

AMELIORATIVE POTENTIAL OF ARTEMISIA CAPILLARIS FORMULA ON NONALCOHOLIC FATTY LIVER DISEASE IN RATS THROUGH REGULATION OF FAT METABOLISM

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Abstract

Background: Artemisia Capillaris Formula (ACF), a traditional Chinese medicinal therapy, has been used clinically in China to treat Nonalcoholic Fatty Liver Disease (NAFLD) for many years. However, the mechanism of action of this treatment on NAFLD is still unknown. The goal of the present study is to test whether Artemisia Capillaris Formula protects against NAFLD through regulation of lipid metabolism.

Methods: Rat models of NAFLD were established through consumption of a high-fat diet (HFD) for 8 weeks. 60 rats were randomly divided into 6 groups (10 rats per group): the control (standard diet) group, the model (HFD) group, the polyene phosphatidylcholine treated HFD group, and the ACF-treated HFD groups (high-, medium- and low-dose). During weeks 5–8 of the HFD regimen, drugs were intra-gastrically administrated to selected groups for a total of 4 weeks. Hepatic changes were observed through pathological examination of Hematoxylin and eosin-stained tissues, quantification of lipid metabolites from sera (ALT, AST, ALP activity and TG, TC, HDL-C, LDL-C), and quantification of related gene and protein expression levels by RT-PCR and Western blotting.

Results: A high-fat diet promoted obesity and the development of hepatomegaly, hepatosteatosis and dyslipidemia in rats after 8 weeks. Treatment with ACF alleviated hepatosteatosis and also protected against high fat diet-induced dyslipidemia. We found that ACF reduced ALT, AST, ALP, TG, TC, and LDL-C and increased HDL-C levels in sera from treated NAFLD rats. In addition, gene and protein expression levels of FAS and ACC were down-regulated following ACF treatment, whereas expression levels of CPT were up-regulated.

Conclusion: ACF ameliorates high-fat diet-induced hepatosteatosis and dyslipidemia in rats by altering lipid metabolism-related gene expression, specifically of FAS, ACC, and CPT.

Key words: Artemisia Capillaris formulation, Nonalcoholic Fatty Liver Disease, lipid metabolic enzyme

Introduction

Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum of diseases that ranges from simple steatosis to steatohepatitis (NASH). NAFLD is the hepatic manifestation of the metabolic syndrome (MetS), which is characterized by obesity, insulin resistance, hypertension, and/or dyslipidemia (Attar et al., 2013). The rapid rise of obesity across ethnic, racial and national boundaries has led to the emergence of NAFLD as one of the most common liver diseases observed worldwide (Pais et al., 2012; Adams et al., 2005; Angulo et al., 2002). The strong association of obesity with NAFLD suggests that obesity contributes to liver injury by steatosis and progression to steatohepatitis (Marchesini et

al., 2003; Eckel et al., 2005; Bugianesi et al., 2005; Del Gaudio et al., 2002). Obese patients comprise the majority of cases of NAFLD (69–100%), and up to one-third of all cases of NASH (25–30%), reported clinically each year (Clark et al., 2006; Dixon et al., 2001; Ratziu et al., 2000).

The pathogenesis of NAFLD is associated with hepatic metabolic disorders, resulting in over-accumulation of fatty acids/triglycerides and cholesterol (Pacifico et al., 2014). Hepatic lipid homeostasis represents a balance between lipid uptake, synthesis, catabolism, and secretion. Therefore, some typical characteristics of NAFLD such as steatosis are caused by disordered lipid metabolism, particularly inhibition of fatty acid oxidation and enhanced lipogenesis (Enjoji et al., 2012). Fatty acid synthesis-associated enzymes, such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), lead to enhanced fatty acid synthesis and overproduction of triglycerides, ultimately resulting in liver steatosis (You et al., 2004). Stefanovic-Racic and colleagues (Stefanovic-Racic et al., 2008) observed that a 60% increase in the hepatic expression levels of carnitine palmitoyltransferase-1a (CPT-1a), a key enzyme in mitochondrial fatty acid oxidation, resulted in a significant increase in the rate of fatty acid oxidation (45%) and a significant decrease in TAG content (70%). Thus, the enzymes associated with lipid metabolism play a key role in NAFLD.

Artemisia Capillaris Formula (ACF) is a traditional Chinese medicinal therapy that consists of a combination of several natural products, including Artemisia Capillaris, Alismatis, Radix Bupleuri and Hawthorn. It has been shown that ACF exhibits significant therapeutic effects on NAFLD in the clinic. However, the mechanism of action associated with ACF treatment in NAFLD is largely unknown. In this study, we investigated the effects of ACF on liver fat metabolism in Sprague Dawley (SD) rats consuming a high fat diet (HFD). This study reveals that ACF regulates expression of lipid metabolic enzymes, and this mechanism of action is therapeutically important for treating NAFLD.

Materials and Methods

Reagents

TRIzol reagent was obtained from Invitrogen (Carlsbad, CA, USA). PrimeScript™ RT reagent Kit with gDNA Eraser was purchased from Takara BIO Inc. (Japan). The kits for analysis of triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine transaminase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) levels were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Unless stated otherwise, all other chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA).

Preparation of Artemisia Capillaris Formulation

Artemisia Capillaris Formula (ACF) was provided by the College of Pharmacy, Fujian University of Traditional Chinese Medicine. For animal experiments, ACF powder was dissolved in saline to make a working concentration of 1 g/ml.

Development of NAFLD Animal Model

Sixty male, 8-week-old Sprague Dawley (SD) rats (SLAC Co. Ltd., Shanghai, China), weighing from 180–200 g, were acclimatized for a week before experimentation. Animals were housed together (five per cage) in an environmentally-controlled room (temperatures ranging from 21–23°C; relative humidity ranging from 40–60%). Air ventilation was carried out 12–18 times/h, and a photoperiod of 12h artificial light and 12h darkness (150-300 lux) was maintained. All animals had free access to food and water. The animal studies were approved by the Fujian Institute of Traditional Chinese Medicine Animal Ethics Committee (Fuzhou, China). The experimental procedures were carried out in accordance with the university's guidelines for animal experimentation.

To establish the animal model, 60 rats were randomly divided into 6 groups (10 rats/group). A control group was fed the standard diet provided to animals in the facility. The remaining groups were fed a high-fat diet (HFD) ad libitum for 8 weeks. The HFD recipe used to induce

NAFLD in SD rats consisted of 87.3% basal fodder, 10% lard, 2% cholesterol and 0.7% swine bile salt. After an 8-week feeding period, the rats fed with the HFD were randomly divided into 5 groups: the untreated HFD model group (model), the polyene phosphatidylcholine-treated group (PP), and the ACF-treated groups (high-, medium- and low-dose) (10 rats/group). The drug regimen involved the intra-gastric administration of either PP (76 mg/kg body weight/day), ACF high-dose (1.848 g/kg body weight/day), ACF middle-dose (0.924 g/kg body weight/day) or ACF low-dose (0.462 g/kg body weight/day) during weeks 5–8 of the 8 week protocol. The HFD model group and the standard diet control group received distilled water. The body weight and food uptake were recorded weekly. All rats were sacrificed by decapitation after 4 hours of food deprivation. Blood samples were collected for analysis of ALT, AST, ALP, TG, TC, HDL-C and LDL-C levels. The liver was sectioned and processed, either by fixation in formaldehyde (4%) in isotonic saline for histological analysis, or by quick-freezing in liquid nitrogen and storage at -80 °C.

Histological Examination

Sections of liver tissue were fixed in formaldehyde (4%) in isotonic saline and embedded in paraffin. 6 serial sections of 5 µm thickness each per tissue were prepared from 10 rats per group and subsequently deparaffinized in xylene, rehydrated in graded ethanol; then stained with hematoxylin and eosin (H&E) for histological observation under a light microscope.

Biochemical Assays

Whole blood samples were incubated at room temperature for 2 h, followed by centrifugation at 3000 rpm for 20 min at 4 °C to separate the fractions. Serum fractions were collected and stored at -20 °C. Serum ALT, AST, ALP, TG, TC, HDL-C and LDL-C levels were measured using commercially available kits and following the manufacturer's instructions.

RNA Extraction and RT-PCR Analysis

Total RNA was isolated from fresh liver tissue with TRIzol Reagent according to the manufacturer's protocol. Oligo(dT)-primed RNA (1 µg) was reverse-transcribed using the PrimeScript™ RT reagent Kit with gDNA Eraser. The resulting cDNA was used for PCR. GAPDH was used as an internal control. The primers used for amplification of FAS, ACC, CPT and GAPDH genes were: FAS F: 5'-CCT TAG TAC TGC GTG GTC GTA T-3', R: 5'-CAG AGG GTG CTT GTT AGA AAG AT-3' (301 bp); ACC F: 5'-TGA GGA GGA CCG CAT TTA TC-3', R: 5'-GAA GCT TCC TTC GTG ACC AG-3' (565 bp); CPT F: 5'-TAT GTG AGG ATG CTG CTT CC-3', R: 5'- CTC GGA GAG CTA AGC TTG TC-3' (629bp); GAPDH F: 5'-AGA TCC ACA ACG GAT-3', R: 5'-TCC CTC AAG ATT GTC AGC AA-3' (308bp). Amplification of each gene was performed with a thermal cycler (GE9600, USA), using the following cycling parameters: denaturing, 95 °C for 3 min, 30 cycles of 95 °C for 30 sec, annealing temperature for 30 sec and 72 °C for 45 sec, followed by a final extension of 10 min at 72 °C. Gene expression was determined for 6-8 samples randomly selected from each group, and each sample was run in triplicate. The PCR products were separated by electrophoresis on a 1.5% agarose gel. The DNA bands were examined using a Gel Documentation System (BioRad, Model Gel Doc 2000, USA).

Immunohistochemical Assay

The paraffin-embedded liver samples were sectioned (3 serial section of 5 µm thickness) from 10 rats per group. Sections were deparaffinized, rehydrated, submerged in 1% hydrogen peroxide; epitope retrieved, and then soaked in goat serum, followed by an overnight incubation with primary antibodies at 4 °C. The primary antibodies used in this study were polyclonal rabbit anti-rat FAS, ACC or CPT (1:200 dilutions, Santa Cruz Biotechnology). PBS served as a negative staining control. After primary antibody staining and washing with PBS, slides were incubated with a biotin-labelled secondary antibody followed by a further incubation with conjugated horseradish peroxidase (HRP)-labelled streptavidin (Dako). Slides were washed with PBS and counterstained with DAB chromogen, according to the manufacturer's

instructions. After staining, five high-power fields (400×) were randomly selected in each slide, and the average proportion of positive cells in each field were counted using the true color multi-functional cell image analysis management system (Image-Pro Plus, Media Cybernetics, USA).

Statistical Analyses

Results are presented as the mean of three measurements, and data sets were analyzed using the SPSS package for Windows (version 11.5, SPSS, Inc., Chicago, IL, USA). Statistical analysis of the data was performed using the Student's *t*-test and ANOVA. Differences between treatment groups with $P < 0.05$ were considered statistically significant.

Results

ACF Ameliorated Hepatic Steatosis in NAFLD Rats.

Histological evaluation is the standard approach for evaluating the presence and severity of NAFLD (Brunt et al., 2005). Thus, we examined H&E-stained liver sections to assess the extent to which ACF attenuated the development of hepatic steatosis. Histological examination revealed that rats fed with a lower-fat diet (control group) had normal liver histology, but liver cells from HFD-fed rats (model group) had extensive hepatocellular degeneration (Figure 1). Model group showed severe degree of micro- and macro-vesicular-steatosis (black arrow), hepatocellular ballooning (red arrow). Moreover, changes in cellular fatty acid content was evident in hepatocytes from HFD-fed rats, with nuclei pushed to the margins of cells by large fatty deposits. On the contrary, treatment with ACF slowed the lower development of steatosis compared to hepatocytes from the model group. This effect was prominently observed in ACF high- and medium-treatment groups, which showed fewer fatty acid deposits and less hepatocellular degeneration than cells from untreated HFD-fed rats (Figure 1). Histological grading of liver sections from each treatment group confirmed that ACF significantly ameliorated hepatic steatosis.

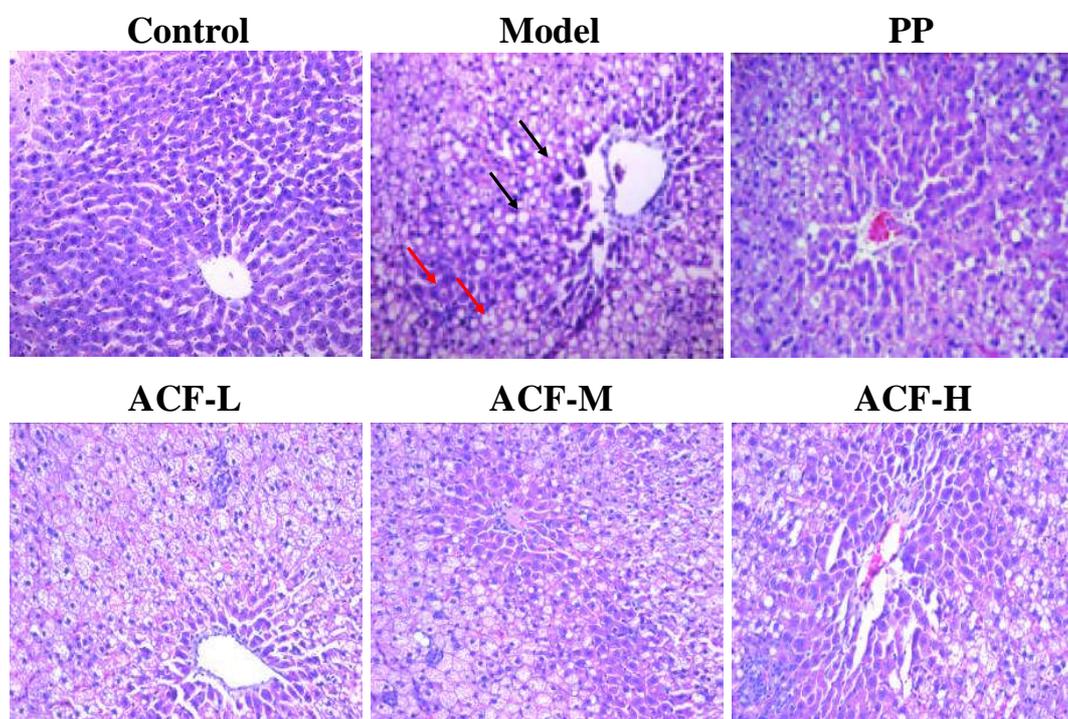


Figure 1: Effects of ACF on the hepatic morphology of liver tissues in rats consuming a HFD. Representative images are shown (200×).

The following abbreviations are used to describe each treatment group: PP (polyene phosphatidylcholine group); ACF-L (ACF low dose group); ACF-M (ACF medium dose group); ACF-H (ACF high dose group). Model group showed severe degree of micro- and macro-vesicular-steatosis (black arrow), hepatocellular ballooning (red arrow).

ACF Altered the Levels of Serum ALT, AST, ALP, TG, TC, HDL-C and LDL-C in NAFLD Rats.

As shown in Fig. 2A, the activities of serum ALT, AST, and ALP were significantly elevated in the HFD-fed model group compared with the control group ($p < 0.05$). Compared to model group rats, the activities of these serum transaminases were significantly decreased in ACF high-, medium- and low-treatment groups, as well as in HFD-fed rats treated with the hepato-protective drug polyene phosphatidylcholine (PP) ($p < 0.05$). Serum TG and TC levels were significantly increased in model group rats compared with control group rats ($p < 0.05$), but TG and TC levels were significantly decreased in ACF or PP-treated rats when compared to the model group ($p < 0.05$). Similarly, serum LDL-C levels were significantly increased in model group rats compared with the control group ($p < 0.05$), but these levels were significantly decreased in ACF or PP-treated rats compared with the model group ($p < 0.05$). In contrast, serum HDL-C levels were significantly decreased in model group rats compared with the control group ($p < 0.05$), but there was no significant difference in HDL-C levels between the model group, ACF treatment groups, or the PP treatment group (Fig. 2B).

ACF Regulates the Expression of FAS, ACC and CPT in Rats.

To determine the mechanism responsible for the ameliorative effect of ACF on HFD-induced hepatosteatosis, we evaluated gene and protein expression levels of three enzymes associated with fatty acid synthesis or oxidation: FAS, ACC and CPT. Gene expression levels were measured from hepatic tissue using RT-PCR, and quantitative immunohistochemistry was used for in situ assessment of protein expression levels in liver sections from each treatment group. The mRNA levels of FAS and ACC, as well as protein levels in hepatic tissue, were significantly increased in the model group compared with the control group ($p < 0.05$) (Fig. 3 and Fig. 4). Compared to the model group, expression levels of these enzymes were significantly decreased in the ACF-treated rats ($p < 0.05$). In contrast, both mRNA and protein expression levels of CPT were significantly decreased in the model group compared with the control group ($p < 0.05$), and increased in the ACF treatment group. Taken together, these observations suggest that ACF treatment significantly reversed changes in expression patterns of ACC, FAS and CPT induced by NAFLD in HFD-fed rats ($P < 0.05$), and ACF treatment was able to restore enzymatic expression in HFD-fed rats to similar levels as in the control group.

Discussion

The prevalence of NAFLD has reached epidemic proportions in recent years due to the increasing prevalence of obesity and metabolic syndrome worldwide. NAFLD is the most common cause of abnormal liver function and chronic liver disease in both developed and developing countries (Yilmaz et al., 2012). It includes variable degrees of simple steatosis (fatty liver), non-alcoholic steatohepatitis (NASH) and cirrhosis. In most westernized countries, approximately 20% of the general adult population has hepatic steatosis and between 2–3% of adults suffer from NASH (Neuschwander-Tetri et al., 2005) whereas NASH is characterized by hepatocyte injury, inflammation and fibrosis. Many of the symptoms appear together and present a complex therapeutic challenge. Pharmacotherapy remains the modality of choice for NAFLD treatment, but undesirable side effects can occur (Giorgio et al., 2013; Carulli et al., 2013; Monsour et al., 2012). Traditional Chinese medicines (TCM) have been effective in the treatment of disease, and the natural products used in the formulae often exhibit fewer adverse side effects. ACF is a traditional Chinese

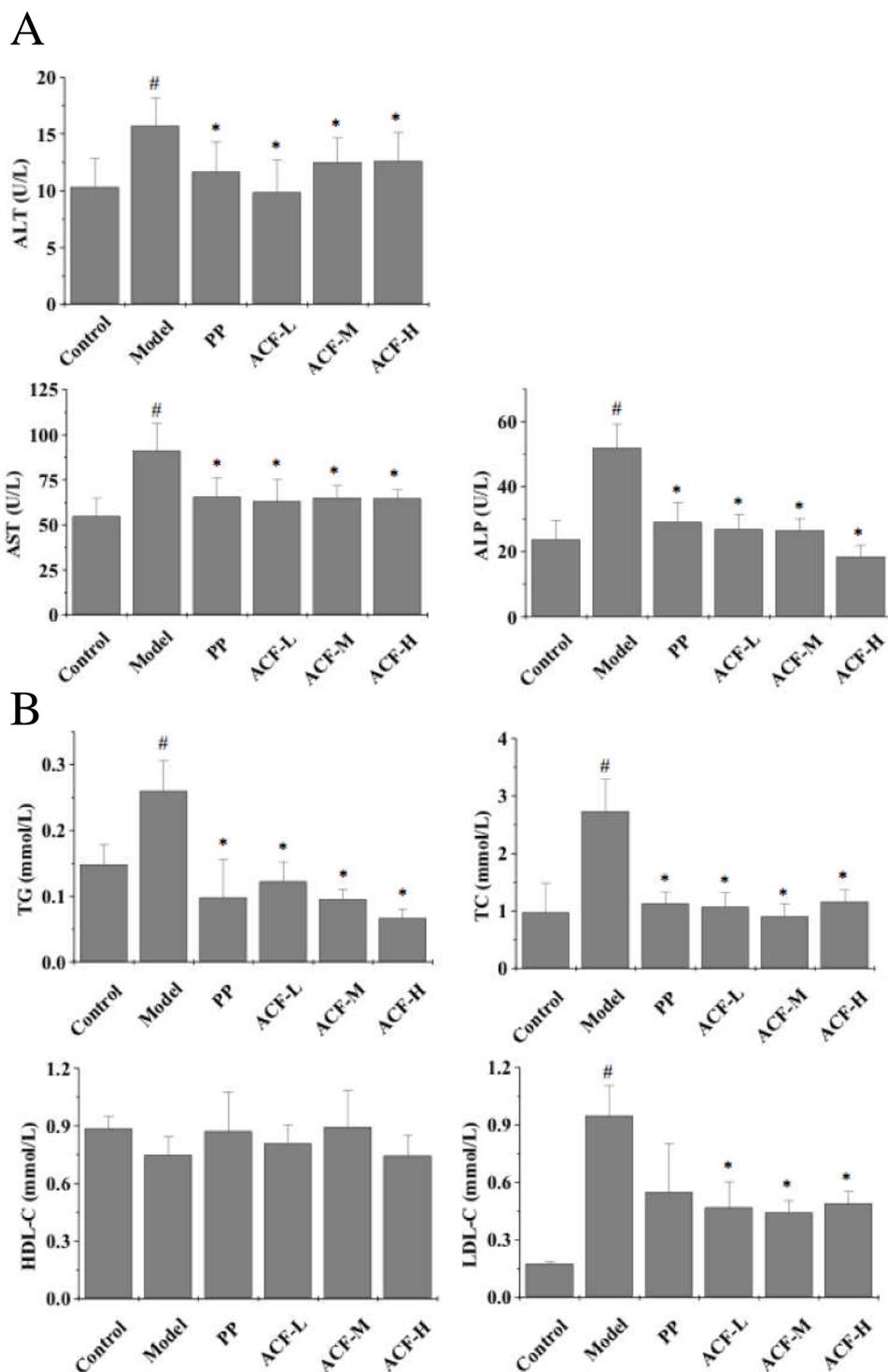


Figure 2: Effects of ACF on the HFD-induced increase in hepatic enzymes and serum lipids in each treatment group. The following symbols indicate statistical relevance: (#) indicates $P < 0.05$, compared with the standard diet control group; (*) indicates $P < 0.05$, compared with the HFD model group, the error bars indicate standard error of the mean. The number of samples shown in each bar of the bar graph ($N = 10$), and each sample was run in triplicate. The following abbreviations describe each treatment group: PP (polyene phosphatidylcholine group); ACF-L (ACF low-dose group); ACF-M (ACF medium-dose group); ACF-H (ACF high-dose group). The following abbreviations describe lipid metabolites: ALT (alanine aminotransferase); AST (aspartate transaminase); ALP (alkaline phosphatase); TG (triglyceride); TC (total cholesterol); HDL-C (high-density lipoprotein cholesterol); LDL-C (low-density lipoprotein cholesterol).

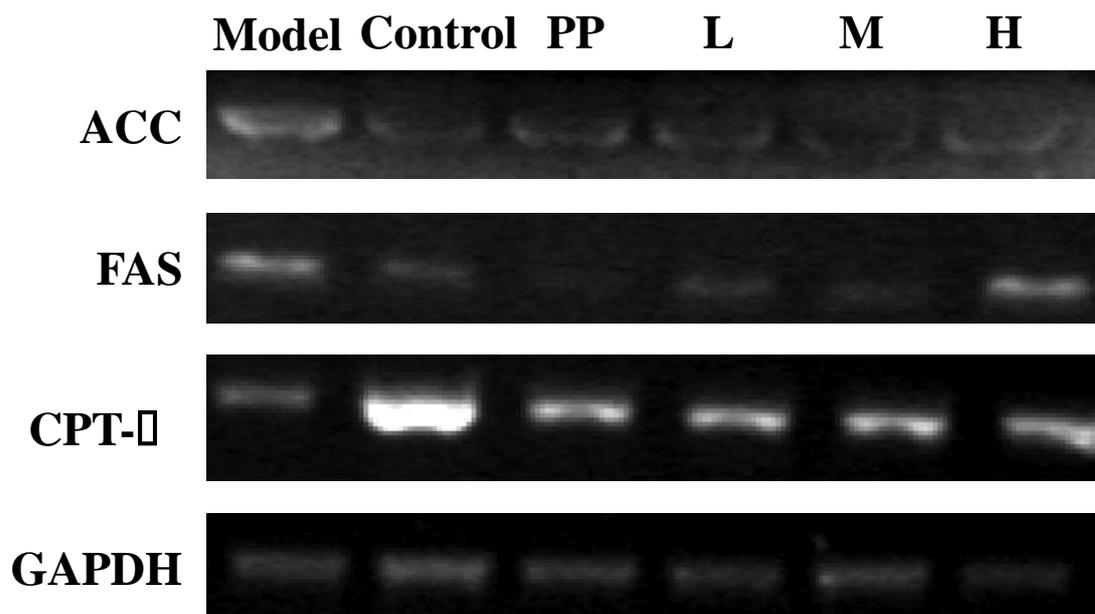


Figure 3: Effects of ACF on ACC, FAS, and CPT-1 mRNA expression in HFD-induced NAFLD rats. GAPDH was used as the internal control for the RT-PCR assay. This experiment was performed in triplicate with similar outcomes, and a representative experiment is shown. The following abbreviations describe the treatment groups: PP (polyene phosphatidylcholine); L (ACF low-dose group); M (ACF medium-dose group); H (ACF high-dose group).

medicinal therapy, and this formulation has been used in clinical practice to alleviate NAFLD for many years. It has been reported that Artemisia Capillaris, one of major components in ACF, exerted a significant anti-inflammatory effect (Jang et al., 2015) on RAW 264.7 macrophages and anti-fibrotic (Wang et al., 2012) in a carbon tetrachloride-induced chronic hepatic fibrosis animal model. Therefore, we speculate that ACF probably also contains the property on NAFLD. As the mechanism of ACF was unknown, we measured the effect of ACF treatment in a rat model of NAFLD and determined the underlying molecular mechanisms that were affected. In addition, PP have demonstrated the hepatoprotective effects on NAFLD in several experiments (Li et al., 2010; Fan et al., 2010; Zhao et al., 2013). It plays an important role in hepatocyte regeneration and lipid metabolism improvement, and as a liver function ameliorating agent and antidote, is frequently used in clinics. So we used PP as positive control treatment.

We successfully established the NAFLD rat model in the present study through administration of a high-fat diet (HFD). Liver histology of HFD-fed NAFLD rats revealed typical hepatosteatosis, hepatocellular ballooning, and vacuolar degeneration (Figure 1), and these results confirm our previous study [26]. When we examined the hepatic morphology of ACF-treated rats, there appeared to be less fatty infiltration or degenerative changes in hepatocytes (Figure 1). Consistent with the amelioration of hepatic steatosis by ACF treatment, expression of serum transaminases such as ALT, AST, and ALP were lower in ACF-treated rats compared to controls and indicate a reduction in NAFLD pathology (Figure 2). ALT is a key enzyme found predominately in the liver, and significantly elevated levels of ALT in the sera often indicate damaged hepatocytes. AST is a hepatic enzyme similar to ALT, but ALT is frequently used to indicate hepatic inflammation whereas elevated AST levels may be indicative of diseases not associated with the liver (Sasidharan et al., 2014). For this reason, ALT is commonly used as a way of screening for liver problems, and elevated ALT levels are strongly correlated with NAFLD (Safwat et al., 2009).

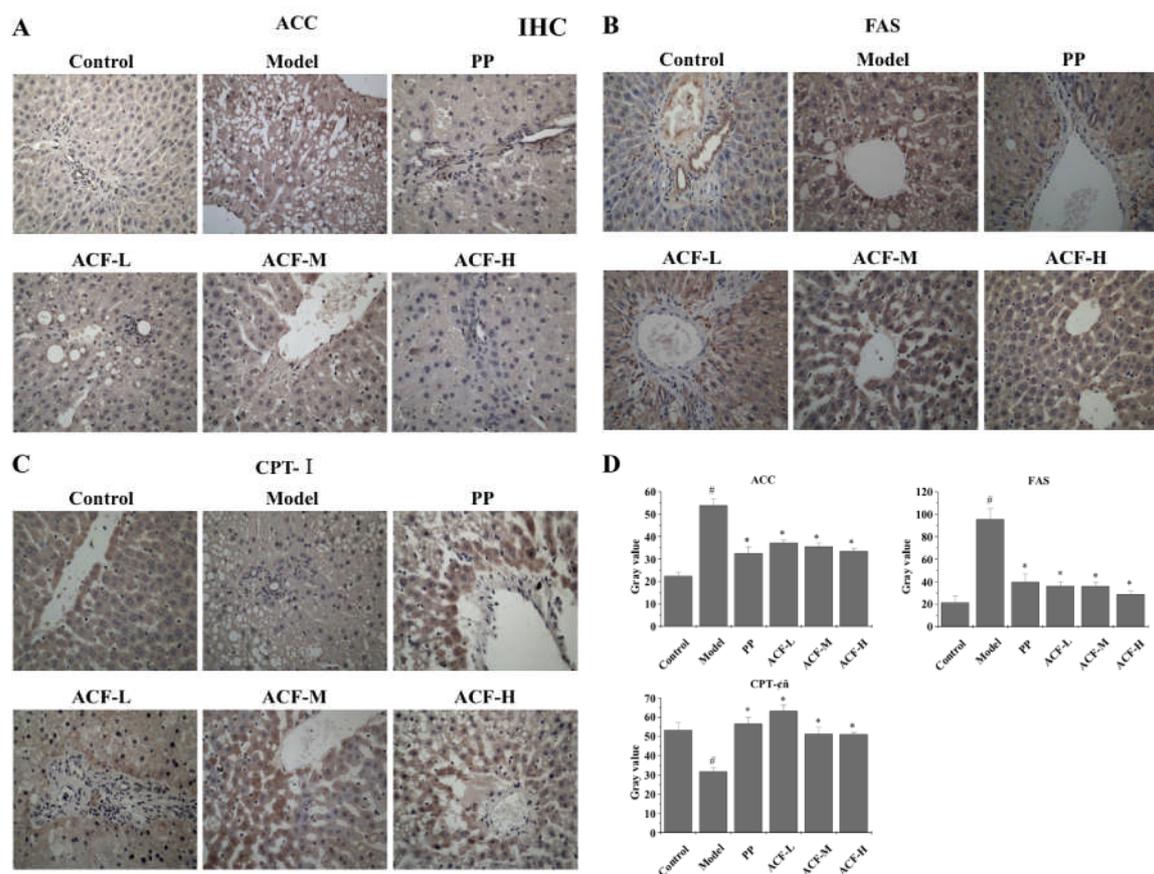


Figure 4: Protein expression and localization of hepatic enzymes in HFD-induced NAFLD models and control groups.

(A) Immunohistochemical analysis of acetyl-CoA carboxylase (ACC) in hepatic tissues from each treatment group. Representative images are shown (400×). The following abbreviations describe the treatment groups: PP (polyene phosphatidylcholine group); ACF-L (ACF low-dose group); ACF-M (ACF medium-dose group); ACF-H (ACF high-dose group). (B) Immunohistochemical analysis of fatty acid synthase (FAS) in hepatic tissues from each treatment group. Representative images are shown (400×), and treatment groups are abbreviated as in (A). (C) Immunohistochemical analysis of carnitine palmitoyltransferase-1 (CPT-1) in hepatic tissues from each treatment group. Representative images are shown (400×), and treatment groups are abbreviated as in (A). (D) Quantification of protein expression as a percentage of positively stained cells. Bars indicate the mean with SD (error bars), calculated from 10 rats in each group. The following symbols indicate statistical relevance: (#) indicates $P < 0.05$, compared with the standard diet control group; (*) indicates $P < 0.05$, compared with the HFD model group.

ALT mainly resides in liver cell endochylema, whereas AST mainly resides in cytochondriomes and to a lesser degree in the endochylema. ALP is localized in liver cell membranes and microvilli of the cholangiole. Thus, the combined detection of ALT, AST, and ALP reflects the extent of damage to liver cells (Wang et al., 2012). The activities of ALT, AST and ALP in sera from HFD-fed NAFLD rats were higher than in control rats that were fed a standardized (lower-fat) diet, which indicates that the NAFLD rats had severe liver cell damage (Figures 1 and 2). After treatment with ACF, the activities of ALT, AST and ALP in sera decreased significantly irrespective of the ACF dosage tested (Figure 2).

Changes in TG and TC are the main pathological characteristics of NAFLD (Salt et al., 2004; Cortez-Pinto et al., 2004). TG are the most common lipids stored in the liver of NAFLD patients. TC is a measure of the total amount of cholesterol in the blood at a given time and is the sum of HDL-C, LDL-C, very low-density lipoprotein cholesterol (VLDL-C), and intermediate density cholesterol (IDL). Excessive lipid accumulation, which usually results from the abnormal activation of lipid metabolism, plays a critical role in the development of NAFLD. In the present study, we demonstrated that ACF significantly reduced elevation of TG, TC and LDL-C levels in HFD-fed rats, indicating that ACF could modulate lipid accumulation (Figure 2).

Hepatic lipid metabolism involves lipid uptake, synthesis, catabolism, and secretion. De novo lipogenesis plays a substantial role in the pathogenesis of NAFLD, accounting for 26% of hepatic triglycerides in human subjects (Donnelly et al., 2005). For de novo lipogenesis, glucose is converted to acetyl-CoA through glycolysis and the oxidation of pyruvate. Acetyl-CoA is then converted to malonyl-CoA by acetyl-CoA carboxylase (ACC). Fatty acid synthase (FAS) catalyzes the formation of palmitic acid from malonyl-CoA and acetyl-CoA. Palmitic acid is then elongated and de-saturated to generate monounsaturated fatty acids, which are the major fatty acid constituents of triglycerides (Kawano et al., 2013). FAS is involved in the last step of the fatty acid pathway by controlling the levels of fatty acid synthetase mRNA (Saha et al., 2003; Postic et al., 2008). Therefore, FAS is a key enzyme in the fatty acid synthesis pathway. Cytosolic ACC1, the major isoform of ACC in the liver, contributes malonyl-CoA for fatty acid synthesis (Mao et al., 2006) and the inhibition of fatty acid oxidation (Savage et al., 2006). Malonyl-CoA produced by ACC inhibits the activity of CPT1, and thereby decreases the rate of β -oxidation by reducing fatty acid entry into the mitochondria. Thus, FAS, ACC and CPT are closely correlated with the occurrence of NAFLD (Saha et al., 2003), and these enzymes may provide the crucial link between NAFLD pathology and lipid metabolism that is targeted by ACF. Our results support this hypothesis through the observation that ACF treatment reduced both gene and protein expression levels of FAS and ACC, as well as increased the expression of CPT in the liver tissues of NAFLD rats (Figures 3 and 4).

In conclusion, the present study demonstrates the ameliorative potential of Artemisia Capillaris Formula on HFD-induced NAFLD in SD rats and indicates a potential mechanism of action that involves regulation of lipid metabolism.

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