

BENEFICIAL EFFECTS OF BERBERINE ON NONALCOHOLIC FATTY LIVER  
DISEASE (NAFLD)

A Thesis

by

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## ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is currently a worldwide problem associated with diabetes, obesity, heart diseases, and insulin resistance. The pathogenesis of NAFLD is divided into simple steatosis, through nonalcoholic steatohepatitis (NASH) to fibrosis and cirrhosis finally, lipid deposition and chronic inflammation are the main markers. There is still no effective medicine for the therapy of NAFLD, specifically in the NASH stage.

Berberine (BBR) is a natural product isolated from plants such as *Coptis chinensis Franch*, and it has been reported it had beneficial pharmacological activities in a series of metabolic diseases, focusing on adenosine monophosphate-activated protein kinase (AMPK) pathway. BBR showed anti-hyperglycemic and anti-hyperlipidemic effects in diabetes and obesity patients and animal models. It improved insulin sensitivity in these reports and our experiment. We found BBR improved insulin resistance and glucose intolerance in HFD mice, without body weight or food intake difference. BBR ameliorates fat deposition in liver, through reducing lipogenesis related genes expression. Meanwhile, BBR decreased inflammation in liver and adipose tissue. BBR inhibited JNK pathway and activated AMPK phosphorylation in hepatocytes, but not in the macrophages. Thus, the function cells may be the main cell type that responds to BBR in liver, and maybe BBR down-regulates lipogenesis through inhibiting Akt.

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## NOMENCLATURE

Abbreviations	Full Name
ACC	Acetyl-CoA carboxylase
AMPK	AMP-activated protein kinase
BBR	Berberine
BMDM	Bone marrow-derived macrophages
CPT-1	Carnitine palmitoyl transferase-1
JNK1	c-jun N-terminal protein kinase 1
DGAT2	Diacylglycerol acyltransferase 2
DMEM	Dulbecco's modified eagle medium
FAS	Fatty acid synthase
FFAs	Free fatty acids
GSH	Glutathione
GSH-Px	Glutathione peroxidase
HFD	High-fat diet
HSL	Hormone-sensitive lipase
IKK $\beta$	Inhibitor of nuclear factor- $\kappa$ B kinase- $\beta$
IL-6	Interleukin-6
IMDM	Iscoe's modified Dulbecco's medium
LPS	Lipopolysaccharide
LFTs	Liver function tests

LDLR	Low-density-lipoprotein receptor
LFD	Low-fat diet
MDA	Malondialdehyde
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NF- $\kappa$ B	Nuclear factor- $\kappa$ B
PUFAs	Polyunsaturated fatty acids
PVDF	Polyvinylidene difluoride
L-PK	Pyruvate kinase
RT-PCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
SAA3	Serumamyloid A3
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gels
SCD	Stearoyl-CoA desaturase
SREBP-1c	Sterol regulatory element-binding protein-1c
SOD	Superoxide dismutase
TZD	Thiazolidinediones
TLR4	Toll-like receptor 4
TNF $\alpha$	Tumor necrosis factor alpha
T2DM	Type 2 diabetesmellitus
VLDL	Very low-density lipoprotein

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## 1. INTRODUCTION

It was reported more than 30 million Americans were affected by Nonalcoholic fatty liver disease (NAFLD), which starts from lipid accumulation in hepatocytes, without consuming excessive alcohol. NAFLD is commonly linked with dyslipidemia, insulin resistance, and accompanied chronic inflammation, and it always represents a bystander and risk (steatosis) for the metabolic syndrome, including hypertension, type 2 diabetes, and cardio-vascular disease(1,2).

By now, there is no specific medicine in the treatment of NAFLD. In clinic guild line books, lifestyle modification such as more physical activity and less caloric intake is still considered as best therapy method, insulin-sensitizing agents including thiazolidinediones (TZD) and metformin will be used as adjuvant drugs. In Asia, because of long history development and utilization of herbs, many research focused on the drugs isolated from natural products.

Berberine is a kind of isoquinoline alkaloid presents in many plants. In the past 10 years, in many clinic trials, it has been proven to be an effective agent in controlling dyslipidemia and dysglycemia of type 2 diabetes and CVD(3). In some research, BBR is reported to up-regulate hepatic LDLR expression in HepG2 cells(4), and reduce fatty liver development in high-fat diet fed mice or obese db/db and ob/ob mice(5). The precise mechanisms of benefits of BBR for NAFLD are unclear. Multiple mechanisms involved in inflammation, lipid metabolism, and insulin resistance pathways, were considered playing important roles.

## **1.1 Nonalcoholic Fatty Liver Disease**

Presently, Nonalcoholic fatty liver disease (NAFLD) is a widely common liver disorder in worldwide. NAFLD may affect persons of all ages and most racial groups. Nonalcoholic fatty liver disease is associated with insulin resistance and hyperinsulinemia even in lean individuals with normal glucose tolerance.

### **1.1.1 Prevalence**

“Nonalcoholic steatohepatitis” has been recognized as a clinical syndrome since 1962. These patients were diagnosed with increased serum alanine aminotransferase levels but not as high as the alcoholic hepatitis patients, and some of them would may incur further liver injury(6). It was reported almost half of them progressed to liver fibrosis, 15% developed cirrhosis, and finally 3% have hepatocellular failure (7).

NAFLD is frequently associated with type 2 diabetes mellitus, obesity, and dyslipidemia. It was reported the prevalence of obesity varied 30% - 100%, among type 2 diabetes the prevalence is 10% - 75%, and hyperlipidemia is 20% - 92%(8).

### **1.1.2 Diagnosis**

Most NAFLD patients had central fat accumulation, increased triglycerides and uric acid, and low HDL cholesterol, irrespective of BMI(8). In the USA, most of the abnormal liver function tests (LFTs) results mean a diagnosis of NAFLD. Around 80% of the fatty liver could be determined by the ultrasound examination, and serum alanine aminotransferase always increased. Serum alkaline phosphatase or  $\gamma$ -glutamyltransferase

will increase, and hyperbilirubinemia, a prolonged prothrombin time, and hypoalbuminemia may also be found in the NAFLD patients. Sometimes the serum ferritin levels and transferrin saturation will elevate, however, the hepatic iron level is normal. The relationship of iron metabolism and fatty liver cannot be confirmed.

### **1.1.3 Pathogenesis**

The mechanism of nonalcoholic fatty liver disease is still not clear and the pathogenesis is still being worked out. Generally, the pathogenesis starts from simple steatosis (fatty liver), through nonalcoholic steatohepatitis (NASH) to fibrosis, and ultimately liver cirrhosis. Day et al.(9) gave a statement of "two-hit" model to describe the NAFLD progression. The first hit is insulin resistance causes lipid accumulation in hepatocytes. This symptom could be alleviated by modifying lifestyle; in the second hit, it was proposed that lipid oxidation, inflammation, and oxidative stress will lead to hepatocyte damage, resulting in irreversible NASH. NASH presents liver injury, hepatocyte carcinoma, and inflammation, thus it is irreversible. It is poorly understood why some of the patients with simple steatosis will develop into steatohepatitis, whereas some other will proceed into severe fibrosis and cirrhosis, and some NAFLD patients were lean and the fasting serum triglyceride level is normal.

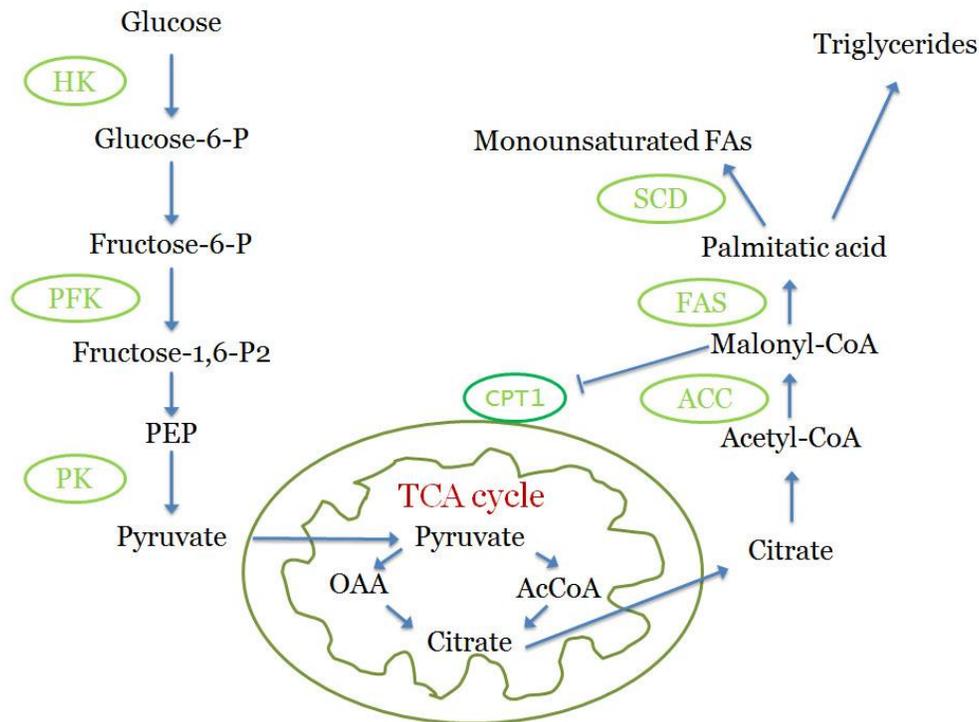
#### **1.1.3.1 Molecular mediators of hepatic steatosis**

NAFLD is always induced by excess dietary fat intake or increased lipolysis in the adipose tissues. Abnormal metabolism of lipid and lipoprotein could be considered as a

mark of NAFLD, and liver produces excessive cytokines and very low-density lipoprotein (VLDL), which result in exacerbated accumulation of triglycerides in the liver.

In the first stage, triglyceride accumulation in the liver is a hallmark of NAFLD. It was commonly understood the main reason is insulin resistance. In the insulin resistant state, hormone-sensitive lipase (HSL) activity is increased which lead to increased release of free fatty acids (FFAs) from adipose tissues. Thus, the plasma FFAs levels increase, it also means the amount of FFAs taken up by liver elevates. FFAs taken up by the liver are either oxidized to generate ATP in mitochondria or esterification to produce triglycerides, which may be stored within the hepatocyte or incorporated into VLDL particles. (10)

In the setting of insulin resistance, insulin will lose its ability to suppress gluconeogenesis genes, while hyperinsulinemia induces expression of a membrane-bound transcription factor, sterol regulatory element-binding protein-1c (SREBP-1c), which will lead to the activation of all lipogenesis genes(11). Increased hepatic SREBP-1c transcription will activate the enzyme Acetyl-CoA carboxylase (ACC), leading to increased malonyl-CoA production. Malonyl-CoA is the inhibitor of carnitine palmitoyl transferase-1 (CPT-1), the protein transporting fatty acid into the mitochondria. Thus, insulin resistance will not only enhance de novo fatty acid synthesis, but also inhibit fatty acid  $\beta$  oxidation.



**Figure 1.1 Processes of glycolysis and lipogenesis in livers of normal individuals.** When glucose intake is excess, more fatty acids are synthesized.

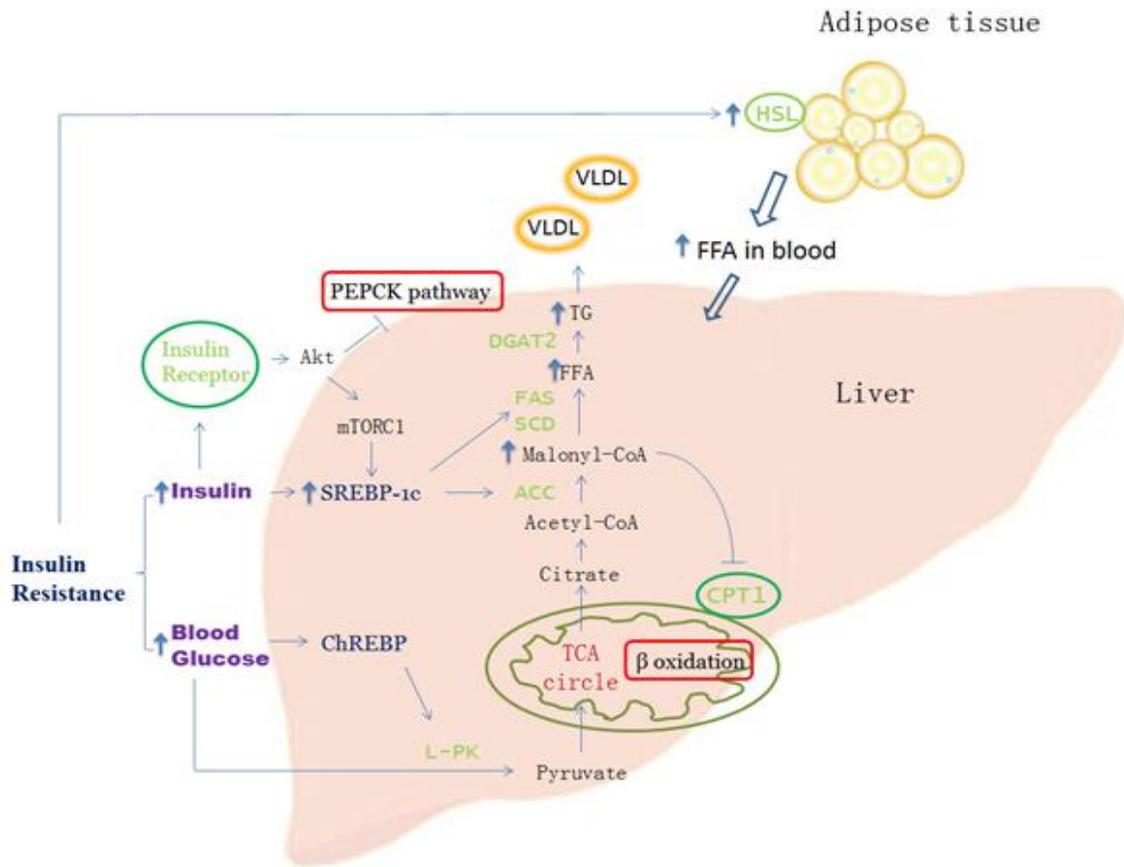
The normal process of the lipogenesis and fatty acid  $\beta$  oxidation are shown in Figure 1.1. When the intake of glucose is excess, glucose is up-taken by liver and converted to pyruvate, which will enter into the TCA cycle in mitochondria, then in the cytosol the citrate generated in the TCA cycle will convert into acetyl-CoA. The acetyl-CoA is the resource of fatty acids synthesis, and it is converted to malonyl-CoA by Acetyl-CoA carboxylase 1 (ACC1), and finally palmitic acid. Palmitic acid may be desaturated to palmitoleic by acid stearoyl-CoA desaturase (SCD). On the contrast, in liver,  $\beta$  oxidation of fatty acids yields acetyl-CoA, NADH and FADH<sub>2</sub>. NADH and

FADH<sub>2</sub> will enter into respiratory electron transfer chain and generate ATP. Acetyl-CoA is used to synthesize ketone bodies, and the ketone bodies travel to non-hepatic tissues as energy. The malonyl-CoA generated in the lipogenesis process is the inhibitor of Carnitine palmitoyl transferase I (CPT1), which is responsible to exchange carnitine for fatty acyl-CoA in cytosol, and this is the first and speed-limited step of  $\beta$  oxidation of fatty acids. Thus, increased fatty acid synthesis also means inhibit of  $\beta$  oxidation of fatty acid.

Insulin resistance means increased hepatic glucose production and elevated plasma glucose concentration. Glucose stimulates ChREBP to bind to an E-box motif of pyruvate kinase (L-PK), a glycolysis regulatory enzyme catalyzes phosphoenolpyruvate to pyruvate, which is converted to acetyl-CoA in TCA cycle(12). Fatty acid synthesis is elevated.

It seems like there are two directions in the insulin signaling pathway when it comes to insulin resistance: the ability of insulin to block glucose production declines, and the ability to stimulate fatty acid synthesis is enhanced. Li et al.(13) indicated the two different insulin-mediated changes will be blocked by inhibiting PI3K and Akt, and a decreased phosphorylation of Akt will lead to decreased mTORC1 activity and, therefore, decreased SREBP-1c mRNA, however, there is no effect on insulin suppression of *PEPCK*.

All the molecular mechanism of hepatic steatosis was summerized in the Figure 1.2.



**Figure 1.2 A series of molecules participate in the development of hepatic steatosis.**

There was another hypothesis: the excess FFAs in liver would supply more energy to promote the excess gluconeogenesis, leading to increased production of hepatic glucose. Thus, in the insulin resistant state, the blocked effect of insulin on gluconeogenesis is inhibited but lipogenesis is not in fatty liver.(14)

AMP-activated protein kinase (AMPK), a sensor of cellular energy storage, is considered a key factor in the pathogenesis of hepatic steatosis as well, via

phosphorylated regulating ACC, reducing the expression levels of SREBP-1c, and inhibiting ChREBP(15). When fasting, cellular AMP levels increase, and AMPK is activated, therefore, stimulates ATP production pathway and inhibit ATP utilization pathway. AMPK regulate lipid accumulation in liver through phosphorylating enzymes involved in lipogenesis and fatty acid  $\beta$  oxidation.

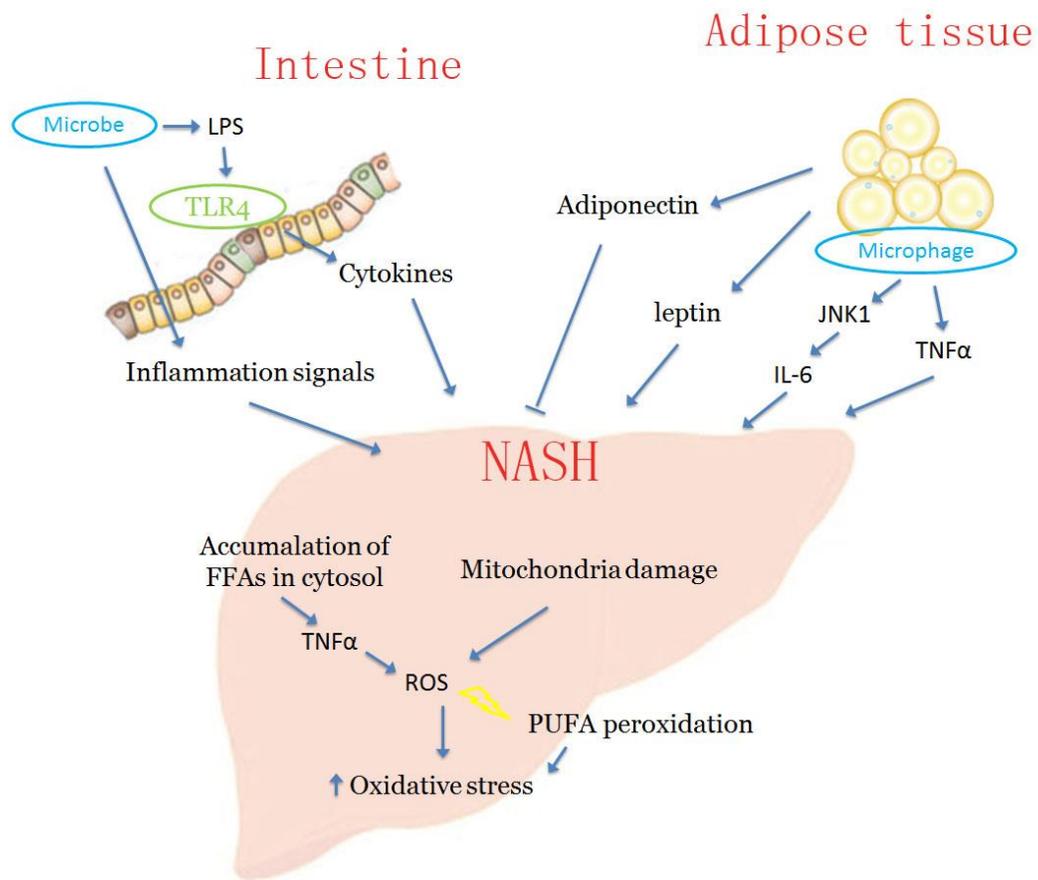
The insulin-induced lipogenesis may lead to triglyceride accumulation in liver in two aspects. One is increased synthesis of triglycerides directly, and another is the increased malonyl-CoA production during lipogenesis which will inhibit CPT-1, reducing fatty acid  $\beta$  oxidation.

#### **1.1.3.2 Development of nonalcoholic steatohepatitis (NASH)**

No more than 20% of the NAFLD patients will develop NASH. Most studies of the mechanism that hepatic steatosis progresses to NASH are always hypothesis. The simple steatosis is a nonprogressive state which could be reversed by modification lifestyle. The irreversible stage of NAFLD that the hepatic steatosis develops to NASH could be considered as another type of disease because of a different pathogenesis. For example, Yamaguchi et al.(16) pointed out that decreased hepatic triglyceride content improves liver steatosis but worsens liver damage, through inhibiting triglyceride synthesis via Diacylglycerol acyltransferase 2 (DGAT2) antisense oligonucleotides. DGAT2 is the enzyme including in the final step of triglyceride synthesis.

It was hypothesized that chronic inflammation aggravates the process of NASH, and the factors of oxidative stress and pro-inflammatory cytokines are the most important factors, shown in Figure 1.3 (17).

Increased triglycerides and cholesterol accumulation in the hepatocyte mitochondria in the early stage of NAFLD can lead to increased oxidative stress. The oxidation of free fatty acids through mitochondrial  $\beta$  oxidation pathway activates tumor necrosis factor alpha (TNF $\alpha$ ) and, therefore, forms reactive oxygen species (ROS)(18), which causes liver damage. It was reported metformin and anti-TNF antibody would reduce steatosis in ob/ob mice (leptin mutation mice), both of them inhibit TNF $\alpha$ (19,20). ROS is the pro-oxidant factor, including superoxide anions, hydroxyl radicals and hydrogen peroxide. Increased ROS leads to DNA damage, destruction of cell membranes, cellular damage and pro-inflammatory cytokines release(21). An additional ROS production is considered to form mitochondrial dysfunction, and mitochondrial damage was found in liver biopsy of NASH patients. The mitochondrial impair interrupts the electron flow in respiratory chain, then hydrogen peroxide and superoxide anions form, and increased cytosolic fatty acids will be oxidized via  $\beta$  oxidation and  $\omega$  oxidation(22,23). On the other hand, polyunsaturated fatty acids (PUFAs) and lipid will be the target of ROS for non-enzymatic peroxidation, producing peroxides and their degradation products.



**Figure 1.3 Molecular mechanisms of nonalcoholic steatohepatitis (NASH).** The inflammatory signalings from other tissues such as adipose tissue and intestine would exacerbate the process of NASH.

The oxidation and peroxidation of fatty acids increase secretion of inflammatory factors, active immune cells infiltration and the stellate cells in the liver, leading to fibrosis and cirrhosis. Park et al.(24) indicated that activation of IL-6 and TNFα will lead to liver inflammation. The deletion of one of these two cytokines reduced liver lipid content and inflammation in mice.

Some researchers suggested inflammatory factors from other tissues such as adipose tissue and gut would play a central role in the process of NASH.

In obesity patients, the infiltration of macrophages (CD11c $\beta$  cells) in adipose tissue increases, and the macrophages in adipose tissue secrete more pro-inflammatory cytokines such as TNF $\alpha$  and interleukin-6 (IL-6). The liver is an important target organ of the cytokines. In adipose tissue, activation of inflammatory signal transducers c-jun N-terminal protein kinase 1 (JNK1) or inhibitor of nuclear factor- $\kappa$ B kinase- $\beta$  (IKK $\beta$ ) will cause insulin resistance, and insulin resistance will aggravate inflammation in NASH (25), additionally, IL-6 secretion is JNK1 - dependent. Human studies suggested liver inflammation is positively related with the amount of fat. Adipocytokines from adipose tissue are also considered to affect lipid accumulation and inflammation in liver(17). Adiponectin is an anti-inflammatory adipocytokine, and it reduces lipid accumulation and improves insulin resistance through activating AMPK in muscle and liver. The leptin will promote fatty acid oxidation and improve NAFLD.

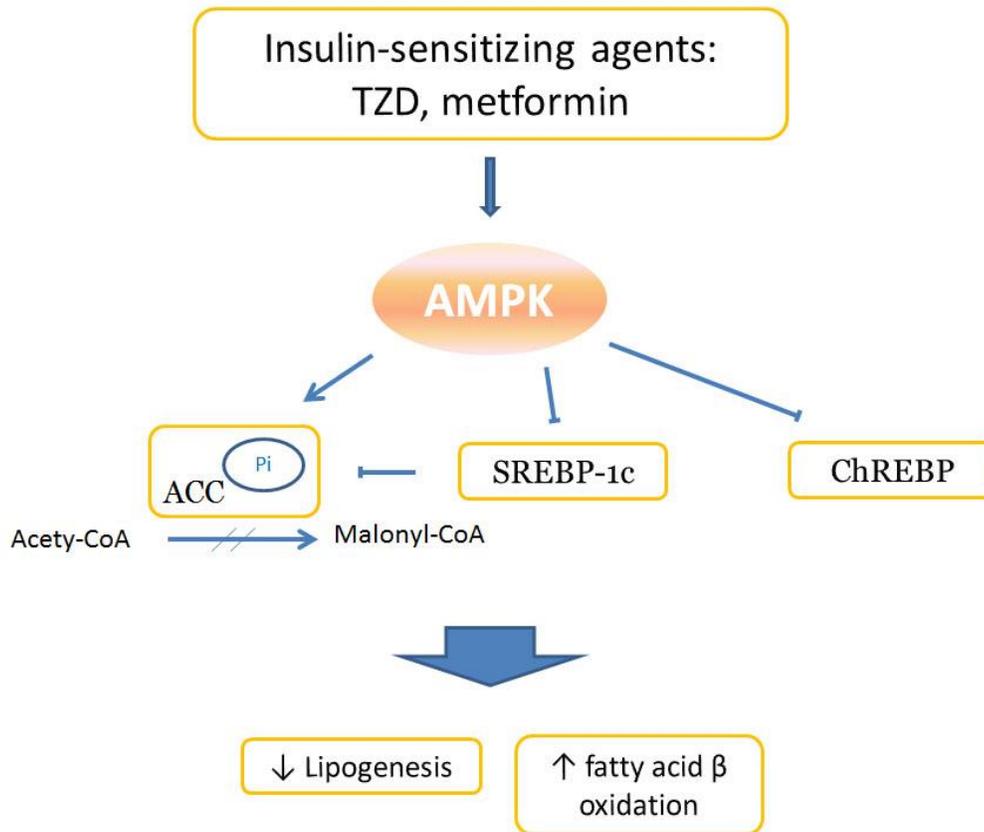
Signals from intestine might induce steatosis and inflammation. Lipopolysaccharide (LPS), named endotoxin as well, produced by a lot of bacteria in humans microbiota in the gut, was introduced to play a key role in the process of NASH(26). It was involved in the dietary factors activating systemic immune responses, when a high-fat or a high-carbohydrate diet might stimulate liver steatosis and worsen NASH. Toll-like receptor 4 (TLR4), expressed on the surface of epithelium and macrophages, may recognize free fatty acids and induce secretion of pro-inflammatory factors(27), therefore it plays a central role in inflammation of obesity and fatty liver.

Furthermore, many studies hypothesized inflammation in liver may play a key role in the NASH development, accompanied with increased inflammation markers, such as TNF- $\alpha$ , interleukin 6 (IL-6), and serum amyloid A3 (SAA3)(5).

#### **1.1.4 Management**

Lifestyle modification is still suggested as the best therapy method of NAFLD in the practice guideline by the American Association for the Study of Liver Diseases(28). For example, caloric restriction and increased physical activity, would achieve weight loss, and lead to decreased aminotransferases and hepatic steatosis. Heavy amounts of alcohol, such as more than 4 drinks/day or 14 drinks/week in men or more than 3 drinks/day or 7 drinks/week in women, are not recommended in NAFLD patients.

Insulin sensitizing agents were suggested to assist with life modification in NAFLD treatment. The antidiabetic drugs thiazolidinediones and metformin improved hepatic steatosis via reducing hepatic fat and decreasing liver size, and it was reported it was through activating AMPK, shown in Figure 1.4 (29,30). They are also effective in hypoglycemic therapy.



**Figure 1.4 Metformin and TZD improved hepatic steatosis via AMPK pathway.**

In addition, a daily dose of 800 IU Vitamin E, an anti-oxidant, also improves liver histology in biopsy-proven NASH patients without diabetes, and it would improve inflammation and ballooning injury. Omega-3 fatty acids are suggested as drugs to treat hypertriglyceridemia in NAFLD patients. (28)

Foregut bariatric surgery could be considered as a potential option for treatment NAFLD or NASH without cirrhosis, but the type and safety are still uncertain.

From the clinic data, metformin would reduce insulin resistance and aminotransferases, but has no significant effect on liver histology in NASH patients. Pioglitazone can be used in biopsy-proven NASH patients, but the adverse effect is promoting weight gain. Therefore, scientists try to find novel and specific drugs to treat NAFLD. In Asian, it is common for the doctors to think about natural compound isolated from traditional herb medicines, because of long-termed and widely utilization of traditional Chinese medicine.

## **1.2 Berberine**

Berberine is the major constituent of a kind of herb named *Rhizoma Coptis*, which has been officially recorded in the ancient traditional Chinese medicine book “*Shen Nong Elite Physicians*” as a powerful drug to treat diarrhea and peptic ulcer for over 2000 years, characterized its antimicrobial and antiprotozoal properties, it could kill most bacteria, viruses, parasites, yeast, fungi, protozoans and *Candida albicans*. However, since from 1988, the first time of discovery of the hypoglycemic effect of berberine in China(31), berberine has been utilized in improvement of many metabolic diseases. It was reported berberine would decrease energy storage and increase fatty acids oxidation, improve insulin sensitivity, protect from CVD, and etc. mostly via AMPK pathway (5,32).

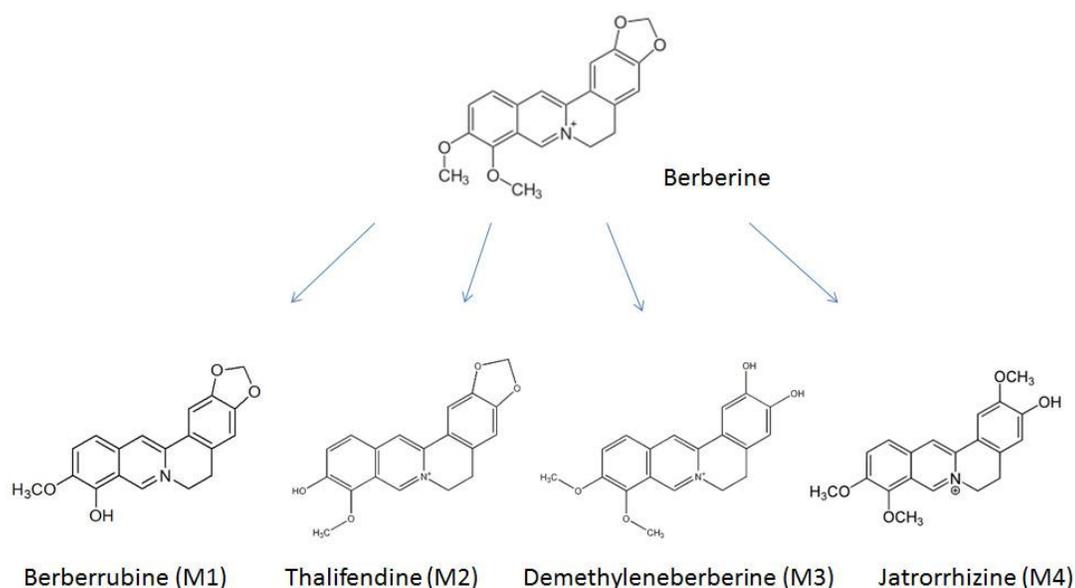
### 1.2.1 Chemical structure and pharmacokinetics

Purified berberine is yellow powder which is difficult to dissolve in water in the room temperature, and tastes extremely bitter. It could be isolated from roots, rhizomes, stems, and bark of various plants, such as *Coptis chinensis* (*Coptis* or *goldenthread*), *Berberis vulgaris* (*barberry*), *Hydrastis canadensis* (*goldenseal*), and *Berberis aristata* (*tree turmeric*), and the content is about 5.2% to 7.7%.

Berberine (BBR, C<sub>20</sub>H<sub>18</sub>NO<sub>4</sub>), an isoquinoline alkaloid, is the protoberberine type, there are many derivatives, including berberine hydrochloride, berberine sulfate, berberine citrate, and berberine phosphate. Generally, berberine sulfate is always used as veterinary medicine in form of injection fluid, because of its good solubility in the water, while humans can only use berberine hydrochloride p.o. pills because the high concentration of NH<sub>4</sub><sup>+</sup> in the blood flux is bad for human.

It was always thought to be poor oral bioavailability because the blood concentration is quite low and hard to be detected in the normal pharmacokinetics method. It was found P-glycoprotein located on the surface of epithelial cells prevent the absorption of berberine molecular. At least 9 types of derivatives have been detected as metabolic products after p.o. Ber administration. Zuo et al. (33) studied the metabolites of berberine in liver and bile in rats after p.o. administration, via liquid chromatography/tandem mass spectrometry (LC/MS/MS) with electrospray ionization. Berberrubine (M1), thalifendine (M2), demethyleneberberine (M3), and jatrorrhizine (M4), together with original BBR are the major metabolites (Figure 1.5) (33) in liver and bile. Previously, it was commonly confused that the plasma BBR concentration was

quite below the effective dose in vitro treatment. Zuo et al. detected the BBR and its major metabolites in liver tissues only 0.5 h and in bile 1h after p.o. administration. They supposed BBR was metabolized in liver fast, thus the clearance of BBR from blood is rapid and the concentration is trace, then the metabolites were excreted into the intestine by bile. The intestinal flora in humans or rats will promote reabsorption of metabolites from jejunum, and the metabolites remained in the rats for a long time to more than 24 hr.



**Figure 1.5 Chemical structures of berberine and its major metabolites.**

M1 is the most powerful of antitumor activity(34), and BBR and M2 were hypothesized the greater active forms of hypolipidemic effect in HepG2 cells(35). In the

experiment plan, we chose BBR alone to survey its effect on lipid metabolism in *vivo* and *vitro*.

### **1.2.2 Novel functions in metabolic diseases**

In the recent several decades, BBR was found to decrease blood glucose and lipid disorders in humans and animals. It was already found 1500 years ago that the herb *Rhizoma Coptidis* was recorded by Hongjing Tao in the book “Note of Elite Physicians” it can be used to treat diabetes. In 1988, it is the first time the hypoglycemic effect of berberine was found when doctors use berberine to treat diarrhea in diabetic patients in the traditional Chinese Medicine hospital. From then, lots of clinic trials proved the safety and effect of BBR in the treatment of type II diabetes mellitus. They used BBR alone or combined with oral hypoglycaemics or anti-diabetic agents, BBR has similar anti-hyperglycemic and anti-dyslipidemic function as metformin and thiazolidinediones (36). From the data analysis of about 700 clinic type 2 diabetes mellitus (T2DM) samples collected from published papers between 2007 and 2011, BBR showed a better dyslipidemic control than metformin, glipizide, or rosiglitazone, but similar anti-glycaemic effect. But combination of BBR and those drugs showed a better glycaemic control than metformin or TZDs alone(3).

In Asian, BBR has been used as an anti-hyperglycemic agent in many clinic trials. It was reported many time BBR would decrease postprandial blood glucose and lower fasting blood glucose in type 2 diabetes patients(3). The insulin resistance index, total cholesterol and serum triglyceride were reduced (3,37). It was supposed anti-

hyperglycemic ability of BBR was due to phosphorylating AMPK, leading to inhibit gluconeogenesis and increase glucose consumption, and BBR may stimulate glycolysis via increase aerobic and anaerobic respiration (38,39).

Berberine improves insulin sensitivity and reduced fat deposition in obese animals. Lee et al. (40) said BBR decreased body weight without altering food intake in db/db mice, an animal model of obesity and insulin resistant due to a mutation in the leptin receptor gene, maybe through increasing AMPK activity and decreasing phosphorylation of ACC in the adipose tissue, as well as increasing GLUT4 translocation in muscle. Cell treatment also showed BBR reduced lipid accumulation in 3T3-L1 adipocytes by inhibiting PPAR $\gamma$  activity. Meanwhile, Berberine altered lipid metabolic genes in adipose tissue, including FAS, AP2, UCP2, and SREBP1c.

In many experiments and clinic trials, BBR showed antioxidant and anti-inflammatory effect in the treatment of T2DM and obesity. Mostly, BBR decreases oxidative stress, through reducing oxidative stress markers malondialdehyde (MDA), increasing antioxidant glutathione (GSH), and promoting activities of antioxidant enzymes glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) (41). Additionally, AMPK is a key factor in the antioxidant effect of BBR, via down-regulating NADPH oxidase (42). BBR reduced inflammation response in tissues of liver, adipose tissue, kidney and pancreas in diabetes mellitus animals. In cultured HepG2 cells, liver cells, macrophages, and pancreatic  $\beta$ -cells, BBR decreased production of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6, IL-1 $\beta$  as well (43,44). It was also reported BBR decrease the serum level of IL-6 in T2DM patients. It was considered MAPK

signaling pathway play a crucial role in BBR anti-inflammatory activity. MAPK phosphorylation was inhibited by BBR in cells treated with inflammatory stimuli like lipopolysaccharide (LPS), FFA and TNF- $\alpha$ . BBR inhibited the nuclear translocation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), by reducing I $\kappa$ B kinase- $\beta$  (IKK- $\beta$ ) in 3T3-L1 adipocytes (45) and liver and adipose tissues in obesity mice, leading to decreased expression of various pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6, iNOS and COX2.

In addition, berberine has been shown benefits in the cardiovascular system, antitumor, immune-regulatory, antimalarial, anti-HIV, antidepressant, and against polycystic ovary and Alzheimer's disease, with significant anti-inflammatory activities.

BBR would inhibit cell proliferation and induce apoptosis in cancer cells, the associated mechanisms include impairing cell cycle, inhibiting the telomere elongation, increasing p53 protein activity, decreasing prostate cancer cells proliferation via AMPK activation, inducing autophagy, and inhibiting folate cycle (46).

### **1.2.3 Berberine in the treatment of nonalcoholic fatty liver disease**

A lot of evidences showed the crucial target of berberine in therapy of most of the metabolic syndromes focuses on AMPK pathway. As we all know, AMPK is an important upstream of most lipid metabolism pathway. Thus, for recent ten years, someone thought maybe berberine had some kind of benefits in ameliorating NAFLD as well as other metabolic syndromes.

Additionally, liver was considered to be the main target organ of BBR in vivo. BBR metabolites are widely distributed in many tissues, such as liver, kidney, spleen, heart,

and brain. In animal studies, neither original BBR nor its metabolites could be metabolized by human or rat intestinal bacteria flora, but by liver homogenate(33). Thus, liver is the primary disposition and metabolic organ for BBR, and BBR has a longer half-life in liver than other tissues. After oral administration, the original protoberberine was characterized by CYP450 isoenzymes via oxidative demethylation and then conjugated by glucuronic acid (47).

A lot of animal and cell experiments proved the hypothesis. Intracerebroventricular injection of  $5\text{mg kg}^{-1}\text{ day}^{-1}$  BBR in db/db mice, has been shown to promoted fatty acid oxidation in muscle and liver, leading to alleviate fatty liver. BBR increased fatty acid oxidation via activating AMPK expression and inactivating ACC in human HepG2 cells (35,48). Similarly, BBR regulates fatty acid metabolism genes in rat primary hepatocytes, including SREBP, acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), and stearoyl CoA desaturase 1 (SCD1)(49). Additionally, BBR significantly inhibited IL-6 and TNF $\alpha$  in HepG2 cells, and reduced ROS in rat livers(5). BBR lowers cholesterol-lowering distinct from statins, through up-regulating the low-density-lipoprotein receptor (LDLR) independent of SREBPs (4). BBR reduces hepatic fat content in the rats of nonalcoholic fatty liver disease, via decreasing methylation of the MTP promoter (50). Berberine improved histological features of NAFLD in high-fat diet fed rats, via up-regulating IRS-2 mRNA and protein levels in liver (51). BBR may also further prevent the development of NASH in liver fibrosis experimental models. BBR supplement may restore liver function, because clinical statistic data demonstrated the serum levels of

alkaline phosphatase (ALP) and aminotransaminases in type II diabetes patients were reduced by BBR (52).

## 2. EXPERIMENTAL AIMS

Conclusively, BBR exhibited the function of anti-hyperglycemic and anti-hyperlipidemic in metabolic diseases, as well as strong antioxidant and anti-inflammatory effect. It could improve insulin sensitivity and have beneficial effect of anti-hyperlipidemia and cholesterol disorder in NAFLD. However, after ten years of study on NAFLD, the molecular mechanism is still unclear. Besides, considering liver is the major site of BBR combination and metabolism, in this project, we focus on determining which cell types in liver may take the major response to Berberine in the NAFLD model.

### 3. MATERIALS AND METHODS

#### 3.1 Diet induced fatty liver mice model

C57BL/6J WT mice from the Jackson Laboratory were bred on a 12/12-h light-dark cycle. Male littermates of 5–6 weeks weighing 20 – 25 g were fed a high-fat diet (HFD, 60% fat calories, 20% protein calories, and 20% carbohydrate calories) for 12 weeks, and in the last 4 weeks they were fed with PBS as a control group or berberine (C<sub>20</sub>H<sub>18</sub>ClNO<sub>4</sub>, Sigma-Aldrich, St Louis, MO, USA) (100 mg kg<sup>-1</sup> day<sup>-1</sup>, dissolved in PBS) via oral gavages. Food intake and Body weight were measured every week. In the final week, all mice were euthanized, and the epididymal fat pads and the livers were weighted and removed, some pieces were stored in -80 °C for QPCR and western blot analysis, and others were fixed in 10% NBF for Oil-Red-O and H&E staining.

It has been proved in the previous experiments performed in our laboratory, that the HFD-fed mice could serve as an appropriate NAFLD mouse model(53), compared with mice fed with low-fat diet (LFD, 10% fat calories, 20% protein calories, and 70% carbohydrate calories). In order to simplify the animal experiment groups, we only set the HFD-induced NAFLD mice groups in this project, our object focus on the therapeutic effects of berberine on fatty liver. Additionally, in order to analyze accurate alternation in molecular metabolism, we used several tissue samples of LFD-fed mice.

All experimental procedures and the animal protocol were approved by Institutional Animal Care and Use Committee of Texas A&M University.

### **3.2 Insulin and glucose tolerance tests**

At 12 weeks of HFD, a glucose tolerance test was performed, the mice were fasted for 4 hours and then injected with 2 g/kg body weight D-glucose, 5 µl blood samples were collected from the tail veins, at 0, 30, 60, 90, and 120 min after the injection. For insulin tolerance test, the mice were injected with 1 U/kg BW insulin, and similarly blood samples were collected at 0, 15, 30, 45, and 60 min after the bolus insulin injection. The plasma glucose levels were all measured via the enzymatic assay kit (Sigma, St. Louis, MO).

### **3.3 Flow cytometry analysis**

Macrophage isolated from adipose tissue of C57BL/6J mice will be analyzed by flow cytometry. 4 fluorescence-colored antibodies, CD11b, F4/80, CD11c and CD206, were used to identify mature macrophage (CD11b<sup>+</sup> F4/80<sup>+</sup>), and ratio of M1 (CD11c<sup>+</sup>CD206<sup>-</sup>) and M2 (CD206<sup>+</sup> CD11c<sup>-</sup>).

### **3.4 Cell culture**

Rat hepatoma cell line H4IIE cells were cultured in high glucose Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum, 100 µg/ml streptomycin, and 100 units/ml penicillin, at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

Primary hepatocytes were isolated from WT C57BL/6J mice. After anesthetizing the mouse by xylazine (AnaSed, USA, mixture of 100ul ketaset and 10ul xylazine in 1ml PBS, 10ul/g body weight of mouse), perfuse the liver with perfusion I (PI, 142mM

sodium chloride, 6.7mM potassium chloride, 10mM Hepes, 2.5mM EGTA, pH 7.4) from the hepatic portal vein for 5- 10 min, until the liver turns white. Then switch to perfusion II with collagenase, it takes 5- 8 min and the liver tissue becomes soft and disintegrated. Collagenase need to be active by incubating in 37 °C for 30 min, 50mg collagenase (Sigma, St Louis, USA) and 1g fatty acid free albumin (BSA, Sigma, St Louis, USA) are dissolved in 100 ml of pre- perfusion II (PII, 66.7mM sodium chloride, 6.7mM potassium chloride, 100mM Hepes, 4.8mM calcium chloride dihydrate, pH 7.6). Gently dissociate cells by shaking the perfused liver, and then suspend the cells in cold Medium 199 (9.2g /L Gibco Medium 199 powder, 23mM Hepes, 26mM sodium bicarbonate, 11mM D- glucose, 100nM dexamethasone, 100nM insulin, 10% fetal bovine serum, 100 µg/ml streptomycin, and 100 units/ml penicillin, pH 7.4). Spin down cells 5 min at 4 °C and wash one time, and re-suspend cells in cold Medium 199 again. Carefully layer 5ml cells onto 5 ml percoll cushion [1:1 mixture of Medium 199 and percoll stock, which containing 45ml percoll (sigma), 5ml 10×PBS, and 0.5ml 1M Hepes, pH 7.4], and spin down cells 5 min at 4 °C. Wash the hepatocytes one time, and then plate them in plates, which already were coated by coating buffer [0.1ml acetic acid, 1ml rat tail collagen type I(Corning, Tewksbury, USA), diluted in 100ml dd water].

To induce lipid deposition, the hepatocytes were pre-treated 2h with 25mM palmitate, and then they were treated by PBS versus berberine (dissolved in DMSO and diluted in PBS).

We isolated bone marrow-derived macrophages (BMDM) from the same background mice for inflammatory response study. Bone marrow will be isolated from the

tibias and femurs of chow diet-fed C57BL/6J male wild-type mice around 12 weeks old. After cultured in Iscove's modified Dulbecco's medium (IMDM) with supplement of 10% fetal bovine serum and 15% L929 for 7 days, BMDM was change to be incubated in normal high glucose DMEM, and treated with LPS (100 ng/ml, 30 min for cell signaling) and BBR.

### **3.5 Western blot analysis**

Cells or tissues were lysed in the lysis buffer (20mM Hepes, 100mM sodium fluoride, 10mM sodium pyrophosphate, 1% NP-40, 0.1% SDS, 2mM EDTA, 2mM Na Vanadate, pH 7.4), and the protein concentration was determined by BCA assay (Thermo scientific, USA). Then the protein samples were separated via 8% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% milk in TBST for 4hr at room temperature. Then the membranes were incubated in 1:1000 primary antibodies (including P-AMPK/AMPK, P-ACC/ACC, Pp46/p46, and Pp65/p65, GAPDH was used as total control) diluted by 5% BSA in TBST at 4 °C overnight. After 3×5 min washes with TBST, the membranes were incubated for 2hr of secondary antibody, and detected with chemi-luminescent reagent.

### **3.6 RNA analysis**

Total RNA of cells and frozen tissues were isolated by STAT-60 and reverse transcribed into cDNA by the GoScript™ Reverse Transcription System (Promega),

and the relative mRNA expression levels were quantified by quantitative real-time polymerase chain reaction (RT-PCR), using SYBR Green (LightCycler® 480 system; Roche). The mRNA expression level was normalized by 18S. The sequences of the primers include: ACC1, FAS, SREPB1c, CPT1a, arginase 1, adiponectin, and resistin, TNFa, IL-1b, IL-6. The results were analyzed by the  $2^{-\Delta CT}$  calculation.

### **3.7 Oil-Red-O staining**

In order to determine the lipid accumulation, the primary hepatocytes and frozen livers were stained with Oil-Red-O. After the treatment, the cells were fixed in 75% formaldehyde in PBS for 15min, and then stained with Oil-Red-O solution [0.5% stock : dd water is 6:4, 0.5% stock (5 mg/ml powder in isoproponal)] for 1h, then washed with ddH<sub>2</sub>O for 3 × 5 min and take pictures.

### **3.8 Statistical analysis**

Data are presented as mean ± SE. Two-group differences were detected by Two-tailed independent Student's t-tests, and P < 0.05 were considered significantly.

## 4. RESULTS

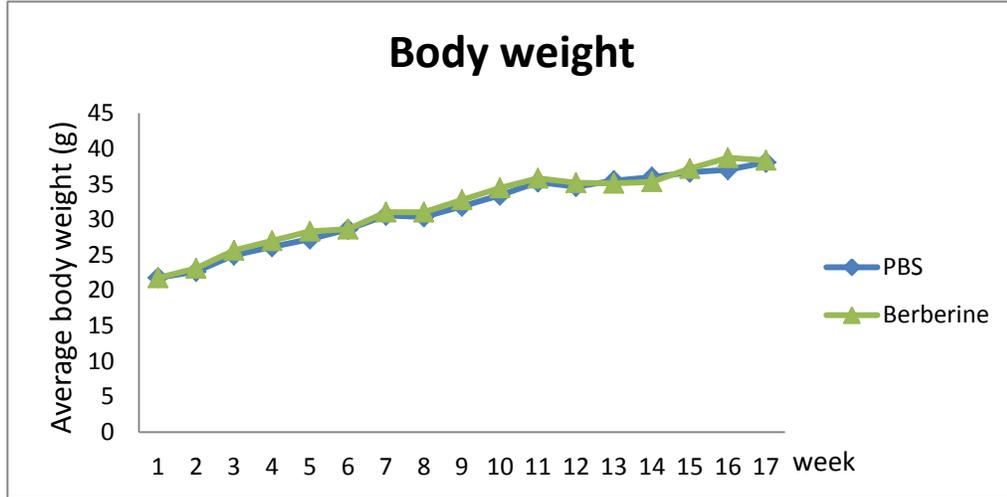
### **4.1 Effect of berberine on the characteristics of the HFD mice**

We induced nonalcoholic fatty liver of C57BL/6J mice with close body weight by 8 weeks high fat diet feeding. The body weight of the male mice increased from 22 g average to around 35g, and the calorie intake remains stable. Then the mice were divided into two groups with 4 weeks oral gavage BBR or phosphate-buffered saline (PBS). Compared with PBS group, berberine did not affect the body weight of HFD-fed mice significantly (Figure 4.1A, HFD-PBS vs. HFD-BBR), as well as the food intake (Figure 4.1B, HFD-PBS vs. HFD-BBR). The data suggested BBR would not affect the mice appetite, even though it tastes bitter.

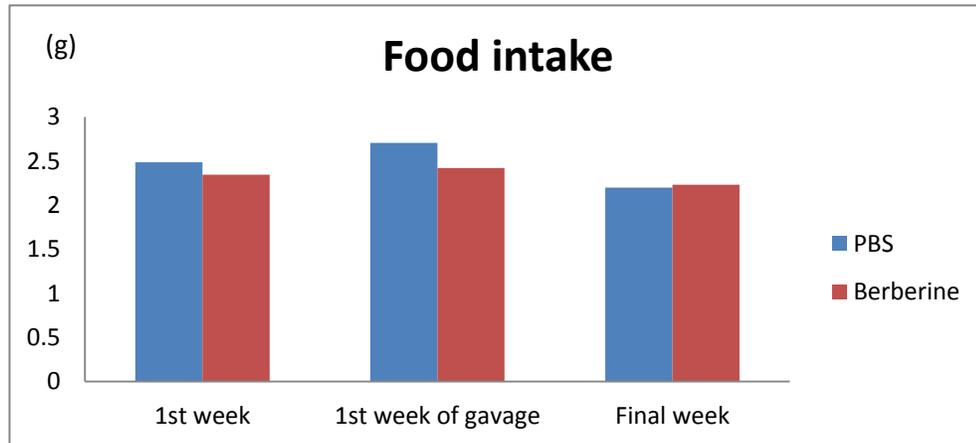
### **4.2 Berberine decreases insulin resistance and glucose intolerance in HFD mice**

Systemic insulin sensitivity was evaluated by glucose tolerance test (GTT) and insulin tolerance test (ITT). The GTT test data shows berberine treatment decrease blood glucose of HFD mice (Fig 4.2A). The response of plasma levels of glucose to glucose peritoneal injection in the BBR – treated mice was more sensitive than the control group. The blood glucose of the BBR group increased modest, and the plasma glucose level decrease faster than the PBS group. In the ITT test, the blood glucose levels in both of the two group exhibited similar decrease after insulin injection, however, the general values of different time point were lower in BBR treatment mice (Fig 4.2B).

A

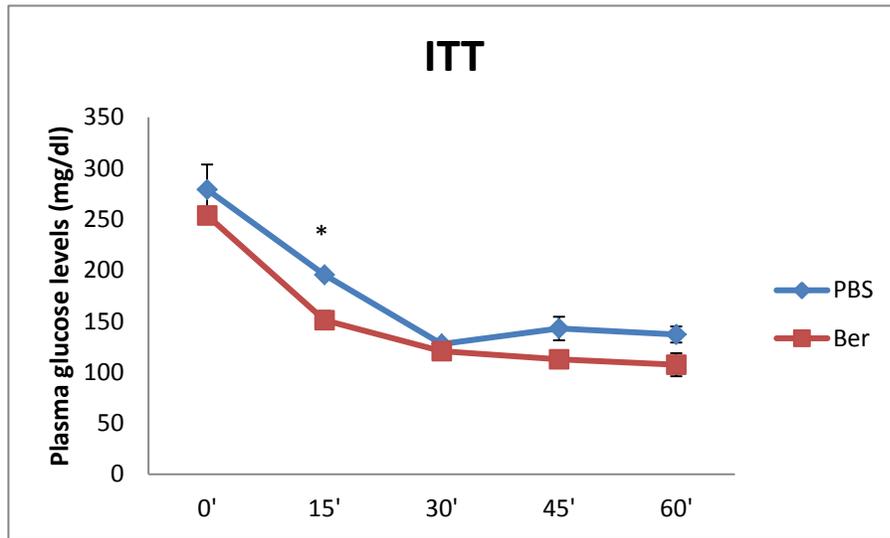


B

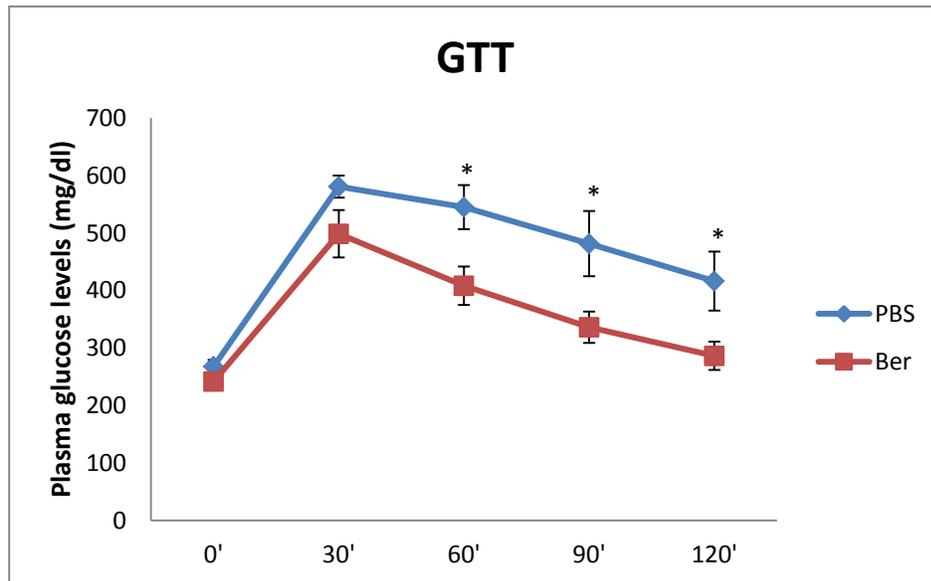


**Figure 4.1 Neither body weight nor food intake were affected by berberine.** Fed 5 ~ 6 weeks male C57BL/6J mice with high-fat diet (HFD) for 12 weeks, and gavage with phosphate-buffered saline (PBS) as control group or berberine (100 mg kg<sup>-1</sup> day<sup>-1</sup>, dissolved in PBS) for 4 weeks. (A) Body weight was measured every week during the HFD period. (B) Food intake was measured weekly and the result stands for food consumption per mouse/ per day.

A



B



**Figure 4.2 BBR reduced fasting blood glucose and improved insulin sensitivity.** After 4 weeks of BBR treatment, (A) Effects of BBR treatment on GTT. The mice were injected with glucose (2 g/kg body weight) after 4 hr fasting. Data are presented as mean  $\pm$  SE (n = 6). (B) Insulin tolerance tests. The mice received an injection of insulin (1 U/kg body weight). Data are presented as mean  $\pm$  SE (n = 6). \*, P<0.05, BBR group compared with PBS group at the same time point.

Therefore, it seems like berberine treatment decreases fasting glucose level, and ameliorates glucose intolerance and insulin resistance without changing body weight and food intake in HFD mice.

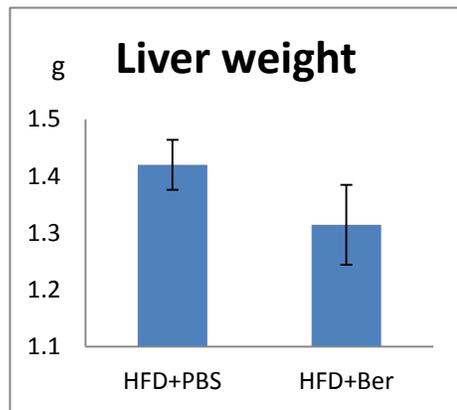
#### **4.3 Berberine decreases HFD-induced lipid accumulation in hepatic steatosis**

At the last week of HFD, we dissected the mice, and the livers were removed and weighed. Compared with PBS-treated mice, liver weights of BBR-treated mice exhibited a slight decrease (Fig 4.3A). The pictures show the fat content is less in the liver sections with Oil-Red-O staining of BBR mice, than the PBS group (Fig 4.3B). Given this, it denominates treatment of BBR ameliorated lipid deposition and steatosis in obese mice.

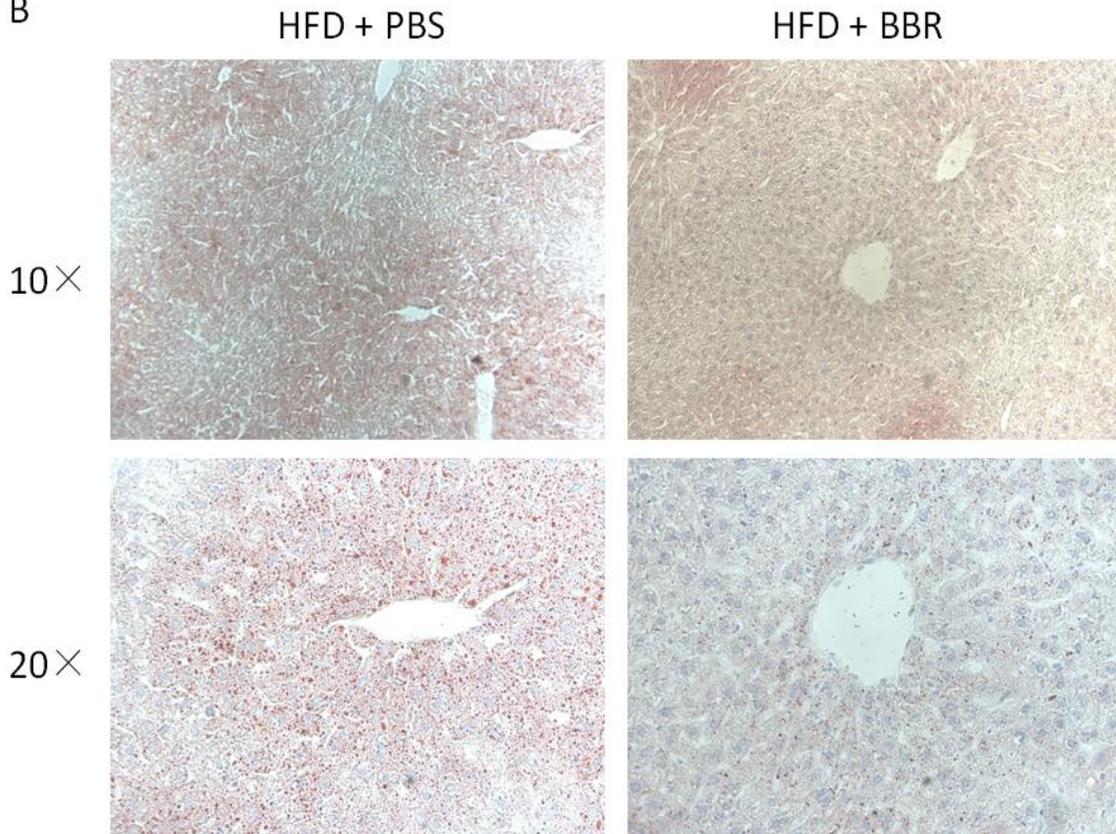
#### **4.4 Berberine altered lipid metabolism and related protein and genes expression**

We isolated total protein of liver and examined AMPK in the liver (Fig. 4.4A). AMPK phosphorylation was reduced in the HFD-fed mice slightly, compared with LED-fed mice. However, we did not observe that BBR treatment reverse decreased AMPK phosphorylation induced by HFD significantly. We also detected the substrate enzyme of AMPK, ACC, which is the key enzyme in lipogenesis. The phosphorylation of ACC in the HFD-fed mice decreased as well as AMPK phosphorylation (Fig 4.4A), thus the active ACC will convert Acetyl-CoA to Malonyl-CoA, and increase production of fatty acid in liver. Consistently, BBR did not alter the phosphorylation of ACC of HFD mice.

A

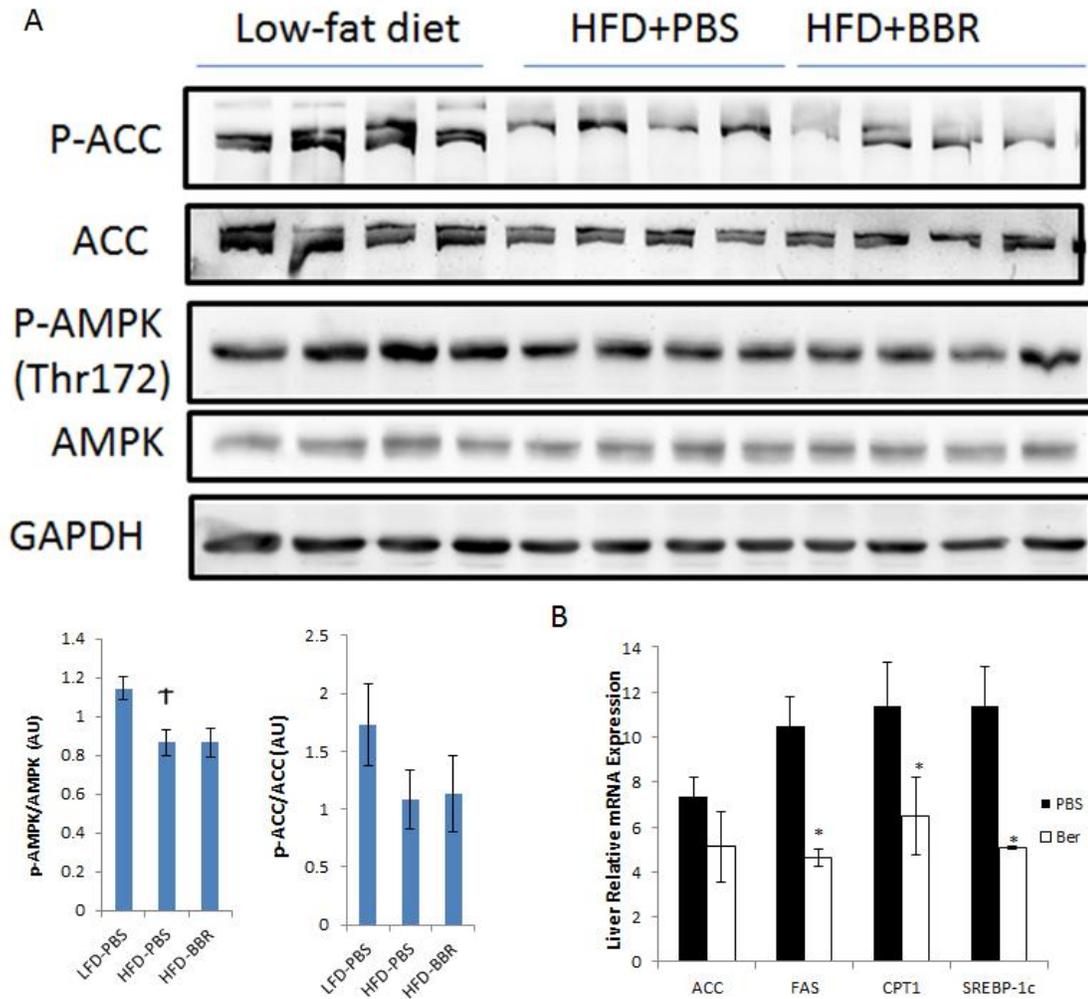


B



**Figure 4.3 Berberine treatment ameliorates fat content in liver.**

The HFD-induced NAFLD animal model was built as described, and all mice were euthanized and the livers were harvested. (A) BBR treatment altered the liver weight. (B) Liver sections with Oil-Red-O staining. The Oil-Red-O marked the lipid drops as the red dot shown in the picture.



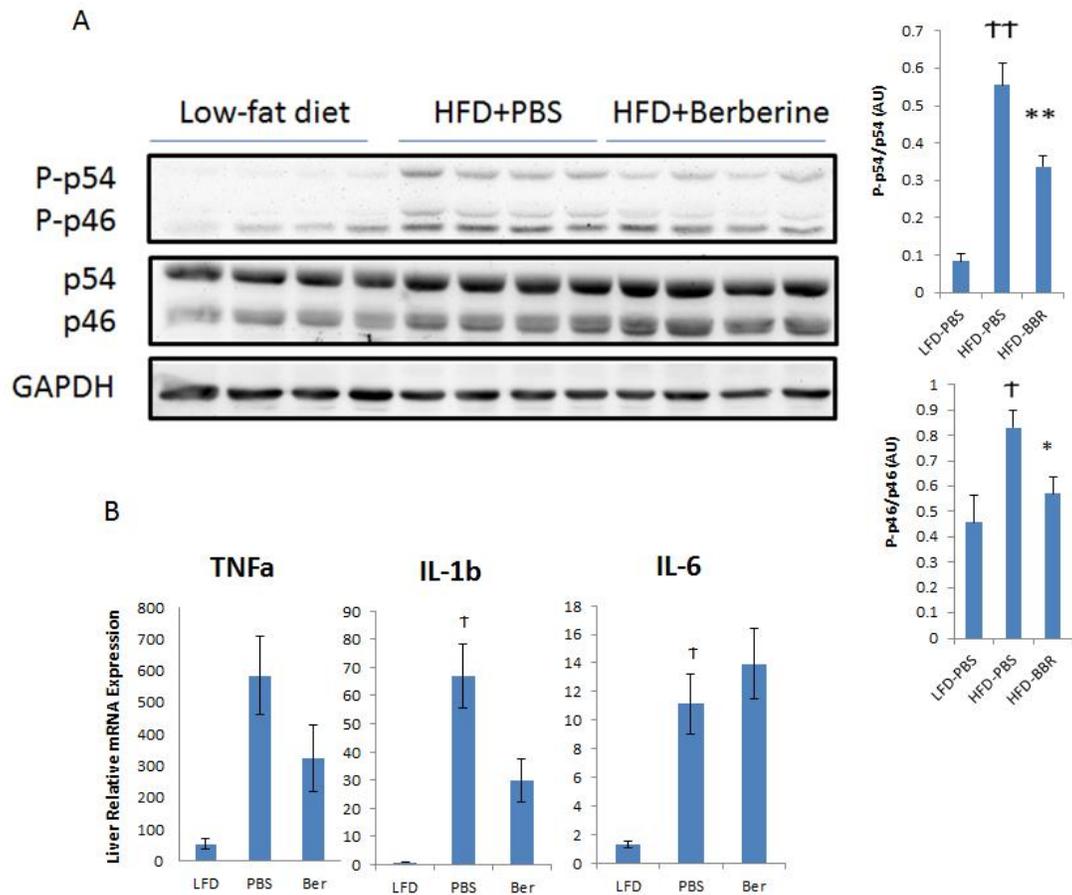
**Figure 4.4 Berberine reduced the expression of lipogenic genes in liver.**

The HFD-induced NAFLD animal model was built as described in Figure 4.1, and expression of protein and mRNA levels in liver was examined. (A) Expression and phosphorylation of AMPK (Thr172) and ACC, loading control is GAPDH. Liver protein expression was measured by Western blot analyses. Ratios of phosphorylated AMPK to total AMPK (P-AMPK/AMPK) and phosphorylated ACC to total ACC (P-ACC/ACC) were quantified using densitometry and normalized by GAPDH (AU, arbitrary unit). (B) mRNA levels of lipid metabolic related genes in liver were analyzed by QPCR. Data are presented as mean  $\pm$  SE (n = 3). \*, P<0.05, HFD-BBR group compared with HFD-PBS group; †, P<0.05, HFD-PBS group compared with LFD-PBS group.

Meanwhile, we measured the mRNA expression of lipogenesis related genes. ACC1, FAS, and the transcription factor SREBP1c play a key role in lipogenesis process. Obviously, BBR treatment decreased the levels of ACC1, FAS, and SREBP1c in high-fat diet mice (Fig 4.4B). These data suggest the lipogenesis pathway may be blocked by BBR, therefore, fatty acid synthesis should be inhibited. But the weird thing is the expression level of CPT1a in the liver did not decrease. Combined with results of Oil-Red-O staining liver, maybe the suppressed effect of BBR in lipogenesis is stronger, and the lipid accumulation amount is less than  $\beta$  oxidation of fatty acids, although the oxidation in BBR group is slow at the same time.

#### **4.5 Berberine inhibits HFD-induced inflammation in liver**

Inflammation was considered to promote steatosis to NASH, and pro-inflammatory cytokines such as TNF $\alpha$  and IL-6 are the most important factors. Firstly, in the experiment we examined the phosphorylation of JNK of liver tissue. We can see the phosphorylation of JNK1 (p46) in livers of HFD-fed mice increased statistically significantly, compared with that of LFD-fed mice (Figure 4.5A). Upon BBR treatment, the phosphorylation of liver JNK1 declined. Secondly, we examined the mRNA expression levels of proinflammatory cytokines including TNF $\alpha$ , IL-1 $\beta$  and IL-6. All of them were greatly activated by the high-fat diet, whereas both of TNF $\alpha$  and IL-1 $\beta$  are reduced under the treatment of BBR (Figure 4.5B). But the induced IL-6 in liver was not affected by BBR. Thus, these results denominate BBR significantly inhibitor inflammatory signaling in liver of HFD mice.



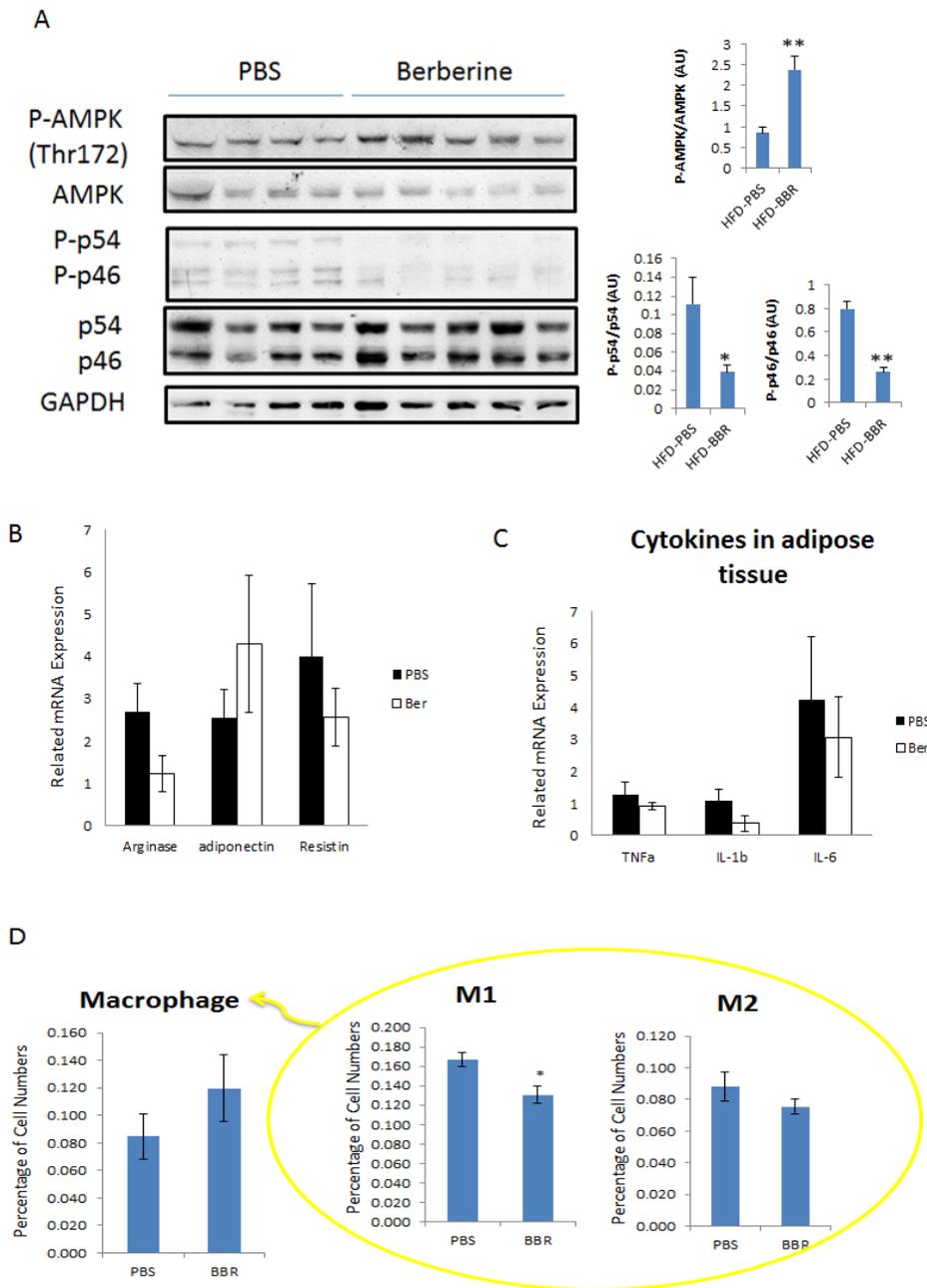
**Figure 4.5 Berberine reduced HFD-induced inflammatory responses in liver.**

The HFD-induced NAFLD animal model was built as described in Figure 4.1, and expression of protein and mRNA levels in liver was examined. (A) Expression and phosphorylation of JNK, loading control is GAPDH. Liver protein expression was measured by Western blot analyses. Ratio of phosphorylated JNK1 to total JNK1 (Pp46/p46) was quantified using densitometry and normalized to GAPDH. (B) mRNA levels of pro-inflammatory cytokines in liver were analyzed by QPCR. Data are presented as mean  $\pm$  SE (n = 2). \*, P<0.05, HFD-BBR group compared with HFD-PBS group, \*\*, P<0.01, HFD-BBR group compared with HFD-PBS group; †, P<0.05, HFD-PBS group compared with LFD-PBS group, ††, P<0.01, HFD-PBS group compared with LFD-PBS group.

#### **4.6 Berberine activated AMPK phosphorylation and inhibited HFD-induced inflammation in adipose tissue**

In obese, pro-inflammatory cytokines secreted by adipose tissue, such as JNK1 - dependent IL-6 secretion, were thought to aggravate inflammation in NASH. Part of epididymis adipose tissues were used for analysis of protein and mRNA levels. It was difficult to get enough adipose tissue from LFD-fed mice to perform these assays, so we only used HFD-fed mice in order to make the result consistent. It is shown (Figure 4.6A) that BBR treatment increase AMPK phosphorylation in HFD-fed mice. Then we examined mRNA levels of arginase 1, adiponectin, and resistin, which are related to functions of adipose tissue and the inflammatory response. It seems like arginase 1 and adiponectin increase, and resistin decrease in the BBR group mice (Figure 4.6B), but the results are not statistically significant.

Next, we examined the phosphorylation of JNK 1 (p46) in adipose tissue, and BBR treatment decrease the phosphorylation of JNK1 obviously (Figure 4.6A). Meanwhile, we measured the mRNA expression of TNF $\alpha$ , IL-1 $\beta$  and IL-6. All of them were reduced in the BBR treatment group (Figure 4.6C).



**Figure 4.6 Berberine activated AMPK phosphorylation and inhibited HFD-induced adipose tissue inflammation.**

The HFD-induced NAFLD animal model was built as described in Figure 4.1, and expression of protein and mRNA levels in liver was examined. (A) Expression and phosphorylation of AMPK (Thr172) and JNK, the loading control is GAPDH. Protein expression was measured by western blot analyses. Ratios of phosphorylated AMPK to total AMPK (P-AMPK/AMPK) and phosphorylated JNK1 to total JNK1 (Pp46/p46) were quantified using densitometry and normalized to GAPDH. (B) mRNA levels of adipocytokines were analyzed by QPCR. (C) mRNA levels of pro-inflammatory cytokines in adipose tissue. (D) FACS analysis of adipose tissue stromal vascular cells. Data are presented as mean  $\pm$  SE (n = 3). \*, P<0.05, BBR group compared with PBS group; \*\*, P<0.01, BBR group compared with PBS group.

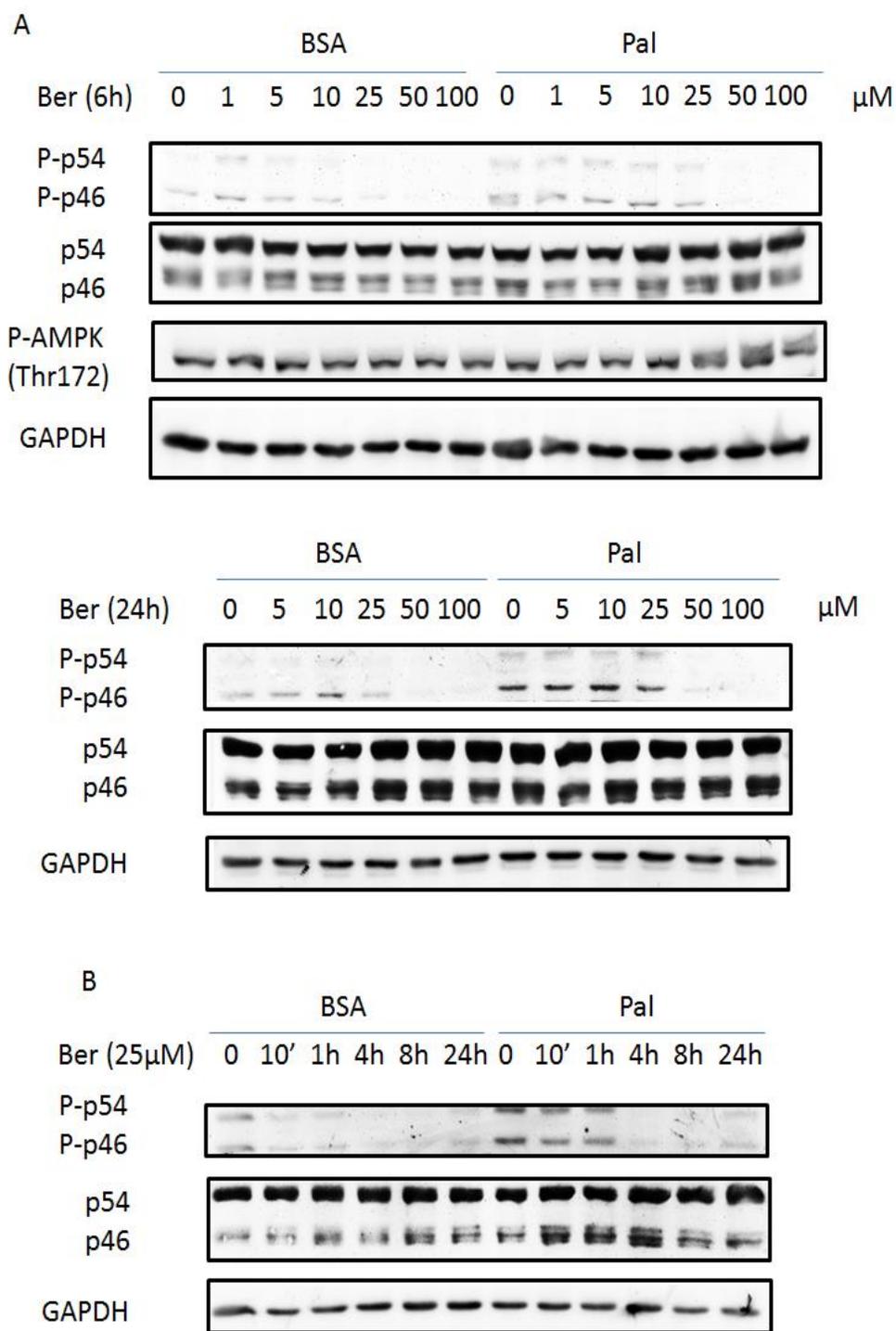
Alternation of the ratio of type 1 macrophages and type 2 macrophages in the adipose tissue was also analyzed. Stromal vascular cells (SVC) were isolated from epididymis fat pads of PBS and BBR group mice and run the FACS analysis. The number of total macrophage increased in BBR treated mice, and this may be related with the function of BBR to activate macrophage reported in the research of antitumor. The ratio of M1 macrophages, the pro-inflammatory macrophages, was reduced by BBR treatment, while the anti-inflammatory activated M2 macrophages had no significant difference (Figure 4.6D).

Totally, treatment of BBR may alter lipid metabolism and inhibit adipose tissue inflammation induced by high-fat diet.

#### **4.7 Berberine dose-dependently suppresses JNK signaling in H4IIE cells**

There are many different types of cells in the liver tissue, the function cell and the kuffer cells are considered the major cells involved in NAFLD. In order to determine which type of cell play a more important role in fatty liver disease and which type of cell will response to the treatment of berberine, we isolated and treated them separately.

At first, we use a rat hepatoma cell line, H4IIE cells, to identify the appropriate dose and time point of BBR treatment in vitro. H4IIE cells were incubated for 6 h and 24h with increasing concentrations of BBR (1, 5, 10, 25, 50, 100  $\mu$ M). When in the absence of BBR, palmitate increased the phosphorylation of JNK 1 (p46), and at both of the two time points, BBR inhibit JNK 1 phosphorylation, and the effective is strength under the increasing doses, either incubated with palmitate or not (Figure 4.7A).



**Figure 4.7 Berberine inhibits JNK phosphorylation dose- dependently and time- dependently in H4IIE cells.** H4IIE cells were pre-treated 2h with BSA or 25mM palmitate (diluted in BSA) before BBR added. (A) H4IIE cells were treated with various concentrations of BBR for 6h or 24h. (B) Incubate H4IIE cells with 25 $\mu\text{M}$  BBR and collect the protein samples at different time points. Protein levels were determined by Western blot.

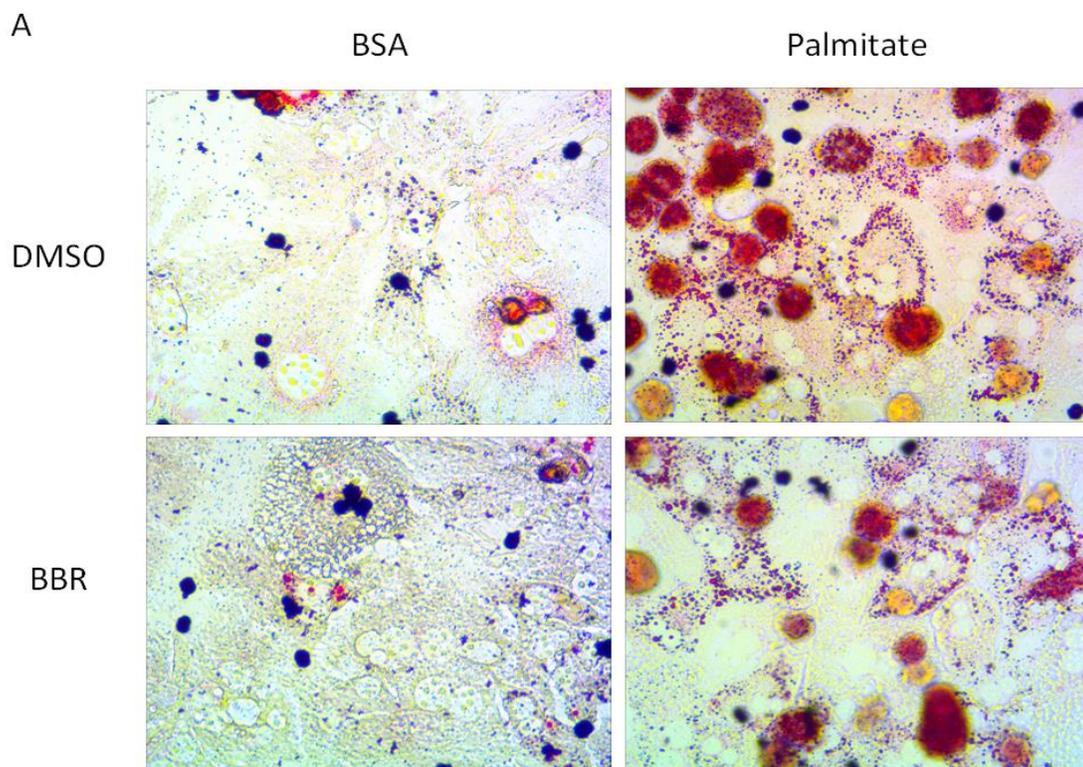
It was suggested the high dose beyond 50  $\mu\text{M}$  was toxic for the hepatocytes and would induce apoptotic program detected by flow cytometry (54), thus we chose 25 $\mu\text{M}$  concentration to decide the time point that BBR have the best anti-inflammation effect. H4IIE cells were treated with BBR for 10 min to 24 hr, and the effective was as more powerful as longer time. Stared from 4h, the phosphorylation of JNK 1 was mostly inhibited under palmitate treated environment, and the effect remained even until 24 h (Figure 4.7B). Therefore, we selected 25  $\mu\text{M}$  and 4h as a suitable BBR treatment condition to treat the cells in ongoing experiments. It should be specifically mentioned that the phosphorylation of AMPK did not affected by BBR in H4IIE cells.

#### **4.8 Berberine reduced fat deposition and inflammatory responses in primary hepatocytes**

As described in the animal experiments above, it seems like the liver is the target of the anti-inflammation effective of berberine in NAFLD obesity mice. In order to try to learn the true response of the hepatocytes to berberine, we isolated the primary hepatocytes of C57BL/6J male mice. After Oil-Red-O staining, we could observe palmitate would induce fat deposition in the hepatocytes, and BBR reversed the effect partly (Figure 4.8A).

The primary hepatocytes were treated by 25 $\mu\text{M}$  BBR for 1h or 4h, in the presence or absence of palmitate. It was obvious phosphorylation of JNK 1 was inhibited at 4h, and the phosphorylation of AMPK of both 1h and 4h increased significantly (Figure 4.8B).

For 4h of BBR treatment, we also identified the effect of various doses of BBR on primary mouse hepatocytes. 5 $\mu$ M BBR is enough to suppress the activity of phosphorylated JNK in absence of palmitate, but the most appropriate concentration still is 25 $\mu$ M upon palmitate incubation (Figure 4.8C). All of the doses significantly activate the phosphorylation of AMPK in the primary hepatocytes.



**Figure 4.8 Berberine reduced fat deposition and inflammatory responses.** Induce fat deposition in primary hepatocytes with 6h treatment of palmitate. (A) Fat deposition in hepatocytes. The red dots were lipid drops stained by Oil-Red-O. (B) Western blot analyses of phosphorylation of AMPK (Thr172) and JNK, GAPDH as control. The primary hepatocytes were treated with 25 $\mu$ M BBR for different hours. (C) Treat the primary hepatocytes with different concentrations of BBR for 4h and analyze protein expression.

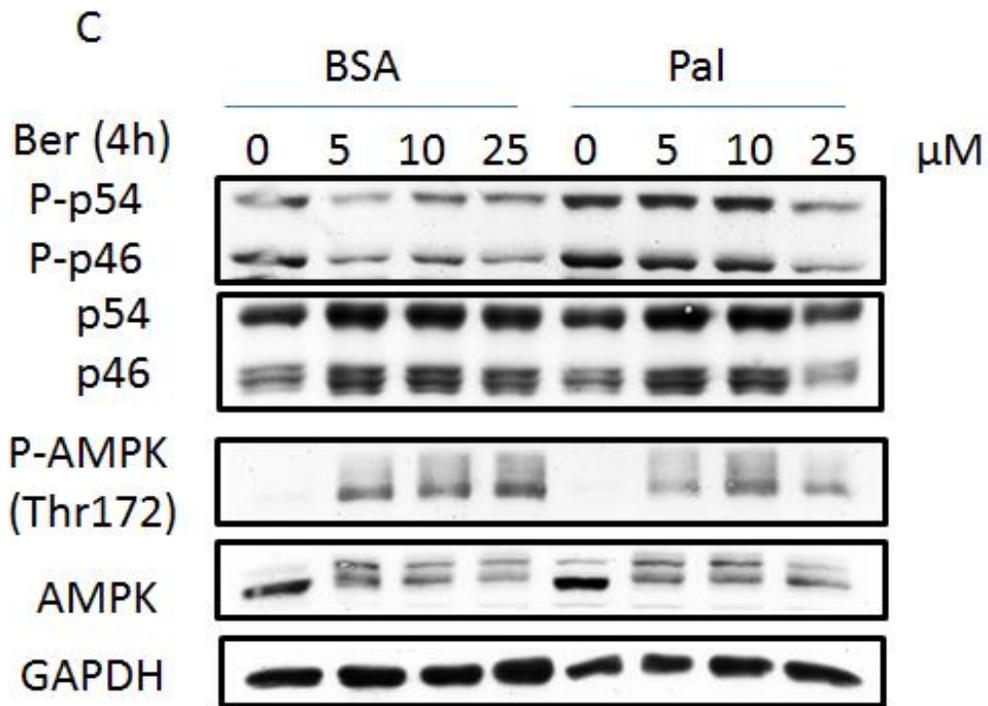
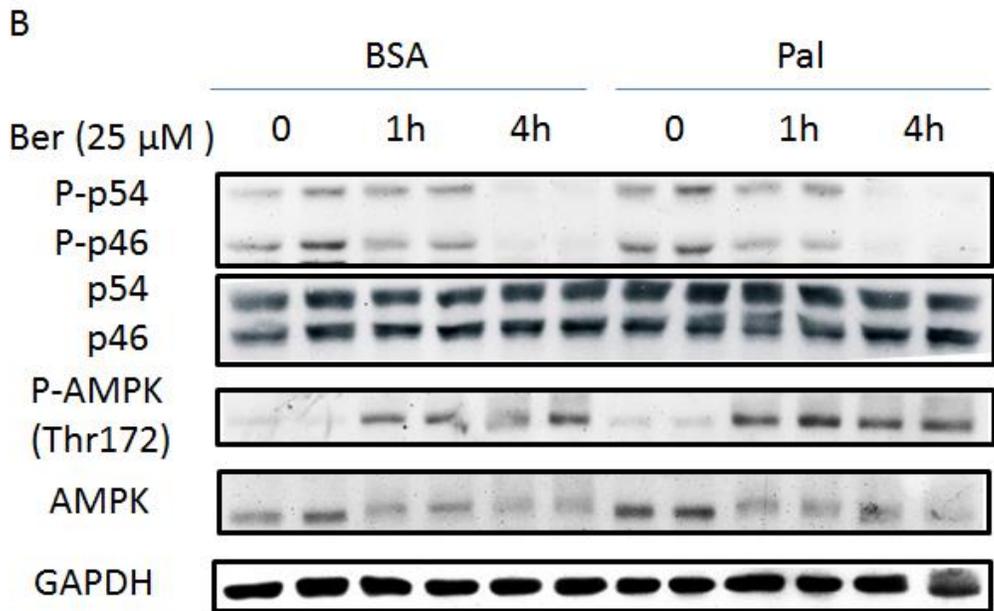


Figure 4.8 Continued

Generally, the anti-inflammation effective of berberine in primary mouse hepatocytes is similar with that in H4IIE cells, the difference is BBR induced the phosphorylation of AMPK.

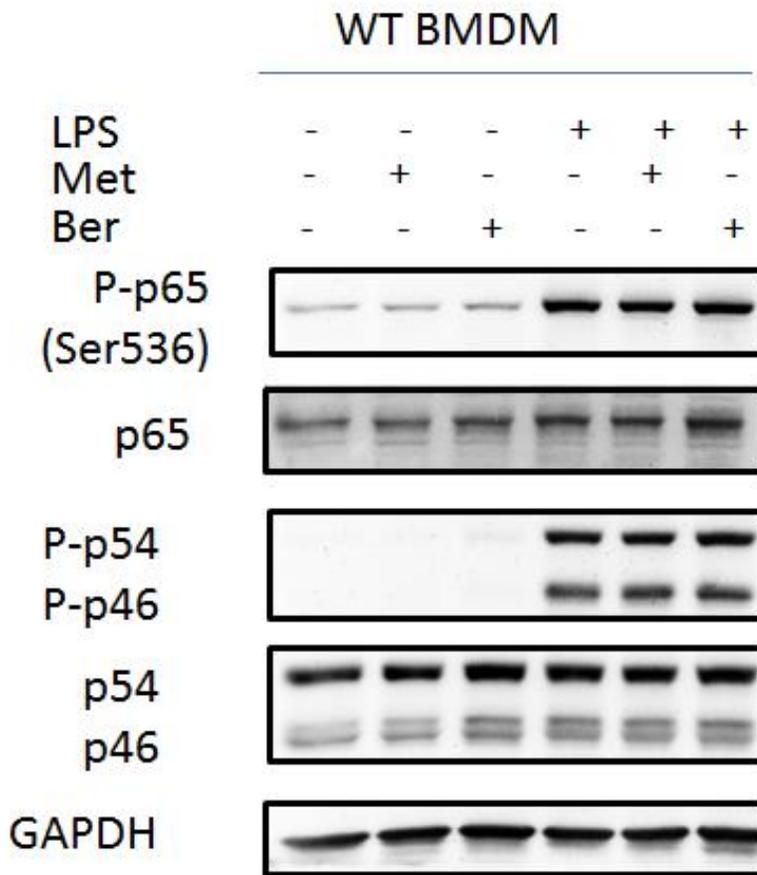
#### **4.9 BBR treatment did not inhibit macrophage pro-inflammatory activation**

It is difficult to separate Kupffer cells from liver tissue, so we bone marrow derived macrophages (BMDM) at first to test the macrophages inflammatory response to BBR. Short-term treatment of LPS is enough to induce strong inflammatory response in macrophages, the phosphorylation of both JNK 1 (p46) and NF- $\kappa$ B p65 (Ser536) was significantly activated (Figure 4.9). However, BBR did not change the activation response at all. This result suggests maybe the macrophages are not the major anti-inflammation target of BBR. Metformin treatment was set as a positive control.

#### **4.10 Effect of BBR treatment in insulin signaling pathway**

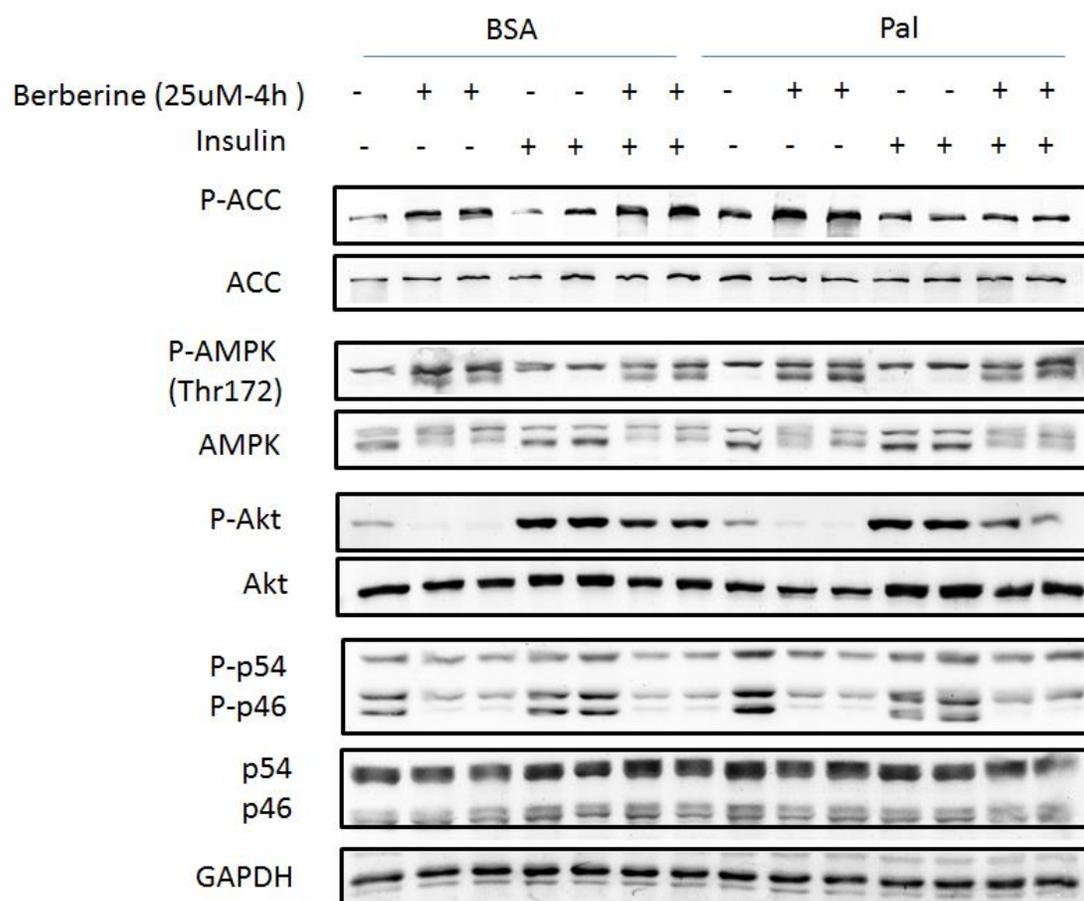
We treated the primary hepatocytes by 25 $\mu$ M BBR for 4h, in the presence or absence of 2h pre-treatment of palmitate. In baseline, the phosphorylation of Akt was induced by 30 min insulin treatment. However, BBR inhibited the activity of p-Akt, independent of palmitate incubation. In the other hand, the alternation of AMPK and JNK 1 (p46) phosphorylation by BBR was not affected by insulin, remains the same increased or decreased level (Figure 4.10). Similarly as described in the liver tissue previously, the phosphorylation of ACC in the primary mouse hepatocytes was enhanced by BBR, in the absence of palmitate. Generally speaking, the activity of ACC was

slightly inhibited by the incubation of palmitate, and it is reasonable in the balance of chemical responses. However, after 30 min treatment of insulin, the inhibit effect of BBR on activity of ACC was abolished, under the incubation of palmitate.



**Figure 4.9 BBR treatment has no effect on macrophage proinflammatory activation.**

Bone marrow-derived macrophages (BMDM) were treated with PBS, BBR(25  $\mu$ M, 4hr), or metformin (500  $\mu$ M, 24hr), and LPS (100 ng/ml, 30 min) was used to induce inflammatory response. Macrophage inflammatory signaling was examined by Western blot analysis.



**Figure 4.10 Berberine change the insulin signaling and related protein expression.** Treat the primary mouse hepatocytes as described in Figure 4.8, and at the last 30 min, add insulin (100nM). Western blot analyses of phosphorylation of Akt, ACC, AMPK (Thr172) and JNK, GAPDH as control.

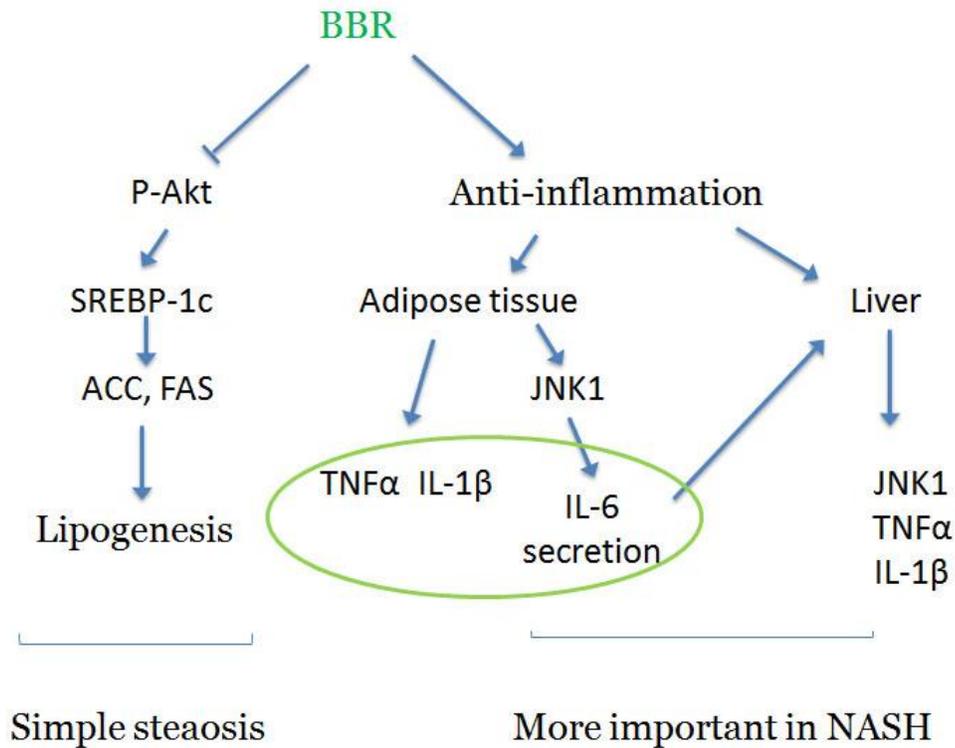
## 5. CONCLUSION

### 5.1 Conclusion

In the results of HFD animal experiments, berberine improved insulin resistance and glucose intolerance in HFD mice, without changing body weight and food intake. One month treatment of berberine ameliorates fat deposition in liver, through reducing lipogenesis related proteins and genes expression. As well as the anti-inflammatory effect showed in other metabolic syndromes, berberine decreased inflammatory signaling in liver and adipose tissue in HFD mice, such as JNK pathway and cytokines including TNF $\alpha$  and IL-6. In cell experiments, we detected responses induced by BBR in macrophages and hepatocytes, which are the major cells associated with fatty liver. However, the cultured macrophages didn't show significant difference in inflammatory responses. Adversely, JNK1 phosphorylation in both of the cell line and primary hepatocytes was inhibited by BBR. Thus, we can hypothesize hepatocytes is the main cell included in the beneficial effect in NAFLD of BBR. Moreover, the phosphorylation of AMPK in primary hepatocytes increase under the treatment of BBR, but the H4IIE cells didn't, maybe because of the different energy metabolism in the cancer cell line. Additionally, BBR inhibited the insulin signaling response in primary mouse hepatocytes. Specifically, berberine reduced the activity of ACC, but the effect was interrupted by increased insulin level, in the high fat model supplemented by palmitate, without affecting the alternation of AMPK.

## 5.2 Discussion

In our cell experiment, BBR blocked the phosphorylation of Akt induced by insulin, and it is consistent with responses in other cell types, such as MDA-MB-231 cells (55) and intestinal cell (56). But it already mentioned that berberine would decrease glucose levels in humans and animals (37,39,57), and increase glucose uptake in related cells, such as in 3T3-L1 adipocytes (58). Someone demonstrated maybe the anti-hyperglycemic effect was not related to insulin pathway. According to the experiments performed by Zhang et al. (57) in KK<sup>Ay</sup> mice, a model of severe obesity, hyperinsulinemia and glucose intolerance by transferring yellow obesity (*Ay*) gene into the KK strain, our results are consistent with their results of insulin signaling molecules showed in the DNA microarrays. They also considered insulin signaling pathway was not important in the effect of berberine on regulating glucose metabolism. It is also reported BBR did not change p-Akt in the liver after 30 minutes insulin challenge in HFD Sprague-Dawley rats (39). Thus, we may suggest BBR would inhibit hyperinsulinemia-induced lipogenesis and associated genes levels through Akt pathway, and it does not mean exacerbated insulin resistant. Together with the increased insulin sensitivity we showed in BBR-treated HFD mice, BBR may ameliorate hyperglycemia via a mechanism distinct from insulin, meanwhile BBR reduce fat storage in hepatic steatosis through inhibition of insulin signaling pathway.



**Figure 5.1 Summary of effect of berberine in NAFLD in our project.**

BBR may improve NAFLD through inhibiting lipogenesis and inflammatory responses in liver and adipose tissue.

In HFD mice, we found BBR activated AMPK and reduced levels of inflammatory cytokines in adipose tissue, specifically inhibited phosphorylation of JNK1 and IL-6 gene expression, which was suggested to play an key role in inflammatory process in NASH development (17). In liver, JNK, TNF $\alpha$  and IL-1 $\beta$  were decreased by BBR as well, but it seems like IL-6 is not be affected. We may hypothesize diet-induced inflammation in adipose tissue is also important in beneficial effect of berberine in improvement of hepatic steatosis, but it needs further experiments. In liver and other

possible related organs, it seems like BBR has good pharmacological effect in inflammation in NAFLD, which is the main reason to develop NASH from steatosis. Maybe BBR will have a better performance in anti-NASH, we can identify it in methionine and choline deficient (MCD) diet fed animals or long-termed HFD mice in further study, shown as Figure 5.1.

Even though the blood concentrations of BBR and its metabolites are low, three months of oral intake of a much lower dose of BBR than in animal experiments is enough to decrease serum triglyceride and fasting and postprandial blood glucose levels in T2DM patients. Therefore, according to obviously higher concentration in organs including the liver and kidney than in blood, BBR has potential effect to treat NAFLD individuals, but it needs more clinic trials data. In our experiments, the effect of BBR in liver seems not to be so sensitive in hepatocytes, maybe because the metabolism of BBR is different in vitro and vivo. In humans clinic data, the blood concentration of BBR and its metabolites after a chronic oral administration of  $15 \text{ mg kg}^{-1} \text{ day}^{-1}$  berberine for three months, were several folds higher than that after a acute administration of a single dose of  $500 \text{ mg kg}^{-1} \text{ day}^{-1}$  (59). It suggested long-termed and low dose treatment of BBR may be more effective than short-termed and high dose.

Because of the low absorption of BBR and the re-absorption of metabolites from jejunum, most of the compound and its metabolic product will stay long time in the intestine. In this case, we could think that berberine may affect intestine flora metabolism, such as secretion of LPS and pro-inflammatory factors (17). Then the inflammatory signaling from the macrophages or epithelial cells, which will affect

NASH development, may be altered. Additionally, in research of bacteria-induced epithelium damage, berberine can ameliorate inflammatory cytokines secretion from intestinal epithelial cells, including TNF $\alpha$  and NF $\kappa$ B (56).

Collect all the results from laboratory research and clinic data, berberine could be considered to use in the treatment of NAFLD, and it surely needs more support. The long history utilization of BBR has identified the safety in humans. No sample of serious side effects of BBR was reported (3) (such as hepatotoxicity or renal failure), except similar gastrointestinal effects as metformin.

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