0.4554***</td>
<td>0.6417</td>
<td>0.5964**</td>
</tr>
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<td>C20:2</td>
<td>0.3121</td>
<td>0.7214</td>
<td>0.6702</td>
</tr>
<tr>
<td>C20:3</td>
<td>0.4024</td>
<td>0.1664</td>
<td>0.3809</td>
</tr>
<tr>
<td>C22:0</td>
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<td>0.1038</td>
</tr>
<tr>
<td>C22:1</td>
<td>0.1652**</td>
<td>0.2335</td>
<td>0.2347</td>
</tr>
</tbody>
</table>

1 Values were compared with One-way ANOVA with Dunnet’s post-test (HF as control). * \(P<0.05\), ** \(P<0.01\), *** \(P<0.001\).

Additionally, we found cocoa supplementation put liver in the position of favoring TAG lipolysis and oxidation. Specifically, cocoa significantly unregulated the expression of two lipolysis enzymes ATGL and HSL, as well as one mitochondrial beta-oxidation transporter, CPT1\(\alpha\). The mobilization of stored intracellular TAG is mediated by HSL and ATGL. These two enzyme proportionally hydrolyze triglycerides and release glycerol and FFAs from hepatocytes.
Recent evidence suggests that ATGL and HSL protein expression is decreased in white adipose tissue (WAT) from obese and insulin-resistant subjects, and over-expression of these two enzyme prevent liver steatosis. Similar results also found in rat, where cocoa polyphenol supplementation suppressed the expression abundance of genes involved in lipogenesis enzyme but activated the expression of those genes dedicated for lipolysis enzymes in mesenteric white adipose tissue. Increased TAG lipolysis didn’t lead to observe fatty acid accumulation in the liver, on the contrary, fat% is significantly decreased in cocoa treated mice compared with HF group. This maybe in part or whole due to two reasons: inhibition of lipid absorption and stimulation of β-oxidation. Firstly, our previous study we found cocoa extracts and procyanidins inhibit intestinal digestive enzyme activity. For example, cocoa suppress pancreatic lipase and phospholipase A2 activity, thereby reduce dietary fat absorption and plasma fat level. A recent study showed that in NAFLD patients plasma FFAs were the primary contributor to the liver triacylglycerol content in the fasted state (50–70% of total FA) by a multiple-stable-isotope labeling approach. Thus, inhibiting the fat absorption help lower the plasma FFAs. Secondly, reduced FFAs in cocoa treated group could be attributed to increases in mitochondrial β-oxidation. During NAFLD, several metabolic adaptations are set up in order to curb fat accumulation. In particular, increased mitochondrial fatty acid oxidation (mtFAO) plays a significant role. This process requires the coordination of several enzymes and FA transporter. CPT1α transfer long chain fatty acid into mitochondrial, where FA undergoes four sequential reactions to generate one acetyl-CoA molecule and a shortened FA. The cycle is repeated to split FAs into several acetyl-CoA and undergo mitochondrial oxidative phospharltion to generate ATP. Previous study found cocoa increased beta-oxidation in rat adipose tissue. However, there is no reports on the increase of CPT1α in liver and co-enhancement of HSL and AGTL. The co-enhancement is important since ATGL and HSL mediated hydrolysis favor mitochondrial beta oxidation. For example, transient adenoviral hepatic overexpression of HSL or ATGL promotes fatty acid oxidation, stimulates
direct release of fatty acid, and ameliorates hepatic steatosis in both ob/ob mice and mice with high fat diet induced obesity. Researches also showed that hepatic overexpression of HSL or ATGL can promote fatty acid oxidation, stimulate direct release of free fatty acid into the medium and activities gene expression related to mitochondrial beta-oxidation\(^{146}\). By contrast, enzymatic inactivation of ATGL in mice increases TG mass in multiple tissue\(^{147}\).

Although the compositions of the lipids that accumulate in the livers of subjects with NAFLD are not well characterized, an increase in the n-6: n-3 fatty acid ratio in total lipids has been described recently\(^{148-150}\). Overnutrition triggers n-3 long-chain polyunsaturated FA (n-3 LCPUFA) depletion, with enhancement in the n-6/n-3 LCPUFA ratio favoring a pro-inflammatory state. Arachidonic acid (C20:4 n-6, ARA) is for the n-6 series and eicosapentaenoic acid (C20:5 n-3, EPA) and docosahexaenoic acid (C22:6 n-3, DHA) is for the n-3 series. EPA and DHA are recognized as major n-3 PUFA in the liver and plays important role in inflammation resolution and lipid metabolism\(^{150-152}\). We found cocoa increased EPA and DHA percentage in obese mice liver, this may have beneficial effects on the resolution of NAFLD. Previous study found changes in hepatic fatty acid composition, particularly lower hepatic n-3 PUFA, predispose to liver steatosis by favoring lipid synthesis over oxidation and secretion\(^{150,153}\). This is consistent with our previous findings where fatty acid oxidation are elevated, lipid synthesis are decreased. In addition, we also found cocoa decreased the ratio of n-6/n-3. Enhancement in the n-6/n-3 LCPUFA ratio in the liver favoring a pro-inflammatory state and changes may lead to hepatic steatosis by different mechanisms: including the insulin resistance depending peripheral lipolysis and FA flux to the liver, n-3 LCPUFA depletion-induced changes in DNA binding activity of sterol regulatory element-binding protein 1c (SREBP-1c) and PPAR-\(\alpha\) favoring lipogenesis over FA oxidation, and hyperinsulinemia-induced activation of lipogenic factor PPAR-\(\gamma\).
2.6 Conclusion

In summary, our results indicate a novel hepatoprotective effect of cocoa supplementation on NAFLD. These beneficial effects are mediated by three mechanisms. First of all, cocoa inhibits SCD1 activity index. This helps restrain the synthesis of PUFA from saturated fatty acids. In addition, cocoa increases TAG hydrolysis by up-regulating gene expression of Atg1 and Hsl. Atg1 and Hsl is important since mediated hydrolysis favor mitochondrial beta oxidation. Based on an understanding of the impact of dietary cocoa on lipid metabolism, it may become possible to make rational dietary recommendations with this food for the modulation of obesity-related fatty liver disease.
Dietary cocoa increases hepatic mitochondrial biogenesis and activates anti-oxidation defense in obese mice

3.1 Abstract

Nonalcoholic fatty liver disease (NAFLD) is an important co-morbidity of obesity. NAFLD is classified into two stages: simple fatty liver (i.e. stetaosis) and non-alcoholic Steatohepatitis (NASH). Mitochondrial dysfunction and oxidative stress act as important triggers for the progression of simple steatosis and NASH. However, the beneficial properties in modulating these two triggers are largely unknown. Under this context, we investigated the effect of dietary cocoa on hepatic mitochondrial biogenesis, oxidative stress, and antioxidant response. In experiments, male C57BL/6J were fed HF (60 % kcal from fat) diet for 8 wks, and then randomized to continue HF diet or to consume HF diet supplemented with 80 mg/g cocoa powder (HF-HFC) for an additional 10 wks. We found cocoa treatment increases hepatic mitochondrial DNA copy number by 48.6% compared to HF-fed mice, suggesting increased mitochondrial biogenesis. Using real-time PCR, we found that mRNA levels of Sirtuin (Sirt)1, peroxisome proliferator-activated receptor gamma coactivator 1α (Pgc1α), and nuclear respiratory factor 1(Nrf1), which can promote mitochondrial biogenesis, were significantly increased in HF-HFC compared with HF-fed mice. We also found that cocoa supplementation decreased hepatic lipid peroxidation by 57% compared to HF-fed controls indicating reduced oxidative stress. Cocoa supplementation also increased the expression of Sirtuin (Sirt)3, an important mitochondrial redox regulator, as well as manganese superoxide dismutase and glutathione peroxidase (Gpx)1.
was increased in cocoa supplemented mice HF-HFC compared to HF-fed controls. Cocoa also increased hepatic GPX activity by 43% compared to HF-fed controls. In summary, our results demonstrate that the beneficial effects of cocoa supplementation on NAFLD may be mediated by increases in mitochondrial biogenesis and related antioxidant response signaling.

3.2 Introduction

The increased incidence of obesity and metabolic syndrome triggers the development of NAFLD. NAFLD refers to the non-physiological accumulation of fat in the liver, which is also called this steatosis. Simple steatosis is a condition with disturbed fatty-acid metabolism and hepatic lipid accumulation. This lipid accumulation alone can be benign, but may also sensitize the liver to more advanced hepatic injury Non-alcoholic steatohepatitis (NASH) when additional hit come into this picture (two-hit hypothesis)

It is well recognized that oxidative stress is potential biochemical mechanism account for NASH. Oxidative stress refers to various deleterious processes resulting from an imbalance between the excessive formation of pro-oxidants (ROS and/or reactive nitrogen species (RNS)) and limited antioxidant defenses. Within the liver, there are three potential sites for ROS generation: mitochondria, endoplasmic reticulum and peroxisomes. In addition, various enzymes, including oxidases and oxygenases, can also generate ROS as part of their enzymatic reaction. Among those sources, mitochondria is considered to be the major source of ROS production in eukaryotic cells, particularly from the redox state of the respiratory chain.

In the respiratory chain, complexes I (NADH: ubiquinone oxidoreductase) and III (ubiquinol:cytochrome c oxidoreductase; cytochrome bc1 complex) are generally considered as the main producers of superoxide anions that are released into the mitochondrial matrix and the intermembrane space, respectively. This superoxide anion production is generated from the
oxidation of FFAs. The oxidation of FFAs in mitochondria is associated with the conversion of oxidized cofactors (NAD+ and FAD) into reduced cofactors (NADH and FADH₂). These reduced cofactors are then re-oxidized by mitochondrial respiratory chain (complexes I, III, and IV), which regenerates the NAD+ and FAD necessary for other cycles of fuel oxidation. This transfer of electrons along the mitochondria respiration chain is coupled with the exit of protons from the mitochondrial matrix into the intermembrane space, creating an electrochemical potential across the inner membrane. A fraction of these electrons directly reacts with oxygen to form the superoxide anion radical and other reactive oxygen species (ROS). In normal condition, superoxide anion is readily dismutated by superoxide dismutase 2 (SOD2, also known as manganese-dependent superoxide dismutase MnSOD) into hydrogen peroxide, which is detoxified further into water by glutathione peroxidase (GPX). However, in patients with NAFLD, mitochondrial oxidative phosphorylation didn’t increase concurrently with mitochondrial oxidative chain activity or antioxidant enzyme activity. Therefore, this imbalance between mitochondrial ROS generation and insufficient antioxidants defenses lead to mitochondrial oxidative stress. In fact, several studies have described that impaired mitochondrial function plays a central role in the development of NAFLD.

Several approaches have been proposed to ameliorate mitochondrial function, such as increased mitochondrial biogenesis, decrease ROS production and boost the endogenous levels of antioxidants. Particularly, mammalian sirtuins family may be good candidates to fulfill those functions. The mammalian sirtuins (SIRT 1-7) are a conserved family of NAD+-dependent deacetylases and ADP-ribosyltransferases. Sirtuins involved in numerous physiological and pathophysiological situations, including metabolic syndrome, cell survival, aging, and calorie restriction-mediated longevity through deacetylation of numerous substrates. Two sirtuins that are central to the control of mitochondrial function are mammalian sirtuin 1 (SIRT1) and sirtuin 3 (SIRT3), which are localized to the nucleus and mitochondria, respectively.
been well studied that SIRT1 plays an important role in the regulation of mitochondrial biogenesis and energy homeostasis through the activation of peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α). Whereas, sirtuin 3 (SIRT3) mainly regulate mitochondrial antioxidant system by activating forehead transcription factor 3a (Foxo3a) and deacetylating its substrate associated with antioxidant properties, such as SOD2 and GPX. Therefore, activate sirt1 and sirt3 activity maybe a feasible way to modulate mitochondrial function in NAFLD and NASH.

Polyphenols, such as resveratrol, quercetin and catechins, have been shown to activate Sirtuins either directly or indirectly in vitro and in vivo. In 2003, a screen for small molecule activators of SIRT1 identified 21 different SIRT1-activating molecules, the most potent of which was resveratrol. Hence, the activation of SIRT1 by polyphenols would be beneficial in therapeutic intervention of fatty liver disease. Cocoa is a polyphenol rich dietary components in western diet. Three groups of polyphenols can be identified in cocoa beans, such as flavanols [(+) catechin and (-) epicatechin] and flavonols [quercetin-3-glucuronide, quercetin-3- glucoside (isoquercitrin), quercetin-3-arabinoside, and quercetin]. In addition, trans-Resveratrol was also found in dark chocolate and cocoa liquor. Therefore, it is reasonable to expect that cocoa may also have sirtuin activating activity, and through this to modulate mitochondrial function. However, studies regarding beneficial effects of cocoa on diet induced liver steatosis, with the emphasis on sirt1 and sirt3 pathway and activity is limited.
3.3 Material and methods

3.3.1 Animal studies

Wild type (C57BL/6J background) was used in this experiment. Mice were housed in a pathogen-free facility (12/12-hour light/dark cycle) and fed by standard diet (LF, 10% Kcal) or high fat diet (HF, 60% Kcal) for 8 weeks. After 8 weeks, half of the high-fed mice were randomly selected and treated with a HF-diet supplemented with 8% unsweetened cocoa powder (HF-HFC). The rest of mice were maintained with their original diet. By the end of 18 weeks, mice were sacrificed, plasma and liver sample were collected for biological experiment. The collected samples were stored at −80°C until further analysis.

3.3.2 Gene expression and mtDNA analysis

Liver RNA and total DNA were extracted according to our lab protocol and quantified using NanoDrop 1000 spectrophotometer. cDNA was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen, Germany) using 1000 ng of RNA and then amplified using SYBR Green PCR Master Mix. Applied Biosystems 7900HT Fast Real-Time PCR System and relative quantification software (Applied Biosystems, Foster City, CA) were used for the real-time analyses. Calculations were performed by a comparative method (2−ΔΔCT) using GAPDH as an internal control. For mtDNA analysis, total DNA was extracted with liver tissue with lab protocol. mtDNA was amplified using primers specific for the mitochondrial cytochrome c oxidase subunit 2 (COX2) gene and normalized to genomic DNA by amplification of the ribosomal protein s18 (rps18) nuclear gene\(^{168}\). mtDNA oxidative damage was estimated as the ratio of 80-bp fragment (260–339 position), compared to a 162-bp fragment (260–421 position) of the same sample\(^{169}\). Both fragments were amplified in a total volume of 10 ul with 15 ng of
total DNA, 5 µl SYBR Green Mix, and 10 umol/l of *HVII-FOR260* and *HVII-L421* for large fragments and 2.5 µmol/l *HVII-FOR260* and *HVII-C339* for short fragments. The PCR conditions were 2 min at 50 °C, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Primers can be found in table 3-1.

Table 3-1: Primer used in chapter 3

<table>
<thead>
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<th>Gene</th>
<th>Forward (5’ → 3’)</th>
<th>Reverse (5’ → 3’)</th>
</tr>
</thead>
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<td>HVII-C339</td>
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</table>
3.3.3. Immunoblotting

Frozen liver sample was homogenized in T-PER™ Tissue Protein Extraction Reagent (Thermal Fisher, Waltham, MA, USA) containing a cocktail of protease inhibitors and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Protein concentration was determined by Bradford assay (Sigma-Aldrich, St. Louis, MO, USA). Protein extracts were then combined with an equal volume of 2X laemmli sample buffer with 5% β- mercaptoethanol (Bio-Rad, Hercules, CA, USA) and denatured at 90 °C for 5 min. 60 ug denatured protein sample was loaded onto a 4-20% SDS-PAGE gel and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) under constant voltage of 90 V for 1 hour in an ice box, and then incubate with primary antibody SIRT1 (1:500 dilution, Santa Cruz Biotechnology, Inc.), SOD2 (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA), SIRT3 (1:1000 dilution, Cell signaling) and GAPDH (1:1000 dilution, Cell signaling) overnight at 4 °C. The immunostaining was detected using a fluorescently-labeled secondary antibody (LI-COR Biosciences, Lincoln, NE, USA) for 1hr at room temperature and imaged with an Odyssey imaging system (LI-COR, Lincoln, NE, USA). Protein loading was normalized to GAPDH Band density was quantified using Odyssey Application Software version 3.0.
3.3.4 SOD activity

SOD level in liver homogenate was measured by the method below. Briefly, 2.4ml Solution A (0.05M Na₂CO₃ buffer, pH 10.2), 0.1 ml Solution B (3mM EDTA in distilled water), 0.1 ml Solution C (1.5mg/ml bovine serum albumin in distilled water), 0.1 ml Solution D (0.75mM NBT in distilled water), 0.1 ml Solution E (3 mM Xanthine in solution A), and 0.1ml test sample (or sod standards) were mixed together, then 0.1ml xanthine oxidase was added to each tube to start the reaction. Then tubes were incubated for 20 minutes at room temperature. After 20 min, 0.1ml Cucl₂ (6mM Cucl₂ in distilled water) was added to each tube to terminate the reaction. The inhibition of NBT reduction in each sample was determined spectrophotometrically at 560 nm. By plotting absorbance with different standard SOD concentration, standard curve was generated and used to calculate sample SOD activity.

3.3.5 Glutathione peroxidase activity assay

Liver homogenate was extracted according to the manufacturer’s protocol using Glutathione Peroxidase Assay Kit (Cayman Chemical, MI, USA). GPX activity was determined by following the rate of NADPH oxidation at 340nm in the presence of substrate cumene hydroperoxide. One unit of activity equaled one nmole of NAPDH oxidized per min. All results were expressed in unit/mg protein.

3.3.6 Nuclear isolation and SIRT1 activity

Nuclear protein fractions from liver were prepared as described on abcam protocol, with minor modifications. All steps were performed on ice. Briefly, 30 mg livers were cut into small pieces and homogenized 300ul with a Dounce homogenizer in buffer containing 10 mM Tris HCl
(pH 7.5), 10 mM NaCl, 15 mM MgCl₂, 250 mM Sucrose, 0.5% NP-40 and 0.1 mM EGTA; and combine the homogenate with 1.2 ml of sucrose cushion containing 30% Sucrose, 10 mM Tris HCl (pH 7.5), 10 mM NaCl, 3 mM MgCl₂ and centrifuge at 1,300 x g for 10 min at 4°C. Then discard the supernatant and wash the nuclei pellet once with cold 10 mM Tris HCl (pH7.5), 10 mM NaCl. The final pellet containing the purified nuclei was resuspended in 50ul of extraction buffer with 50 mM Hepes NaOH (pH7.5), 420 mM NaCl, 0.5 mM EDTA-Na₂, 0.1 mM EGTA, 10% glycerol. Protein concentration were determined by Bradford method or equivalent and stored the crude nuclear extract at -80°C until use. Sirt1 activity were determined by SIRT1 activity assay fluorometric kit from abcam (ab156065) and normalized to protein content for each sample.

3.3.7 Data Analysis

All statistical analyses were conducted using one-way ANOVA with Dunnett test in GraphPad Prism, where \( p < 0.05 \) will be considered significant.

3.4 Results

3.4.1 Cocoa increases mitochondrial biogenesis

Cocoa up-regulates mitochondrial biogenesis factors. Mitochondrial biogenesis involves the integration of multiple transcriptional pathways controlling both nuclear and mitochondrial gene expressions. The PPAR coactivator \( Pgc1α, Nrf1 \) and \( Nrf2 \) are considered key regulators of mitochondrial biogenesis in multiple tissues. RT-PCR measurements revealed that the expression
of the mitochondrial biogenesis factors \( Pgc1\alpha \) and \( Nrf1 \) was increased by 62.4% and 52.7% in HF-HFC compared with HF. The expression of Nrf-2 and Tfbm1 are not affected.

Figure 3-1: Effects of cocoa supplementation on the gene expression of (a) \( Pgc1\alpha \) (b) \( Nrf1 \) (c) Nrf2 and (d) Tfbm1. Values are mean ± SEM (n = 6-12). *P < 0.05 vs. obese group according to one-way ANOVA with Dunnett’s post-test (HF as control).
3.4.2 Cocoa increased mitochondria DNA copy number

Regarding the mitochondria biogenesis, we found HF feeding cause significantly increase DNA copy number compared with lean mice. Cocoa supplementation slightly, but not significantly increase DNA copy number compared with HF mice (P value=0.07, student t test). In addition, dietary cocoa also unregulated *Sirt1* gene expression, sirt1 activity determined in nuclear extracts are not affected.

![Figure 3-2](image)

**Figure 3-2:** Effects of cocoa supplementation on (a) DNA copy number and the gene expression of (b) *Sirt1* and (c) SIRT1 activity. Values are mean ± SEM (n = 6-12). *P < 0.05 vs. obese group according to one-way ANOVA with Dunnett’s post-test (HF as control).

3.4.3 Cocoa increased the expression of mitochondria complex I and III

In order to find whether cocoa is able to modulate mitochondria complex expression. We did gene expression for five mitochondria complex. RT-PCR reveals that expression of *Ndufs8* (complex I) and *Uqcrcl* (complex III) were significantly suppressed by HF treatment. Cocoa treatment significantly reverse this trend with significantly increase of *Ndufs8* and *Uqcrcl* gene expression compared with HF group.
3.4.4 Cocoa increase the gene expression of Sod2 and SOD activity

In mammals, there are three forms of SODs localized in various cellular compartments. SOD2 is located in the mitochondria. To determine whether SOD2 plays a major role in reduction cellular ROS, we analysis gene expression for three SODs. Interestingly, we only detected a significant increase in SOD2 in cocoa treated mice compared with HF feeding mice. In line with the change in Sod gene expression, SOD activity of liver homogenate was significantly increased.
We found cocoa treatment significantly elevated GPX activity in the liver lysate compared with HF mice. To figure of which subclass of GPX contribution to the observed effect, we determine the gene expression of five GPX isoforms. Interestingly, only GPX1, the one mainly present in the mitochondria, are significantly induced by cocoa treatment.

**3.4.5 Cocoa increase Gpx1 gene expression and GPX activity**

![Figure 3-4](image)

Figure 3-4: Effects of cocoa supplementation on gene expression of (a) *Sod1* (b) *Sod2* (c) *Sod3* (d) SOD activity. Values are mean ± SEM (n = 6-12). *P < 0.05 vs. obese group according to one-way ANOVA with Dunnett’s post-test (HF as control).
3.4.6 Cocoa decrease lipid peroxidation marker and mitochondria DNA oxidative damage

We analyzed mtDNA oxidative damage by quantitative reverse transcription-PCR and observed that, as expected, high-fat intake induced oxidative damage in mtDNA, which was completely reversed by cocoa treatment. HF diet cause a significant increase in TBAR level, indicating an increase in lipid peroxidation. Cocoa treatment significantly decrease MDA content to the level near LF control group.
3.4.7 Cocoa increase protein level of SIRT3 and SOD2

To gain a better insight into the mechanism that could explain the effect of cocoa against oxidative damage, gene and protein expression of SIRT3 were determined. *Sirt3* gene expression were not affected by cocoa supplementation and HF feeding, and gene expression in these three group present in the similar level. However, we found an increasing trend of SIRT3 protein expression in HF-HFC and HF compared with LF lean mice, and HF-HFC induced more elevation than HF. In addition, in line with what we thought in SOD activity, cocoa treatment moderately increased *Sod2* gene expression when compared with HF controls.

![Figure 3-6](image-url)

Figure 3-6: Effects of cocoa supplementation on (a) marker of lipid peroxidation and (b) Oxidative DNA damage. Values are mean ± SEM (n = 6-12). *P < 0.05 vs. obese group according to one-way ANOVA with Dunnett’s post-test (HF as control).
Figure 3-7: Effects of cocoa supplementation on (a) *Sirt3* gene expression (b) SIRT3 protein relative expression and (d) SOD2 protein relative expression. Values are mean ± SEM (n = 6-12). *P < 0.05 vs. obese group according to one-way ANOVA with Dunnett’s post-test (HF as control).

### 3.4.8 Cocoa increase gene expression of *Foxo3a*

As shown in Figure 3-8, HF feeding slightly decreased the gene expression of *Foxo3a*. Cocoa up regulated the mRNA levels of this gene to levels even higher than those observed in lean controls.
3.5 Discussion

Nonalcoholic fatty liver disease is now considered the main hepatic manifestation of obesity and metabolic syndrome. In fact, our previous data have shown that high-fat feeding increased body weight gain and subsequently induced liver steatosis. This is considered as the early event in the development of NASH. Mitochondrial dysfunction, such as elevated oxidative stress and decrease in antioxidant defenses, induce the development of NAFLD to more advanced liver damage. Moreover, some researchers described the ability of cocoa to reverse the effects of high fat diet on liver steatosis. However, underlying mechanism is still largely unknown. In this study, we have found that the beneficial effects of cocoa on obesity associated fatty liver could be mediated through increasing mitochondrial biogenesis and activating anti-oxidant defense system.

To begin with, cocoa increases the transcript levels of SIRT1 and PGC-1α, mitochondrial transcription factors, and components of the electron transport chain. Those beneficial effects of cocoa on liver are likely due to sirt1-mediated increases in mitochondrial biogenesis. Indeed,
SIRT1-PGC1α-NRF1&2 is proven pathway to regulate mitochondrial biogenesis and plant polyphenols has been found to be activator for SIRT1\textsuperscript{171}. SIRT1 promotes mitochondrial biogenesis through deacetylation and activation of PGC-1α, a master regulator of mitochondrial biogenesis, co-activates the nuclear respiratory factors (NRF-1 and NRF-2), which induce the transcription of genes involved in mitochondrial biogenesis. Similar results were found in resveratrol, medium dose resveratrol induce sirt1 expression at gene and protein level in skeletal muscle\textsuperscript{104}. In our experiment, sirt1 activity were slightly increased but didn’t meet statistical difference. In this case, our data do not support the notion that these metabolic effects are mediated by direct SIRT1 activation. Rather, cocoa may indirectly activate sirt1 through other mechanisms such as AMPK activation\textsuperscript{172–174} or increasing intracellular NAD+ levels\textsuperscript{175}. In addition, it is also possible that the current dose we use in our experiment probably too low to activate sirt1 activity. Similar result were found in one study where they found only medium dose resveratrol is potent to activate sirt1 activity, low and high dose remains useful on sirt1 activity\textsuperscript{168}.

Figure 3-9: Summarized effects of cocoa on mitochondria biogenesis
Furthermore, additional evidence on mitochondria biogenesis is increased mtDNA copy number in cocoa treated group. mtDNA copy number regulation is one way to preserve mitochondrial integrity. Various molecular and cellular pathways are activated in eukaryotes to control the quality and integrity of mitochondria. For example, mtDNA dynamics plays a significant role in preventing biochemical defects of mitochondrial mutations. Increased mtDNA replication of wild-type copies can shift the mtDNA mutation below the phenotypic threshold level. In addition, cocoa treatment increased mRNA expression of the mitochondrial electron transport chain (*Ndufs8* and *Uqcrcl*). Patients with NASH have decreased hepatic mitochondrial DNA levels, decreased protein expression of several mitochondrial DNA-encoded peptides, and lower activity of complexes I, III, IV and V (ATP synthase), which are partly encoded by mitochondrial DNA, and also complex II, which is only encoded by nuclear DNA. It is worthwhile to mention, increased mtDNA copy number factor was detected in HF group compared with LF lean mice. However this increase is unlikely due to the activating of mitochondrial biogenesis pathway, maybe due to the trigger of oxidative stress.

![Figure 3-10: Summarized effects of cocoa on mitochondria complex expression](image-url)

Figure 3-10: Summarized effects of cocoa on mitochondria complex expression
In addition, enhanced mitochondrial β-oxidation (mtFAO) need concomitantly with mitochondrial respiration chain activity and/or anti-oxidation response\textsuperscript{177}. mtFAO in NAFLD is thought to be an adaptive way to counteract excessive fat storage due to increased hepatic uptake and synthesis of FFAs. For example, an increase in mtFAO has been observed in NASH patients as well as in the liver of genetically obese-diabetic (ob/ob) mice with massive steatosis or in the liver of diet-induced obese rodents\textsuperscript{178}. However, excessive mtFAO may produce more ROS, and lead to mitochondrial dysfunction. Mitochondrial dysfunction will in turn block mtFAO and result in cytosolic fatty acid accumulation. Therefore, enhanced mtFAO without concomitant up-regulation of the antioxidant activity induces ROS overproduction within mitochondria. It is preferable if therapeutics can increase mtFAO and activate antioxidant defense system in a coordinated manner. By doing this, steatosis will be ameliorated without harm of the of oxidative stress.

As we expect, we found cocoa induces an increase of mtFAO and antioxidant defense system in a coordinated manner. To begin with, cocoa treatment significantly increases activity of two major anti-oxidative enzymes (SOD and GPX). Both SOD and GPX has multiple isoforms, and each isoform present in a specific cell compartment. For example, SOD has three isoforms: SOD 1 to 3. Among them, SOD2 is the major SOD present at mitochondrial. In addition, GPX1 is major gene encoded mitochondrial glutathione peroxidase in mouse liver\textsuperscript{179}. To find out which isoform contributes to activity, we screen the gene expression of each isoform. We demonstrated that SOD2 and GPX1, which mainly exists in the mitochondria, were significantly increased in response to cocoa treatment. In line with Sod2 gene expression, a moderate elevation in SOD2 protein were also detected in the liver lysate in HF-HFC compared with HF (with p value 0.07, determined by t test). Therefore, we conclude that cocoa active the mitochondrial antioxidant enzyme activity.
The activation of mitochondria antioxidant enzyme activity by cocoa help reduces lipid peroxidation. When ROS attacks the unsaturated lipids of fat deposits, it will form lipid peroxidation products. Lipid peroxidation products are harmful, since they can directly attack and inactivate respiratory chain components, including cytochrome c oxidase, the terminal oxidase of the respiratory chain\textsuperscript{180,181}. For example, mitochondria have a substantial concentration of phospholipids containing docosahexaenoic acid, which may be essential for functional assembly of the electron transportation chain. Peroxidation of these mitochondrial membrane components could lead to further impairment of the activity of the ETC and ROS overproduction\textsuperscript{182}. As a consequence, lipid peroxidation will secondarily aggravate mitochondrion.

In addition to lipid peroxidation, cocoa also reduced oxidative mtDNA damage caused by high fat diet. In fact, mtDNA is more susceptible to oxidative attack than nuclear DNA, possibly because of its proximity to the respiratory chain in the mitochondrial inner membrane, the lack of protective histone-like proteins, and its poor repair activity against damage\textsuperscript{183,184}. One detrimental effect of mtDNA damage is the formation of Oxidative mitochondrial DNA lesions which may impair the synthesis of respiratory chain polypeptides. In line with those findings, we found HF-diet suppressed the gene expression of mitochondrial complex I and III, this probably due to mtDNA damage.

Moreover, several lines of evidence described that SIRT3 stimulates antioxidant defenses by different ways. One of the most important mechanisms is the deacetylation of mammalian Foxo factors that control various biological functions, including detoxification of ROS and repair of DNA damage. Particularly, the ability of Foxo3a to stimulate mitochondrial antioxidant defenses such as SOD2 has been described in different studies. Studies suggest that increasing SOD2 expression alone can only modestly reduce cellular ROS. The ability of SOD2 to reduce cellular ROS is greatly enhanced by SIRT3 deacetylation\textsuperscript{185}. Moreover, Jacobs et al. suggested that Foxo3a is a mitochondrial protein, whose function could be regulated through the interaction
with SIRT3 in the mitochondria. In agreement with this hypothesis, other research described that activation of Foxo3a through deacetylation is specifically stimulated in response to oxidative stress stimuli. In this context, our results demonstrate for the first time that acetylation levels of Foxo3a are strongly increased in steatotic livers whereas LA treatment completely reversed this effect.

3.6 Conclusion

In summary, our results indicate a novel protective effects of cocoa supplementation on obesity-associated fatty liver diseases, through increasing mitochondrial biogenesis (Fig 3-9), activating anti-oxidant enzyme gene expression (Fig 3-11). Cocoa may also have SIRT1 and SIRT3 activating activity to modulate mitochondrial function. Our data indicate that beneficial effect of cocoa against oxidative stress are due to at least three mechanisms. Firstly, as discussed before, cocoa significantly increase Cpt1a gene expression which is a marker for mitochondrial beta-oxidation. This increased b-oxidation is thought be adaptation to attenuates oxidative stress within the cell by reducing fat deposition and decreasing alternative fat oxidation pathways in peroxisomes and microtomes\textsuperscript{186}. Secondly, cocoa activate mitochondrial antioxidant enzyme activity. SOD and GPX, two important antioxidant enzyme in cell, both significantly increased after cocoa treatment. Finally, maintenance the integrity of mitochondrial complex also plays an important role in scavenging ROS. While further work is needed to fully determine the importance of SIRT1 and SIRT3 in the ability of cocoa to prevent metabolic syndrome and other age-related diseases, this study provides the first in vivo evidence that beneficial effects of cocoa on mitochondrial function require SIRT1 and SIRT3.
Figure 3-11: Summary of anti-oxidant effect of cocoa
Chapter 4

Dietary Cocoa directs a phenotypic switch of liver macrophage polarization in high fat-fed mice

4.1 Abstract

Nonalcoholic fatty liver disease (NAFLD) is one of the most common forms of chronic liver disease and is the major cause of liver-related mortality in western-countries. It has been demonstrated that activation and polarization of Kupffer cell (KCs) governs the central event in chronic liver injury. The local balance between classical (M1) and alternative (M2) activation pathway is considered as a predominant mechanism in the resolution of NAFLD. In this study, we demonstrated that dietary cocoa is able to alternatively activate M2 KCs in high fat-fed (HF) mice, and this phenotypic switch protects mice from ongoing of liver damage compared with HF treated alone. In experiments, male C57BL/6J were fed with either low-fat (LF, 10 % kcal from fat) or HF (60 % kcal from fat) diet for 8 weeks. After 8 weeks, mice from HF group were randomized to HF diet or HF diet supplemented with 8% cocoa powder (HF–HFC group) for 10 weeks. Our results show that HF-HFC significantly decreased the hepatic expression of (macrophage inflammatory protein 1) *Mip1a* at mRNA level and monocyte chemotactic protein-1 (*Mcp1*) at protein level which are associated with monocyte infiltration. Interesting, cocoa treatment significantly increase the gene expressions of M2 surface marker (*e.g.* *Cd163*) and M2 related anti-inflammatory cytokine interleukin 10 (*Il10*). These data reveal a novel hepatoprotective effect of dietary cocoa in the context of obesity-related fatty liver disease.
4.2 Introduction

The worldwide rising prevalence of obesity and insulin resistance is associated with a parallel increase in nonalcoholic fatty liver disease (NAFLD). NAFLD is characterized by excess accumulation of triglyceride in the hepatocyte. This mainly due to increased inflow of free fatty acids and/or de novo lipogenesis caused by western diet. The association of steatosis with other liver lesions is called steatohepatitis (NASH). Nowadays, a “two-hit model” has been proposed in order to explain potential mechanism responsible for NASH pathogenesis. It was found that inflammatory response is important second hit for the development of NASH. Therefore, it is reasonable to think KCs, the major inflammatory cell in the liver, may participate in the transition from NAFLD and NASH.

KCs are a major immune effector in the pathogenesis of NASH which secrete cytokines and chemokines in responding to inflammation and metabolic stresses by orchestrating local immune responses. More importantly, KCs can undergo a phenotypic switch in response to various local stimuli and express distinct patterns of surface markers. The phenotype switch of KCs depends on the triggering stimuli, it can undergo either a “classical” (Th1 dependent; M1) or “alternative” (Th2 dependent; M2) activation pathway. Macrophage M1 response is an essential part of innate immunity and act as the first responders to pathogens, toxins, and tissue damage. M1 macrophage can produce a class of M1 (Th-1) proinflammatory cytokines, such as tumor necrosis factor-a (TNF-α), γ-interferon (IFN-γ), and interleukin (IL)-1β. However, if excessive, the inflammatory response becomes detrimental and undesirably result in tissue damage. To counteracted toxic effects generated by M1 macrophage, an “alternative” (M2) activation pathway is opened. The activation of M2 macrophage results in a protective phenotype. It promotes maturation of alternatively activated macrophages to counteract excessive inflammation, enhance tissue repair and may have a beneficial role in regulating nutrient
homeostasis. For example, recent studies\textsuperscript{64,65} demonstrate that blocking the anti-inflammatory or alternative (M2 or Th-2) activation program of KCs exacerbates obesity-induced insulin resistance and decreases hepatocyte fatty acid oxidation. Those study indicate the beneficial role of M2 macrophage activation in the resolution of NAFLD. Therefore, it expects that increase the population of M2 or shape an environment favors M2 macrophage activation would be a pharmacological strategy to reduce hepatic inflammation.

Cocoa has been shown to possess anti-inflammatory, antioxidant properties, and attenuates hepatic lipid metabolism as we discussed in chapter 2. Although cocoa has been suggested to reduce hepatic fat accumulation and modulate mitochondrial function, the anti-inflammatory effect is unclear in our study. To date, results regarding the beneficial effects of cocoa on KCs differentiation has not been reported. Therefore, the purpose of this study was to investigate the effectiveness of cocoa supplementation on KCs infiltration, and inflammation induced genes in high fat diet mice.

\section*{4.3 Materials and methods}

\subsection*{4.3.1 Animal experiment}

Wild type (C57BL/6J background) was used in this experiment. Mice were housed in a pathogen-free facility (12/12-hour light/dark cycle) and fed by standard diet (LF, 10% Kcal) or high fat diet (HF, 60% Kcal) for 8 weeks. After 8 weeks, half of the high-fed mice were randomly selected and treated with a HF-diet supplemented with 8% unsweetened cocoa powder (HF-HFC). The rest of mice were maintained with their original diet. By the end of 18 weeks, mice were sacrificed, plasma and liver sample were collected for biological experiment.
4.3.2 Real time PCR

Liver RNA was extracted according to our lab protocol and quantified using NanoDrop 1000 spectrophotometer. cDNA was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen, Germany) using 1000 ng of RNA and then amplified using SYBR Green PCR Master Mix. Applied Biosystems 7900HT Fast Real-Time PCR System and relative quantification software (Applied Biosystems, Foster City, CA) were used for the real-time analyses. Calculations were performed by a comparative method (2-ΔΔCT) using GAPDH as an internal control. Primer sequence were listed in table 4-1.

Table 4-1: Primers used in chapter 4

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’ → 3’)</th>
<th>Reverse (5’ → 3’)</th>
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<tbody>
<tr>
<td>Cd206</td>
<td>ATGGCAGACGATGATCCCTAC</td>
<td>TGTTGACAGTGTTTTCTGGTG</td>
</tr>
<tr>
<td>iNos</td>
<td>CCAAGCCCTCACCTACTCC</td>
<td>CTCTGAGGGCTGACACAAGG</td>
</tr>
<tr>
<td>Tnf-a</td>
<td>ACGGCATGGATCTCAAAGAC</td>
<td>AGATAGCAAAATCGCTGACG</td>
</tr>
<tr>
<td>Il1b</td>
<td>GCAAAGTTCTGAACTCAACT</td>
<td>ATCTTTTGAGGTCCGTCAACT</td>
</tr>
<tr>
<td>Il10</td>
<td>GCTCTTACTGACTGGGATGAG</td>
<td>CGCAGCTCTAGGAGCATGTG</td>
</tr>
<tr>
<td>Cd163</td>
<td>TGGGTGGGGAAAGCATACT</td>
<td>AAGTTGTCGTCACACACGT</td>
</tr>
<tr>
<td>Arg1</td>
<td>CTCCAAGCCAAATCCTTAGAG</td>
<td>AGGAGCTGCTATTAGGGACATC</td>
</tr>
<tr>
<td>Gapdh</td>
<td>TGAAGCGAGCATCTGAGGG</td>
<td>CGAAGGTGAAAGGAGTGGGAG</td>
</tr>
<tr>
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<td>CGTGGAAATCTCTCCCCGCTGTAG</td>
</tr>
<tr>
<td>Chi313</td>
<td>AGAAGGGAGTTTCAACCTGGA</td>
<td>GTCTTGCTCATGTGTAAGTGA</td>
</tr>
</tbody>
</table>
4.3.3 MCP1 protein level determination

MCP1 protein level in the liver were determined using commercially available ELISAs (R&D Systems, Minneapolis, MN) according to the manufacturer’s protocols.

4.3.4 Data analysis

All pathophysiological data are presented as the mean ± standard error (SEM). *P < 0.05, **P < 0.01, ***P < 0.001 vs. obese group according to one-way ANOVA with Dunnett’s post-test (HF as control). The correlation between Fat% levels, TG level and liver weight was assessed by GraphPad Prism 7.0 (San Diego, CA, USA).

4.4 Results

4.4.1 Effects of Cocoa on Emr1 gene expression

Figure 4-1: Effect of cocoa supplementation on Emr1 gene expression. Values are expressed as
mean ± SEM (n = 6-12). *P < 0.05 vs. obese group according to one-way ANOVA with Dunnett’s post-test (HF as control).

Figure 4-1 shows the change of Emr1 in the liver of LF, HF and HF-HFC group. The Emr1 expression was not significantly affected in HF group compared with LF group. Similarly, we didn’t observe significant difference in Emr1 expression in HF-HFC group compared with HF group.

4.4.2 Effects of cocoa on gene expression of iNOS

Figure 2 shows the mRNA expression M1 marker iNOS was also unaffected upon cocoa supplementation. Cocoa treatment seems like induce the expression of iNOS with slightly elevated gene expression in HF-HFC compared with HF.

Figure 4-2: Effect of cocoa supplementation on iNOS gene expression. Values are expressed as mean ± SEM (n = 8). *P < 0.05 vs. obese group according to one-way ANOVA with Dunnett’s post-test (HF as control).
4.4.3 Expression of M2 markers

Figure 2 shows the mRNA expression of two M2 markers: *Cd163*, *Chi313* and Arginase 1. The mRNA expression of M2 macrophage specific marker *Cd163*, was significantly decreased in HF compared to LF group (p < 0.05). Cocoa treatment reverse this trend with marked increase of *Cd163* expression in HF-HFC group compared to the HF group (p < 0.05). *Chi313*, another surface marker for M2 macrophage was modestly, but not significantly increased in HF-HFC compared with HF group.

Figure 4-3: Effect of cocoa supplementation on gene expression of (a) *Cd203* (b) *Cd163* (c) *Arg1* and (d) *Chi313*. Values are expressed as mean ± SEM (n = 8). *P < 0.05 vs. obese group according to one-way ANOVA with Dunnett’s post-test (HF as control).
4.4.4 Changes of inflammatory marker

We determined several inflammatory cytokines that mainly produced by M1 macrophage. The mRNA expression of *Mcp1* was not significant difference in HF and HF-HFC group. However, MCP1 protein level in the blood sample was significant reduced in HF-HFC group compared to HF group. In addition, gene expression of *NF-κB* and *Il-1β* didn’t significantly differ in HF-HFC compared with HF group. Nevertheless, we found 10 weeks HF treatment significantly induce the expression of *Mip1α*, a member of the C-C subfamily of chemokines compared to LF group. The response caused by high fat diet was significantly suppressed by cocoa supplementation.

![Figure 4-4: Effect of cocoa supplementation on expression of (a) *Mip1α* (b) MCP1 (c) *NF-κB* (d) *Tnfa* and (e) *Il-1β*. Values are expressed as mean ± SEM (n = 8). *P < 0.05 vs. obese group according to one-way ANOVA with Dunnett’s post-test (HF as control).](image-url)
4.4.5 Changes of anti-inflammatory cytokines

mRNA expression of *Il10* was significantly increased in the HF-HFC group compared to HF group (p < 0.05). No significantly difference was found in HF group compared to LF group.

![Figure 4-5: Effect of cocoa supplementation on gene expression of Il10. Values are expressed as mean ± SEM (n = 8). *P < 0.05 vs. obese group according to one-way ANOVA with Dunnett’s post-test (HF as control).](image)

4.5 Discussion

Our previous study showed cocoa significantly liver weight, liver triglycerides content and as well as plasma cytokine markers such as MCP1 and IL-6, which suggests protective effects of cocoa supplement in obesity-related metabolic syndrome in high fat-fed mice. In current study, we focus on the beneficial effects of cocoa on liver inflammation, especially kupffer cell differentiation in mice model of diet induced steatosis.

To begin with, cocoa supplementation prevents monocytes infiltration. Liver macrophage has two origins: circulating monocyte precursors and resident kupffer cells. Monocytes
infiltration of is considered as the early event in NASH development, and it can happen from days to weeks after high fat feeding. Monocytes recruitment is induced by small molecular weight chemokine MCP1. MCP-1, also known as CCL2, is a potent monocyte chemokine produced mainly from hepatic macrophages and hepatic stellate cell. MCP-1 levels appear to be increased in NASH patients and in diet-induced NASH models. Serum levels of CCL2/MCP-1 was found gradually increased from healthy controls to simple steatosis, reaching the highest levels in NASH. We found cocoa significantly reduce protein level of MCP1 in the liver. MCP1 is small molecular weight chemokine responsible for monocytes recruitment. This result is consistent with our previous study, where we found cocoa supplementation reduced plasma MCP-1 production by 25.3% (P < 0.05).

In addition to MCP1, gene expression of the chemotactic chemokine Mip1α was significantly reduced in HF-HFC compared with HF group. MIP1α is a member of C-C subfamily of chemokines, which primarily act on lymphocytes and monocytes. Although no reports on the association between plasma MIP1α and hepatic steatosis or NASH, it was found that MIP1α mRNA in human liver is positively associated with liver fat. SIRT1 deficient (LKO) mice was associated with hepatic TAG accumulation, and gene expression of MIP1α was increased by 70-80% at the same time. This results indicates cocoa may modulate the crosstalk between lipid metabolism and inflammation.

Even though cocoa reduce the monocyte infiltration in the liver, the total macrophage population only slightly decrease and didn’t meet statistical significant. Interestingly, similar result was found in one study, where resveratrol supplementation was not enough to reduce F4/80 in high fat induced liver steatosis. However, these authors failed to give any explanation to the phenomena. We think these may be due to two reasons. First of all, compared with the infiltrated monocyte, local KCs pool still remains to be the major contribution of total macrophage population. KCs represent up to 80–90% of the total body macrophage pool. Thus, it is unlike
that the infiltrated monocyte will impact the total macrophage population. In other words, the amount of infiltrated monocytes is too low to affect the total macrophage population. Secondly, KCs have the capacity to self-renew and hence in the liver the macrophage pool remains populated by embryonically derived macrophage\textsuperscript{193}. In fact, literature regarding the effect of HF diet on the macrophage infiltration to the liver is not uniform. Some studies\textsuperscript{194–196} report a slight increase in liver macrophages but most of the authors found no macrophage infiltration in response to HF diet\textsuperscript{197–201}.

Furthermore, we found cocoa has no effect on limiting early cytokines production: TNF-\(\alpha\) and IL-1\(\beta\) gene expression. These two cytokines are mainly produced during the early phase of macrophage activation. For instance, activation of KCs might enhance hepatic lipid accumulation and liver injury through local secretion of IL-1\(\beta\) and TNF-\(\alpha\) on the MCD diet\textsuperscript{28}. In addition, infiltrated monocytes produce the higher amount of TNF-\(\alpha\) than KCs during the ongoing progression of NASH development, with KCs has little contribution. Consistent with these results, we found cocoa has no effect on plasma TNF-\(\alpha\) level our previous published paper\textsuperscript{135}. There results indicating effects of cocoa on the production of IL-1\(\beta\) and TNF-\(\alpha\) may be independent of monocyte infiltration in the liver.

Another possible anti-inflammatory mechanism of cocoa is through the increasing of M2 macrophage population and its anti-inflammatory cytokine production. The successful resolution of inflammatory processes requires the inhibition of pro-inflammatory signaling. M2 macrophages typically fulfill this function, owing to their high capacity to counteract the pro-inflammatory functions of M1 macrophage\textsuperscript{202,203}. We found that cocoa treated mice favoring M2 KC polarization and that might protect against fatty liver disease. CD163, a marker for M2 macrophage significantly increased in cocoa supplemented group compared with HF control. This results indicating increase M2 KCs population in the liver. In line with this result, we found anti-inflammatory cytokine, IL10, significantly increased in HF-HFC compared with HF control,
IL10, which mainly produced from M2 macrophage, displays potent anti-inflammatory properties\textsuperscript{202-205}. Recent studies identify a new mechanism for M1 macrophage elimination that relies on IL10 induced M1 macrophage apoptosis\textsuperscript{206}.

4.6 Conclusion

In summary, we have observed that dietary supplementation with cocoa ameliorates obesity-liver inflammation in HF-fed obese mice, principally mediated by increasing M2 macrophage population, down-regulation of pro-inflammatory gene expression and up-regulation of anti-inflammatory gene expression. These effects appear to be due in the inhibition of macrophage infiltration and the elevation of M2 macrophage in liver. With future works, we need to investigate the underlying mechanism why cocoa is able to prompt the increase differentiation of M2 KCs.
Chapter 5

Conclusions and prospects

5.1 Achievements

Our previous studies have preliminary results regarding the beneficial effects of dietary cocoa on obesity related mitochondrial syndrome\(^{112,135,135-137}\). In those study, we found dietary cocoa significantly improved key NAFLD criteria, including liver weight, ALT level and triglycerides contents compared with HF-fed mice. These results motivate us to investigate the mechanisms, which is very rare to study to the best of our knowledge. For this dissertation, we test the hypothesis that cocoa supplementation will ameliorate obesity related NAFLD through modulating lipid metabolism pathway, boosting endogenous antioxidant defense capacity, increasing mitochondria biogenesis and reduce inflammation.

In chapter 2, we demonstrate that cocoa-treated obese mice decrease Triglycerides (TAG), an important diagnostic marker of NAFLD, by enhance fatty acid disposal and reduce lipogenesis in the liver. We found cocoa supplementation significantly decreased the hepatic gene expression of Agtl and Hsl which are associated with lipolysis. By contrast, gene expression of Cpt1 were significantly increased in HF-HFC compared with HF group. In addition, dietary cocoa significantly reduced SCD1 activity expressed as product/precursor ratio (C16:1/C16:0 and C18:1/C18:0). SCD1 is major enzyme related to fatty acid de no lipogenesis. It catalyzes delta-9 - desaturation of long-chain fatty acids, leading to biosynthesis of palmitoleic (C16:1) and oleic (C18:1) acids as major products\(^{132}\). Finally, we found cocoa increased the profile of two important w-3 fatty acids (EPA and DHA), may also contribute to anti-inflammatory effects.
These data reveal a novel hepatoprotective effect of dietary cocoa in the context of obesity-related fatty liver disease.

In chapter 3, we demonstrate that the beneficial effects of cocoa supplementation on NAFLD is through increasing mitochondrial biogenesis and related antioxidant response signaling. We found cocoa treatment increases hepatic mitochondrial DNA copy number by 48.6% compared to HF-fed mice, suggesting increased mitochondrial biogenesis. Using real-time PCR, we found that mRNA levels of sirtuin (sirt)1, peroxisome proliferator-activated receptor gamma coactivator 1a, and nuclear respiratory factor 1 were significantly increased in HF→HFC compared with HF-fed mice. These genes have been reported to promote mitochondrial biogenesis. We found that cocoa supplementation decreased hepatic lipid peroxidation by 57% compared to HF-fed controls indicating reduced oxidative stress. Cocoa supplementation also increased the expression of Sirt3, an important mitochondrial redox regulator, as well as manganese superoxide dismutase and glutathione peroxidase (Gpx)1 was increased in cocoa supplemented mice HF→HFC compared to HF-fed controls. Cocoa also increased hepatic Gpx activity by 43% compared to HF-fed controls.

Furthermore, a local balance between classical (M1) and alternative (M2) activation of Kupffer cell (KCs) is considered as a predominant mechanism in the pathogenesis and resolution of NAFLD. In chapter 4, we demonstrated that dietary cocoa is able to alternatively activate M2 KCs in high fat-fed (HF) mice, and this phenotypic switch protects mice from ongoing of liver damage compared with HF treated alone. Male C57BL/6J were fed with either low-fat (LF, 10 % kcal from fat) or HF (60 % kcal from fat) diet for 8 weeks. After 8 weeks, mice from HF group were randomized to HF diet or HF diet supplemented with 8 % cocoa powder (HF–HFC group) for 10 weeks. Our results show that HF-HFC significantly decreased the hepatic expression of (macrophage inflammatory protein 1) mip1a at mRNA level and monocyte chemotactic protein-1 (Mcp1) at protein level which are associated with monocyte infiltration. Interesting, cocoa
treatment significantly increase the gene expressions of M2 surface marker (e.g. CD163) and M2 related anti-inflammatory cytokine interleukin 10 (IL10). These data reveal a novel hepatoprotective effect of dietary cocoa in the context of obesity-related fatty liver disease.

In conclusion, cocoa supplementation did mitigate obesity-induced inflammation and hepatic injury, and could be useful in preventing the progression of hepatic steatosis to NASH. The effects seen may be due three mechanisms: modulating lipid metabolism pathway, boosting endogenous antioxidant defense capacity, increasing mitochondria biogenesis and reduce inflammation. While the underlying mechanism requires further investigation, it is clear that dietary cocoa could be a potential dietary intervention to modulate obesity-related fatty liver disease.

5.2 Future works

5.2.1 Investigating mechanism of cocoa induced mitochondrial biogenesis

In chapter 3, we found cocoa supplementation significantly increased mitochondrial biogenesis through SIRT1-PGC1-NRF1 pathway, which is a proven pathway to regulate mitochondrial biogenesis. At the beginning, we think those results maybe the direct activation of sirt1 activity by cocoa. Indeed, many plant polyphenols has been found to be activator for SIRT1171. We found a significant increase in the gene expression of sirt1. Inconsistent with gene expression, SIRT1 protein level and nuclear SIRT1 activity remains unchanged. In this case, our data do not support the notion that these metabolic effects are mediated by direct SIRT1 activation. Rather, cocoa may indirectly activate sirt1 through other mechanisms, for example, the activation of AMPK signaling pathway172–174. AMPK has been shown to activate SIRT1 through two indirect pathways. Firstly, AMPK enhances SIRT1 activity by increasing cellular NAD+
levels, resulting in the deacetylation of transcriptional factors, such as PGC1α, FOXO1 and FOXO3α. In line with these finding, we observed significant increase in those three transcriptional factors, which may be regulated by boosting cellular NAD+ levels. Secondly, liver kinase B1 (LKB1) has been identified as a major AMPK kinase in the liver. Increasing evidence has shown that the AMPK phosphorylation by natural compounds is dependent on upstream LKB1 activation for the protection of the liver from hepatotoxicity. LKB1 signals the activation of AMPK phosphorylation, this AMPK activation will in turn activate SIRT1 activity by increasing cellular NAD+ levels. I observe a significant increase in hepatic gene expression of Lkb1 in cocoa supplemented group compared with HF controls (data not shown). It would be interested to study whether AMPK is a crucial components of increased mitochondrial biogenesis by cocoa treatment.

5.2.2 Investigating the effects of cocoa supplementation on plasma oxylipins profile

The key role of oxidative stress in disease progression of NAFLD is well established. Multiple studies have documented the increase oxidative stress and circulating lipids oxidation product in different animal model or patients with NASH. A recent study compare plasma lipidomic signature in patient with NAFLD and NASH, and showed that circulating oxylipids, especially lipoxygenase metabolites and products of nonenzymatic (free-radical-mediated) oxidation of arachidonic acid (AA), is increased. Oxylipids are produced by polyunsaturated fatty acids (PUFA), particularly by arachidonic acid (AA) and linoleic acid (LA). Lipoxygenase (LOX) and cyclooxygenase (COX) convert AA to eicosanoids, which are strong inflammatory modulators. This family includes prostaglandins (PG), leukotrienes (LT), lipoxins (LX), hydroxyeicosatetraenoic acids (HETE) and hydroperoxyeicosatetraenoic acids (HpET).
Our previous data suggests cocoa prevents liver oxidative damage through the inhibition of lipid peroxidation and the stimulation of mitochondrial antioxidant defenses. We found HF significantly inhibits GPX (glutathione peroxidase) activities (48.5%) in the liver, this trend is reversed by cocoa treatment with significant increase by 43.7% and in HF-HFC. HF-HFC also showed an increase in superoxide dismutase (SOD) activities (30%) compared with HF group. One beneficial effect of those activated antioxidant system is inhibition in lipid peroxidation determined by TBAR assay. AA can be enzymatically metabolized by three main pathways: CYP450s, COXs and LOXs. The COX pathway metabolizes AA to form prostanoids, including the PGs. The LOXs are more numerous and convert AA into diverse eicosanoids including leukotrienes (LT)s. Blocking one of these pathway can prompt the body to switch to other available alternatives. A decrease of GPX activity in HF group will prompt AA get metabolized by COXs and CYP450s. In the future, the effects of cocoa on plasma oxylipins in a mouse model of NAFLD can be investigate. Levels of plasma oxidized lipids including PGs, LTs, HETEs can be quantified by mass spectrometry. We may observe global shift of oxidized lipid metabolites from COXs or CYP450s family in the HF group to LOXs metabolites in HF-HFC group.

5.2.3 Investigate the effects of cocoa supplementation on gut permeability

Emerging evidence suggests that patient or animal model of NAFLD has different degrees of intestinal leakiness which facilitate gut-derived pathogen or pathogen components translocate to the liver and generate inflammatory response. For example, Lipopolysaccharide (LPS), cell wall of gram negative bacterial, translocate into intestinal capillaries through a toll-like, receptor 4 (TLR4)-dependent mechanism and rushes into the liver via portal vein. Many liver cells can sensor LPS through TLR4 recognition manner and result in the production of several pro-inflammatory cytokines. These produced cytokines sensitizes the
fatty liver to other additional “hits” such as oxidative stress which lead to necroinflammation and fibrosis\(^{21,218,26,22}\). Thus, gut barrier integrity plays an important role in maintaining the health condition of NAFLD.

In the future, a multi-sugar assay will be administered before sacrifice to assess specific intestinal permeability and plasma and liver LPS levels will be measured. Two hours before sacrifice mice will be gavaged (4mL/kg body weight) with a solution of L-rhamnose (0.1 mg/mL), lactulose (0.2 mg/mL), erythritol (0.2mg/mL), sucrose (0.2 mg/ml) and sucralose (0.2mg/mL). Plasma and urine will be collected and analyzed via gas chromatography-mass spectrometry. The area under the curve for each compound will be analyzed and appropriate sugar ratios will be calculated to determine specific intestinal permeability. In human and animal studies, differential sugar-absorption has been used to assess relative permeability of the intestinal tract. This assay elucidates gut barrier function by comparing the transcellularly absorbed monosaccharide and sugar alcohol, rhamnose and erythritol, to the paracellularly absorbed disaccharides, lactulose and sucralose. Sucrose is rapidly hydrolyzed by sucrose in the duodenum and used to assess the mucosal permeability of the stomach and duodenum\(^{215}\). Mannitol absorption is more dependent on intestinal surface area, whereas lactulose absorption is more affected by changes in epithelial tight junctions. Thus, lactulose/rhamnose (L/R) ratio were used for small intestinal permeability. Sucralose/erythritol (L/R) were used to assess colonic permeability. A decrease in ratio of lactulose/rhamnose (L/R) and sucralose/erythritol (L/R) indicate improved intestinal and colonic permeability. This decreased gut permeability will lead to a decrease in plasma and liver LPS level.
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• Mingyao Sun, Joshua D. Lambert. Dietary Cocoa improves hepatic mitochondrial function in obese mice. In preparation.
• Mingyao Sun, Joshua D. Lambert. Evaluate the polyphenol contents and inhibitory potency against key digestive enzymes of teas in the United States market. In preparation.
• Mingyao Sun, Ling Tao, Yeyi Gu, Joshua D. Lambert. Dietary cocoa improves hepatic mitochondrial function in obese mice. Oral and Poster presentation, Experimental Biology 2015 at Boston, Massachusetts, USA. (Oral and Poster presentation)
• Ling Tao, Mingyao Sun, Weslie Khoo, Jong-Yung Park, Soo-Yeon Lee, Shannon Kelleher, Joshua D. Lambert. The Role of Metallothioneins in the Differential Pro-oxidant Effects of (-)-Epigallocatechin-3-gallate (EGCG) in Oral Cells. Experimental Biology 2015 at Boston, Massachusetts, USA. (Oral and Poster presentation)
• Mingyao Sun, Ling Tao, Yeyi Gu, Joshua D. Lambert. Dietary cocoa improves hepatic mitochondrial function in obese mice. Poster presentation, Food Systems for Gut Health Conference 2015 at State College, Pennsylvania, USA. (Poster presentation)