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The Effect of Sitagliptin on Lipid Metabolism of Fatty Liver Mice and Related Mechanisms

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Background: In clinics, patients with type 2 diabetes complicated with non-alcoholic fatty liver disease (NAFLD) have been shown to receive significant improvements in blood glucose levels, lipid levels, and liver function after sitagliptin treatment, although the mechanism of drug action remains poorly understood. This study investigated the possible mechanism of sitagliptin on lipid metabolism of NAFLD mice.

Material/Methods: Male C57/BL6 mice were induced for NAFLD via 16 weeks of a high-fat diet, and were treated with 15 mg/kg/day sitagliptin for 16 consecutive weeks. Blood lipid levels were measured and samples were stained with hematoxylin and eosin (H&E) and oil red staining for liver pathology and lipid deposition. Serum levels of fibroblast growth factor (FGF)-9 and FGF-21 were quantified by enzyme-linked immunosorbent assay (ELISA). Peroxisome proliferator-activated receptor (PPAR)- α , and cAMP reactive element binding homolog (CREBH) were measured by Western blotting, while fatty acid synthase and carnitine palmitoyltransferase 1 (CPT1) mRNA levels were assayed by RT-PCR.

Results: Compared to the control group, the NAFLD model mice had liver fatty disease, lower serum FGF-21 and FGF-19 levels, elevated serum lipid levels, depressed PPAR- α , CREBH, and CPT1 expression, and enhanced FAS expression ($p < 0.05$). Sitagliptin treatment depressed blood lipid levels, increased serum FGF-21 and FGF-19 levels, PPAR- α , CREBH, and CPT1 expression, and suppressed FAS expression ($p < 0.05$).

Conclusions: Sitagliptin can protect liver tissue and modulate lipid metabolism in NAFLD mice via elevating FGF-21 and FGF-19, upregulating liver PPAR- α and CREBH levels, and mediating expression levels of key enzymes for lipid metabolism.

MeSH Keywords: **Fatty Acid Synthases • Fibroblast Growth Factors • Liver Diseases**

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Background

Dipeptidyl peptidase IV (DPP-4) inhibitors are novel diabetic drugs that work by inhibiting DDP4 enzyme activity and degrading/inactivating glucagon-like peptide-1 (GLP-1) [1,2]. Currently approved DDP4 inhibitors include sitagliptin, saxagliptin, and linagliptin, among which phosphate sitagliptin was the first approved DDP4 inhibitor for treating type 2 diabetes [3,4]. Clinical studies have confirmed that sitagliptin significantly improves blood lipid levels and liver function of patients with type 2 diabetes complicated with non-alcoholic fatty liver disease (NAFLD) [5,6]. As type 2 diabetes shares common insulin resistant mechanism with NAFLD, sitagliptin can extend the pro-insulin function of GLP-1 via inhibiting its degradation. With improved insulin resistance, fatty acid hydrolysis is decreased, thus improving incretin hormone levels that affect metabolism. Studies have shown that sitagliptin could affect high-fat diet induced-fatty liver via decreasing intrahepatic lipogenesis and increasing lipid oxidation [7,8], although its detailed mechanism is still unclear. Metabolic disorders and insulin resistance both play important roles in NAFLD pathogenesis [9,10]. Fibroblast growth factor (FGF)-19 and FGF-21 play critical roles in liver fatty disease metabolism, and are recognized as novel adipocyte factors [1]. The correlation between type 2 diabetes and elevated serum FGF-21 level in NAFLD patients might be related to a compensatory mechanism after insulin dysfunction. Serum FGF-19/FGF-21 levels in the plasma of NAFLD patients are associated with liver fatty disease, as lowered FGF-19 levels occurred in such patients [12]. This study used a high-fat diet-induced NAFLD mouse model, in which the effect of sitagliptin on metabolism and related mechanism were investigated.

Material and Methods

Animals and grouping

Healthy male C57/BL6 mice (8 weeks of age, body weight 16~18 g) were provided by Laboratory Animal Center, Shanghai University of Traditional Chinese Medicine (Certificate No. SYXK-2013-0025). The animals were kept in an SPF grade facility with food and water ad libitum. Ten mice were provided with a normal diet while another 20 mice were given a high-fat diet (1% cholesterol, 68.5% basic diet, 0.5% bile salt, 15% lard oil, and 15% dextrin) for 16 consecutive weeks to induce NAFLD model as previously described [13]. Ten of these high-fat diet mice were given 15 mg/kg/day of sitagliptin by gavage for 16 consecutive weeks, while the other 10 mice plus 10 control mice received equal volume of saline by gavage.

Mice were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Puto Hospital, Shanghai University of Traditional Chinese Medicine.

Drugs and reagents

Drugs and reagents included: phosphate sitagliptin (0.1 g pill dissolved in 0.9% physiological saline, MSD, USA); normal- and high-iron, high-fat diet (Radiology Institute of Tianjin, China); assay kits for total cholesterol (TC) and triglyceride glycerol (TG, Beikong Biotech, China); pentobarbital (Shanghai Chem., China); assay kits for aspartate aminotransferase (AST) and alanine aminotransferase (ALT), (Jiancheng Bio-engineering, China); injection water (Kelun Pharmacy); ELISA kits for FGF-21 and FGF-19 (R&D Systems, USA); TRIZOL kit and reverse transcription kit (Invitrogen, USA); monoclonal antibody against peroxisome proliferator-activated receptor (PPAR)- α and cAMP reactive element binding homolog (CREBH, CST, USA); horseradish peroxidase labelled goat anti-rabbit secondary antibody (CST, USA); Primers (Aibosi, China).

General condition of mice

Food/water intake, body weight, behavior, and fur status of mice during the experiment were observed and recorded.

Liver function and blood lipid level

At 4, 8, 12, and 16 weeks after preparing the animal model, venous blood samples were collected for separation under centrifugation at 3,500 rpm for 15 minutes. A fully automatic biochemical analyzer was employed to test liver function index and blood lipid level.

H&E and oil red staining for liver morphology

Mice were sacrificed and liver tissue was extracted for paraffin embedding. Tissue slides were then stained by oil red or hematoxylin and eosin (H&E). The pathological condition of the liver was observed under light field microscope after mounting. Under oil red staining, the distribution of fatty tissue was observed. Fatty liver disease was classified as grade zero, grade one (less than 33% hepatocytes with fatty disease), grade two (33~66% cells having fatty lesion) and grade three (more than 66% fatty hepatocytes) [14].

Serum FGF-19 and FGF-21 levels by ELISA

Enzyme-linked immunosorbent assay (ELISA) was employed to test serum levels of FGF-19 and FGF-21. Samples of 2 mL venous blood were collected and centrifuged at 3,500 rpm for 15 minutes ($r=10$ cm) to separate serum. Assays for FGF followed standard ELISA protocols.

Table 1. Primer sequence.

Target gene		Sequence (5'-3')	Fragment length (bp)
FAS	Forward	ATCTGGGCTGCCTGCCT	101
	Reverse	GATATAATCCTTCTGAAGCAG	
CPT1	Forward	TTCAGCTCTGGCAAGAACAA	125
	Reverse	GTCAAACCACCTGTTATAG	
GAPDH	Forward	TGGAGTCTACTGGCGTCTT	138
	Reverse	TGTCATATTCTCGTGGTTCA	

RT-PCR for liver mRNA levels of fatty acid synthase (FAS) and carnitine palmitoyltransferase 1 (CPT1)

Total RNA was extracted from liver tissue and then synthesized for cDNA via reverse transcription. RT-PCR followed standard TRIzol protocol. UV spectrometry was used to quantify mRNA levels using primers (see Table 1 for primer sequence). Amplification products were employed for agarose gel electrophoresis in triplicates, and were presented as relative expression levels as target gene gray values against GAPDH.

Western blotting for liver expression of peroxisome proliferator-activated receptor (PPAR)- α and cAMP reactive element binding homolog (CREBH)

Mice liver tissue was lysed in lysis buffer, and cells were collected after centrifugation at 4,000 rpm for 10 minutes. BCA protein quantification assay kit was used to measure protein concentration. Protein samples were separated by dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene difluoride (PVDF) membrane, which was blocked for 1 hour in blocking buffer, and incubated in primary antibody (1: 2,000 dilution) at 4°C overnight. After TBST (Tris-buffered Tween-20) rinsing (three times), secondary antibody was added for 1 hour incubation, followed by TBST rinsing. Enhanced chemiluminescence (ECL) reagent was used to develop the membrane, which was exposed in a dark room for analysis of colored bands using Quantity One software. Relative expression levels of target proteins (PPAR- α and CREBH) were normalized against internal reference protein bands.

Statistical method

SPSS19.0 software was employed for statistical analysis. Measurement data were presented as mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) was employed for comparing means across multiple groups. LSD (least significant difference) test was used to compare between two groups. A statistical significance was defined when $p < 0.05$.

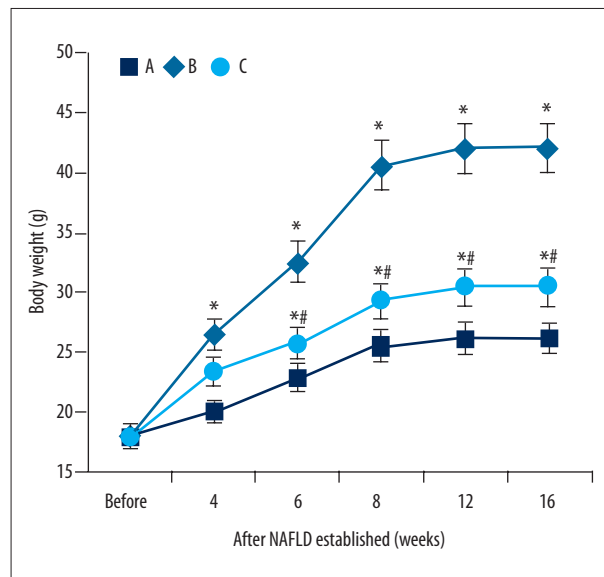


Figure 1. The effect of sitagliptin on body weight of NAFLD mice. A – Control group; B – Model group; C – Sitagliptin group. * $p < 0.05$ compared to control group; # $p < 0.05$ compared to model group.

Results

Effect of sitagliptin on general conditions of mice

The control mice group had normal feeding/drinking behavior and smooth fur. The model mice group had increased food/water intake, lower activity, worsened fur color, and significantly higher body weight compared to the control group ($p < 0.05$). Compared to the model group, sitagliptin treatment significantly depressed body weight ($p < 0.05$, Figure 1).

Effect of sitagliptin on blood lipid level and liver function in NAFLD mice

Results showed significantly higher blood lipid, ALT, and AST levels in the model group compared to the control group ($p < 0.05$). Magnitudes of these increases were lower with elongated time. The sitagliptin-treated mice had significantly lower

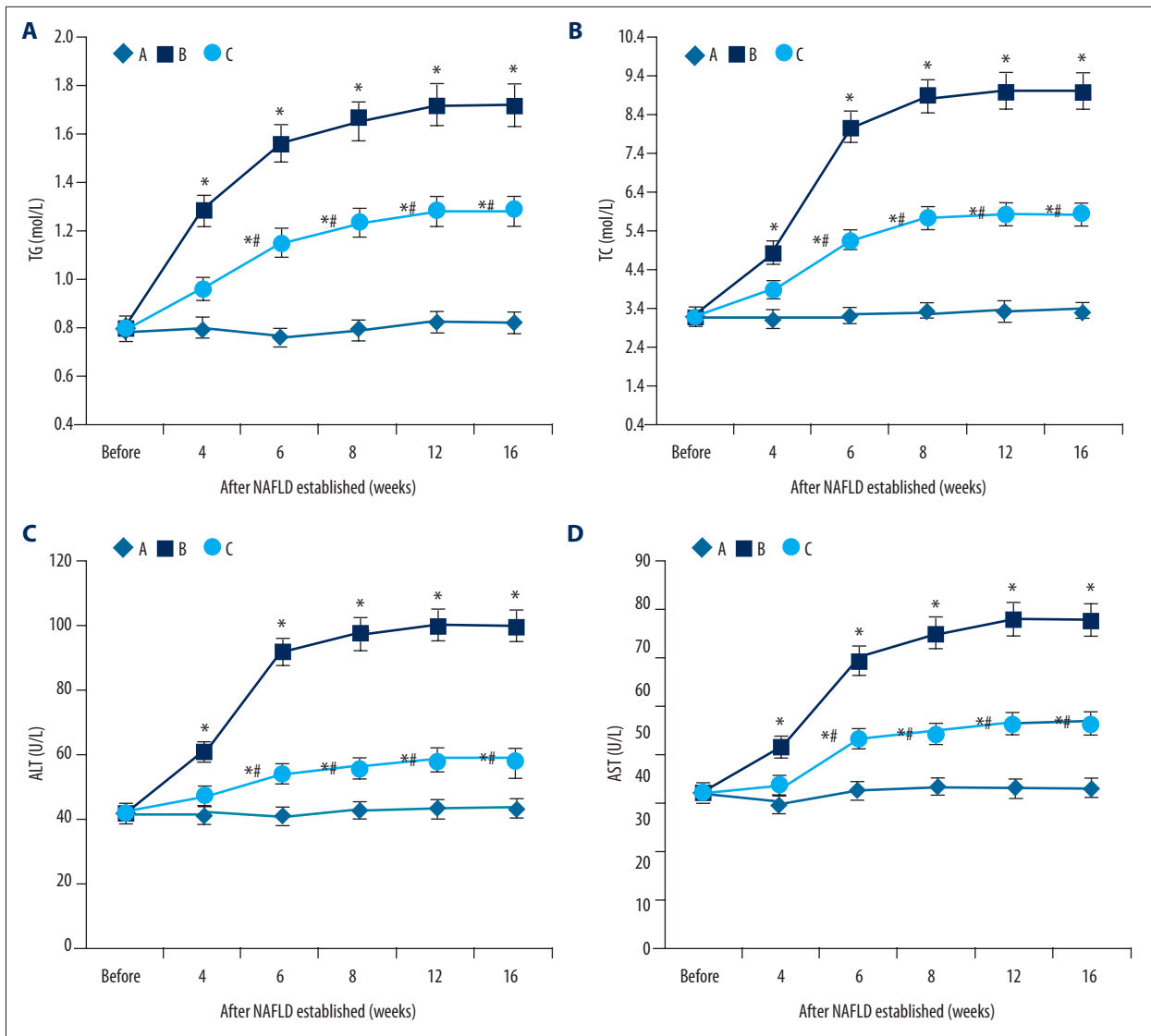


Figure 2. (A–D) Effect of sitagliptin on blood lipid and liver function in NAFLD mice. A – Control group; B – Model group; C – Sitagliptin group. * $p < 0.05$ compared to control group; # $p < 0.05$ compared to model group.

blood lipid, ALT, and AST levels compared to the model group ($p < 0.05$, Figure 2).

Effect of sitagliptin on liver disease morphology of NAFLD mice

H&E staining showed regular hepatic lobes in the control group, with small lipid droplets and arranged hepatocytes, which had evenly distributed cytoplasm. The model group showed increased size of hepatocytes, which showed swelling and different size lipid droplets. Inflammatory cell infiltration was observable in hepatic lobes, with a palette of necrotic lesion in the hepatic portal area. The sitagliptin-treatment group had smaller size hepatocytes compared to those in the model group, along with fewer intracellular lipid droplets and

alleviated inflammatory infiltration (Figure 3). Oil red staining showed fewer lipid droplets in the control group hepatocytes without lipid denaturation. In the model group, severe lipid denaturation was observed in hepatocytes, in which different sizes of lipid vacuoles were observed, with significance presence in portal and sinus areas. Sitagliptin treatment appeared to alleviate lipid disease of hepatocytes, as shown by smaller lesions compared to the model group ($p < 0.05$, Figures 3, 4).

Sitagliptin and FGF-19/FGF-21 levels in mice

With elongated treatment time, FGF-19 and FGF-21 levels in the model mice group were gradually decreased, and significantly lower than in the control group ($p < 0.05$). The magnitude of decrease was suppressed with elongated time. Compared to

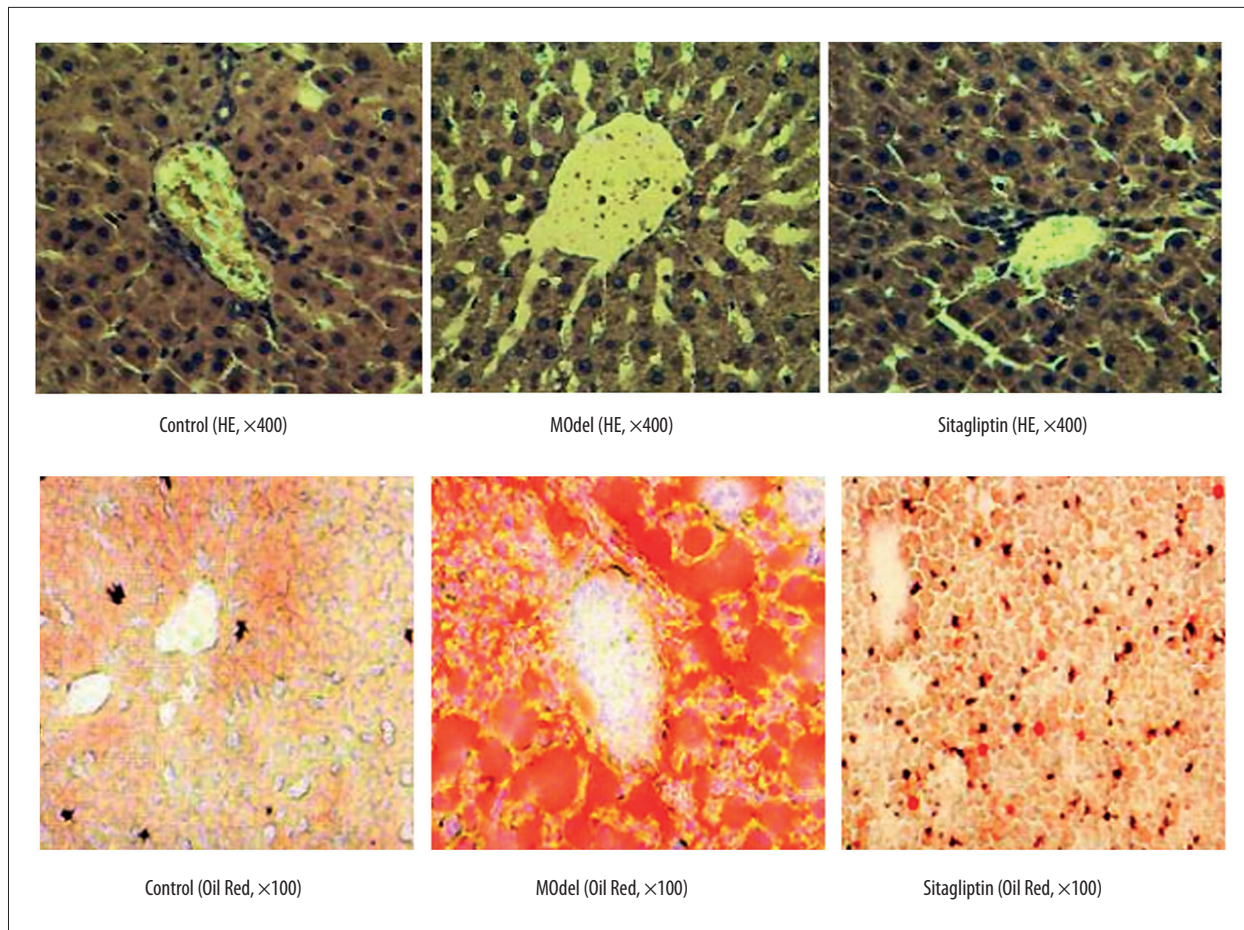


Figure 3. Hepatic morphology of NAFLD mice after sitagliptin treatment.

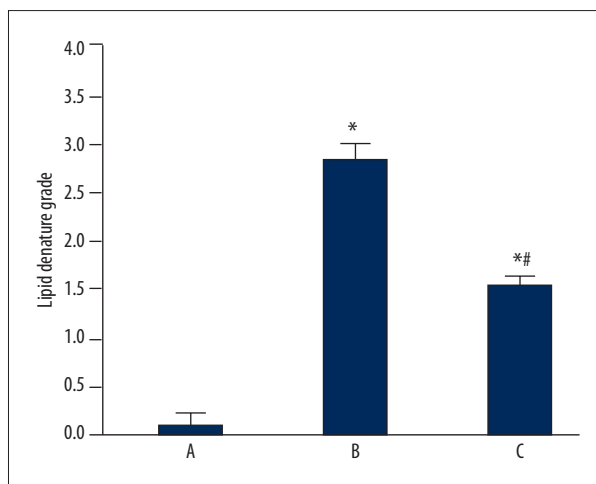


Figure 4. Hepatic lipid disease grade of all groups. A – Control group; B – Model group; C – Sitagliptin group. * $p < 0.05$ compared to control group; # $p < 0.05$ compared to model group.

the model group, FGF-19 and FGF-21 levels in the sitagliptin-treatment group were significantly elevated ($p < 0.05$, Figure 5).

FAS and CPT1 mRNA expression levels in hepatic tissues

The model group had significantly lower CPT1 mRNA levels than the control group, while FAS mRNA levels were higher ($p < 0.05$). Liver tissue of the sitagliptin-treatment group had lower FAS mRNA and higher CPT1 mRNA expression than liver tissue of the model group ($p < 0.05$, Figure 6).

PRAR- α and CREBH expression levels in mice hepatic tissues of all groups

Compared to the control group, the model group had depressed expression of PRAR- α and CREBH ($p < 0.05$). Hepatic tissues after sitagliptin treatment had upregulation of PRAR- α and CREBH expressions ($p < 0.05$) compared to the model group, Figure 7).

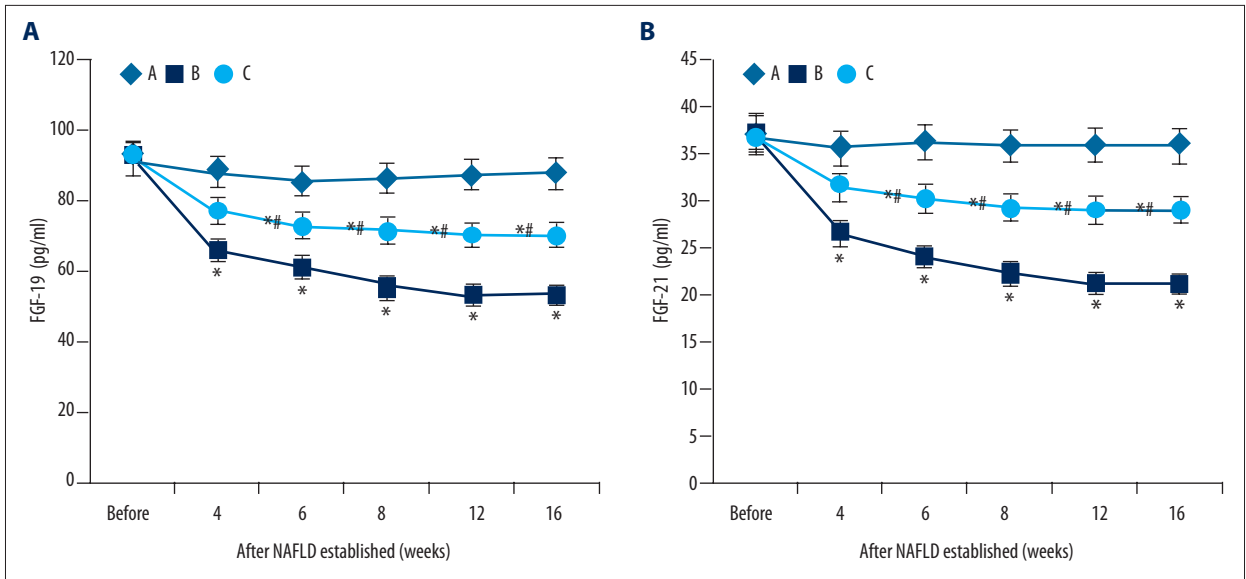


Figure 5. Effect of sitagliptin on FGF-19 (A) and FGF-21 (B) levels in mice. A – Control group; B – Model group; C – Sitagliptin group. * $p < 0.05$ compared to control group; # $p < 0.05$ compared to model group.

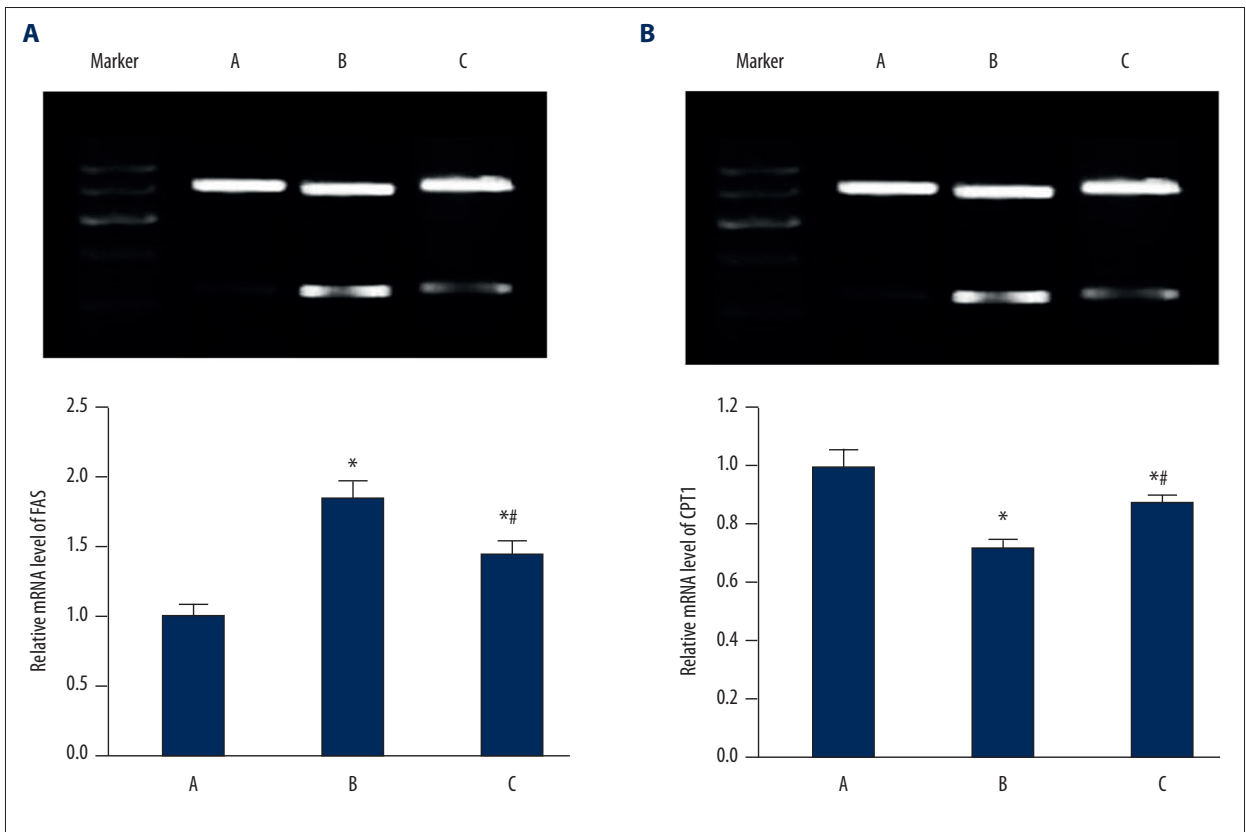


Figure 6. (A, B) FAS and CPT1 mRNA expression levels in mice hepatic tissues. A – Control group; B – Model group; C – Sitagliptin group. * $p < 0.05$ compared to control group; # $p < 0.05$ compared to model group.

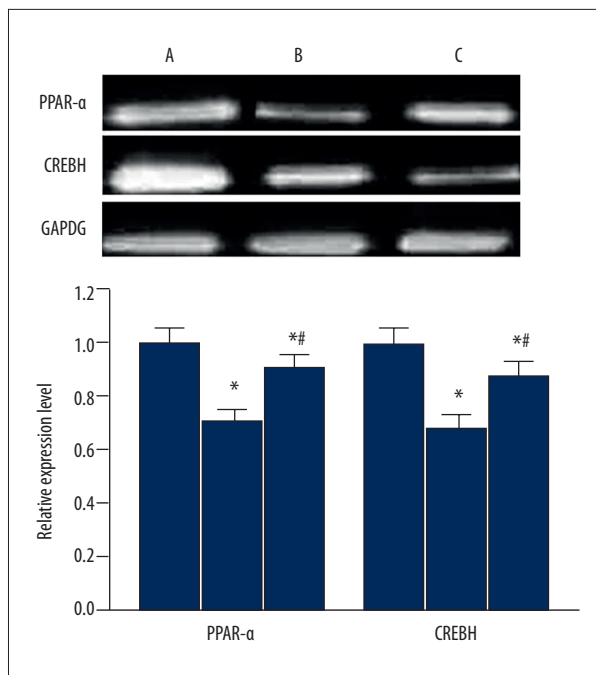


Figure 7. Expression level of PPAR- α and CREBH in mice hepatic tissues. A – Control group; B – Model group; C – Sitagliptin group. * $p < 0.05$ compared to control group; # $p < 0.05$ compared to model group.

Discussion

NAFLD is closely correlated with hyperinsulinemia, type 2 diabetes, and obesity; it mainly presents as lipid deposition and denaturation of hepatocytes [15]. A high-fat diet could interfere with insulin-induced glucose transport and insulin binding, thus affecting liver lipid metabolism [16–18]. The proliferation of adipocytes decreases insulin receptor numbers and activity. The study of NAFLD pathogenesis has revealed that both hyperinsulinemia and insulin resistance can induce lipid deposition [19–21]. Various factors can cause oxidative stress response, lipid over-oxidation, and mitochondrial injury, all of which facilitated hepatocyte necrosis. Sitagliptin is one of several novel DPP-4 inhibitors approved for treatment of diabetes. It can inhibit DPP4 enzymes with high efficiency and elevate GLP-1 levels within physiological range. GLP-1 mimics could reduce fatty acid content in hepatic adipocytes and increase phosphorylation of insulin signal pathway proteins, including AKT and PDK-1, and possibly improve hepatic lipid denaturation [22]. In our study, we showed significant lipid lesion in the livers of model mice that had remarkably higher blood lipid levels. Compared to the model group, sitagliptin significantly decreased blood lipid levels and alleviated fatty denaturation of hepatocytes. The degree of fatty denaturation was significantly lower in the sitagliptin-treatment group than in the model group, indicating that sitagliptin could downregulate blood lipid level of NAFLD and protect liver function.

Most members of the FGF family have higher affinity with proteoglycan or heparin via paracrine factors, and FGF-19 and FGF-21 play important roles in modulating blood lipid levels and insulin sensitivity [20,21]. Cholesterol is metabolized into bile acid inside the liver for accelerating the absorption of lipids. FGF-19 participates in the regulation of bile acid metabolism via mediating the expression level of liver cholesterol hydroxylase. Transgenic studies showed that FGF-19 could enhance the energy metabolic rate and decrease white fat independent of insulin growth factor or leptin [17]. FGF-19 could potentiate beta-oxidation of fatty acid inside the liver, as the critical enzyme for beta oxidation of fatty acid, CPT1, has elevated activity. FGF-21 modulates glucose intake in the insulin-independent pathway, via activating the extracellular signal pathway to improve the function of islet beta cells. FGF-21 can also protect against high-fat diet-induced lipid disease via governing lipid metabolism related genes [17,20]. The tight regulation of lipid metabolism is closely correlated with transcriptional factor, nuclear receptor and intracellular related enzymes. PPAR- α plays an important role in modulating lipid metabolism. The activation of CREBH is associated with multiple factors including inflammatory response or ER stress response. After activation, CREBH is involved in the generation and degradation of hepatic fatty acid, and the expression of fatty acid oxidation related genes. Among downstream target genes of PPAR- α , FGF-21 plays an important role [23]. As transcriptional activating factor, PPAR- α and CREBH potentiate the expression of FGF-19 and FGF-21, and participate in liver fatty acid metabolism. A previous study showed the critical function of CREBH-PPAR- α -FGF-21 axis in the metabolic stress of the liver [24]. FAS is one critical enzymes catalyzing the synthesis of palmitic acid. When its activity is elevated, excess fatty acids are synthesized to produce lipids via esterification. When FAS is upregulated, the deposition of the glycerol triglyceride is increased. When FAS expression is inhibited, the generation of lipids is decreased as the blockade of lipogenesis pathway. CPT1 has critical functions for liver lipid disease [24]. Our study showed significantly lower serum FGF-19 and FGF-21 levels, and lower hepatic CPT1 mRNA, PPAR- α , and CREBH levels in the sitagliptin treatment group compared to the control group. FAS mRNA expression was, however, remarkably higher in the sitagliptin-treatment group than the control group. In the sitagliptin-treatment group, serum FGF-19 and FGF-21 levels, and hepatic CPT1 mRNA, PPAR- α , and CREBH levels were all significantly higher than the model group, while FAS mRNA was downregulated, suggesting that sitagliptin could suppress cholesterol and glycerol triglyceride levels, enhance energy metabolism, decrease deposition of lipid inside liver tissues, and improve blood lipid metabolism via inhibiting DPP4 enzymes, elevating GLP-1 enzymatic activity, and facilitating FGF-19/FGF21 expression. Sitagliptin could also facilitate lipid oxidation via upregulating PPAR- α and CREBH expression levels and facilitating secretion of FGF-19/FGF-21, and could also improve fatty liver lesion in NAFLD mice via potentiating CREBH-PPAR- α -FGF-21

axis. It might also regulate biosynthesis and absorption of triglyceride and cholesterol, affect lipid metabolism of NAFLD mice, and protect liver functions via mediating expression levels of critical enzymes for liver lipid metabolism.

Conclusions

Sitagliptin could affect lipid metabolism and protect liver function of NAFLD mice via elevating FGF-19/FGF-21 levels,

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upregulating liver expression of PPAR- α and CREBH genes, and modulating the expression level of critical enzymes in lipid metabolism.

Disclosure of conflict of interest

The authors declare no competing financial or commercial interests in this manuscript.