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Ganoderma lucidum Polysaccharides Ameliorated Metabolic Disorder through AMPK Signaling Pathway in Hepatic Steatosis Mice

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***SUMMARY*. Disorders of hepatic lipid metabolism could result in hepatic steatosis. The *Ganoderma lucidum* polysaccharides (GLP) has been employed for centuries in Asian countries to treat diverse diseases. Herein, we examined the effect and the mechanism of GLP on a high-fat diet (HFD) induced model of hepatic steatosis in *db*/*db* mice. The *db*/*db* mice were randomly assigned to treatment with GLP and metformin or vehicle for 8 weeks. Age-matched non-diabetic db/+ mice acted as controls. Results show that showed that GLP was significantly decreased the parameters of glucose and lipid metabolism along with reduced lipid droplets accumulation in hepatocytes. Moreover, GLP treatment significantly decreased the expression of genes or proteins such as SREBP1c, SCD1and phosphorylated mTOR. etc, whilst, up-regulated the expression of PPAR-α, Acox1, and phosphorylated AMPK etc, in a dosage-dependent manner. Collectively, these findings demonstrated that GLP might significantly ameliorate lipid accumulation through activation of AMPK signaling in *db*/*db* mice.**

RESUMEN. Los trastornos del metabolismo de los lípidos hepáticos pueden provocar esteatosis hepática. Los polisacáridos de *Ganoderma lucidum* (GLP) se han utilizado durante siglos en países asiáticos para tratar diversas enfermedades. En este documento, examinamos el efecto y el mecanismo de GLP en un modelo de esteatosis hepática inducida por una dieta alta en grasas (HFD) en ratones *db*/*db*. Los ratones *db*/*db* se asignaron aleatoriamente al tratamiento con GLP y metformina o vehículo durante 8 semanas. Los ratones *db*/+ no diabéticos de la misma edad actuaron como controles. Los resultados muestran que el GLP disminuyó significativamente los parámetros del metabolismo de la glucosa y los lípidos junto con la reducción de la acumulación de gotitas de lípidos en los hepatocitos. Además, el tratamiento con GLP disminuyó significativamente la expresión de genes o proteínas como SREBP1c, SCD1 y mTOR fosforilado. etc., mientras que regulaba positivamente la expresión de PPAR-α, Acox1 y AMPK fosforilada, etc., de una manera dependiente de la dosis. En conjunto, estos hallazgos demostraron que el GLP podría mejorar significativamente la acumulación de lípidos mediante la activación de la señalización de AMPK en ratones *db*/*db*.

INTRODUCTION

Hepatic steatosis is a multifactor influenced disease, such as genetic susceptibility, diet, lifestyle, lipotoxicity, mitochondrial dysfunction, oxidative stress, and intestinal derived bacteria endotoxins initiated inflammation, etc., which resulted in increased free fatty acids (FFAs) accumulation and lipid droplet formation [1,2]. It was reported that over 24% of the global population suffered from the disease [3]. Besides, a study has revealed that the risk of hepatic steatosis was up to 70% in patients with type 2 diabetes mellitus (T2DM) [4]. In normal physiological conditions, fatty acids derived from dietary fats and sugar were either lipid oxidation or converted into cholesterol or triacylglycerol (TAG) combined with very low-density lipoproteins (VLDL) or chylomicron. While, in pathologic status, the nonesterified fatty acid (NEFA) induced lipotoxicity through hepatic oxidative stress, which produced reactive oxygen

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species (ROS) and endotoxins to the involvement of lipid peroxidation and inflammation, thus destroying the glucose and lipid metabolism in the liver and resulted in hepatic steatosis [5,6].

More interestingly, in the process, many hepatic metabolism enzymes played an important role in the activity of lipogenesis and lipid oxidation, for example, the lipid synthesized nuclear transcription factor sterol regulatory element binding protein 1c (SREBP1c) mediated its downstream signals, FAS, SCD1, DGAT1, Cpt1, *etc* to control the hepatic lipid metabolism through AMPK signaling pathway [7-9]. Besides, the peroxisome proliferator-activated receptors, PPARs also contributed to the activity of lipid metabolism [10]. A study had shown that the transcription factor PPAR-γ being activated by the upstream signal mTOR facilitated the lipid esterification and inhibited the production of free fatty acid through the AMPK signaling pathway [11]. And the PPAR-α was also identified to be a transcriptional activator of ACOX1 in regulating its downstream signals SCD1, Cpt1, Hmgcs2 in the activity of fatty acids beta-oxidation and ketogenic metabolism [12,13]. Therefore, understanding hepatic metabolic disorder pathogenesis and finding proper therapies have become increasingly important.

Nowadays, traditional Chinese medicine has been more and more accepted for its broad medicinal properties. *Ganoderma lucidum* polysaccharides (GLP) as the major component of *G. lucidum* (Chinese name "lingzhi") had been proved its biomedicinal properties by numerous studies, such as anti-tumor [14], immunoregulatory [15,16], anti-oxidation [17], and hypolipidemic activities [18]. Therefore, there is a great medicinal utilization of GLP in treating varied diseases. Thus, in this study, we attempted to investigate the effects and the underlying molecular mechanisms of GLP on hepatic metabolic disorder through the AMPK signaling pathway in *db/db* mice with hepatic steatosis.

MATERIALS AND METHODS

Animals treatment

A total of 24 male *db/db* mice (32.5 ± 2.5g) and the other six Age-matched non-diabetic *db/+* mice (18.2 ± 1.2 g), six weeks old, were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). Animals were maintained at 20-25 °C, 40%-70% relative humidity environment with a 12 h light/dark cycle for one week adapted cultivation in the animal experimental research center of Zhejiang Chinese Medical University (Zhejiang, China). Besides, the mice were free to access the regular chow diet. After acclimation, mice were fed either a regular diet (RD) or a high-fat diet (HFD) (21% fat, 0.15% cholesterol) for 14 weeks, we calculated the weight of the mice and collected the blood via the tail vein to evaluate the glucose levels. The mice were selected as an eligible model when the glucose production in overweight mice was over 11.1 mmol/L. Then, the eligible *db/db* mice model were randomly divided into four groups (n = 6), including the model group, GLP (100 mg/kg) group, GLP (400 mg/kg) group, and metformin (300 mg/kg/day) group and were administrated with GLP (100 mg/kg, 400 mg/kg, 95% purification, Johncan International Bio, tec, Hangzhou, Zhejiang), and metformin (300 mg/kg/day, BMS, Shanghai) for consecutive eight weeks. The control and model groups received the same volume of CMC-Na solution. After eight weeks of administration, all the mice were evaluated the food and water intake, then the mice were anesthetized with 2 % isoflurane. Then, we weighed their body weight and they were sacrificed. Afterward, blood samples were collected for serum assessment. Liver tissues were removed and weighed, and frozen at -80 °C or immersed in formalin for experiments. Additionally, the animal study was performed in accordance with the experimental animal guidelines of the animal medical center institution.

Biochemical parameters analysis in serum

After 8 weeks of administration, blood samples were collected after 12 h of overnight starvation and were centrifuged at 3500 rpm for 15 min. Subsequently, the serum glucose levels were determined by using a 7020 full-automatic biochemical analyzer (Hitachi, Tokyo, Japan) according to the manufacturer's instructions. Also, the glycosylated hemoglobin levels were analyzed with a GHb ELISA kit (mlBIO, shanghai, China). Besides, the levels of ALT, AST, Scr, BUN, TG, TC, LDL-C, and HDL-C in serum were quantified with commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

Histopathology analysis

The formalin-fixed liver tissues were washed, dehydrated, transparent, and embedded in paraffin, then cut the tissues with 4 μm thickness and dried. Afterward, dewaxed the slices and stained them with hematoxylin and eosin (HE) (Beyotime, Shanghai, China), then made the stained slices dehydration and transparency, finally,

sealed the slices with gum. At last, the pathological morphology features of the slices were observed with a light microscope (Nikon Eclipse E100 microscope; Nikon, Tokyo, Japan).

Oil-Red-O staining

To further detect hepatic lipid distribution, Oil Red O staining was performed according to the manufacturer's instructions. Briefly, the liver tissues frozen in liquid nitrogen were cut into 5-10 μm thickness, then mounted the slices in microslide and dried. Afterward, fixed them with formalin and dried again, then the slices were stained with 0.2% (w/v) Oil-Red O solution (ab150678, Abcam) for 15-20 min at room temperature. Later, washed them with isopropanol and dyed the nuclear with hematoxylin; at last, sealed the slices with glycogelatin and visualized the lipid droplet accumulated in hepatocyte with inverted microscopy (Leica). Images of the same magnification (200×) were collected.

Real-time quantitative PCR

Total RNA was extracted with Trizol Reagent (Thermo Fisher Scientific) from frozen liver tissues according to the manufacturer's instructions, then the RNA was reverse-transcribed into cDNA using SuperScript™ III First-Strand Synthesis System (Thermo Fisher Scientific). And, qRT-PCR using SYBR Green PCR Master Mix (Takara, Tokyo, Japan) was run in the ABI Prism 700 thermal cycler (Applied Biosystems, Foster City, CA). Afterward, the mRNA expressions were calculated according to a comparative method ($2^{-\Delta\Delta Ct}$) using GAPDH as control. The primers were synthesized in Sangon Biotech (Shanghai, China), and the primer sequences were listed in Table 1.

Genes	Gene-specific primers
SREBP1c	forward: 5'-GTCGGCGATCCTGAGGAA-3' reverse: 5'- CTCTTCTGCACGGCCATCTT-3'
FAS	forward: 5'-TGAAGGACCTTATCGCATTGC-3' reverse: 5'- GCATGGGAAGCATTTTGTTGT-3'
SCD1	forward: 5'-GGCGTTCCAGAATGACGTTT-3' reverse: 5'-TGAAGCACAACAGCAGGACA-3'
PPAR-γ	forward: 5'-TTTTCAAGGGTGCCAGTTTC-3' reverse: 5'-GAGGCCAGCATGGTGTAGAT-3'
DGAT1	forward: 5'-CCCATACCCGGGACAAAGAC-3' reverse: 5'-ATCAGCATCACCACACACCA-3'
PPAR-α	forward: 5'-CAAACCAACCATCCTGACGAT-3' reverse: 5'-GGAGGTCAGCCATTTTTTGGA-3'
ACOX1	forward: 5'-AAGCCAGCGTTACGAGGT-3' reverse: 5'-CTGTTGAGAATGAACTCTTGG-3'
CPT1	forward: 5'-GGGTTGCCCTTATCGTCACA-3' reverse: 5'-TACAACATGGGCTTCCGTCC-3'
Hmgcs2	forward: 5'-TCGACCCAACAATAACAGATGC-3' reverse: 5'-TCTCGTATCTTTCTTGGCGACT-3'
GAPDH	forward: 5'-CATGGGTGTGAACCATGAGAAGTA-3' Reverse: 5'-CAGTAGAGGCAGGGATGATGTTCT-3'

Table 1. Sequence of primers for qRT-PCR. SREBP1c: sterol regulatory element binding protein-1c; FAS: fatty acid synthase; SCD1: stearoyl-CoA desaturase 1; PPAR-γ: peroxisome proliferator-activated receptor γ; DGAT1: diacylglycerol acyltransferase 1; PPAR-α: peroxisome proliferator-activated receptor α; ACOX1: acyl-CoA oxidase 1; CPT1: carnitine palmitoyltransferase 1; Hmgcs2: 3-hydroxy-3-methylglutaryl-CoA synthase 2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Western blot

Total proteins from the isolated liver tissues were extracted with RIPA buffer (Sigma) containing protease inhibitors, then the proteins were denatured and the bicinchoninic acid (BCA) method was used to determine protein concentration. Subsequently, 20 μg proteins were added into each hole lane and separated with 10 or 8% SDS polyacrylamide gels (Beyotime, Shanghai, China), and then transferred onto PVDF membranes (Millipore). After the membranes were blocked with 5% non-fat milk blocking buffer for 2 h at room temperature, then dividedly incubated the membranes with primary antibodies: anti-AMPK (1:1000, ab32047, Abcam), anti-pAMPK (1:2000, ab23875, Abcam), anti-pmTOR (1:1000, ab109268, Abcam), anti-mTOR (1:1000, ab134903, Abcam), anti-Srebp-1c (1:2000, ab191857, Abcam) and anti-PPAR-α (1:500, ab24509, Abcam) at 4 °C overnight. Then, the membranes were washed thrice with TBST and were immunoblotted with anti-mouse IgG antibody (1:5000, ab205719, Abcam) for 1h at room temperature. Besides, the GAPDH (1:5000, D190090, Sangon Biotech, Shanghai, China) was as a control. At last, protein bands were visualized after development using an enhanced chemiluminescence solution (ECL, Beyotime, Shanghai, China) for 5 min. Western blots were quantified using Image J Software (National Institutes of Health, NY).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). The results were analyzed in SPSS 19.0 software with one-way ANOVA analysis, the significant differences among groups were assessed if $p < 0.05$.

RESULTS

GLP mitigated glucose and lipid metabolic disorder in db/db mice with hepatic steatosis

The *db/db* model mice have an increased food intake, water intake, body weight, and liver index compared with the normal mice. However, as shown in Figs. 1A-1D, upon the treatment with GLP for eight weeks, we found that the GLP and metformin reduced the mice's food intake, water intake, body weight, and liver index compared with the model mice. Moreover, GLP and metformin have significantly decreased the levels of blood glucose and glycosylated hemoglobin compared with the model mice (Figs. 1E-1F). Meanwhile, the serum levels of biochemical metabolic parameters, ALT, AST, Scr, BUN, TG, TC, LDL-C were higher in the model mice compared with the normal mice (Figs. 2A-2E). However, the metformin and GLP treatment significantly decreased the serum production of ALT, AST, Scr, BUN, TC, TG, and LDL-C compared with the model mice without treatment. Conversely, the amount of HDL-C in serum was increased in the metformin and GLP groups compared with the model mice (Fig. 2E).

GLP alleviated the pathological lesion of liver tissue in db/db mice

To visualize the pathological injury in the liver, the HE staining results showed that there was an obvious increase of lipid vacuoles and hepatocellular hypertrophy in the model mice compared with the normal mice, as shown in Fig. 3, while, these characteristics were alleviated with GLP and metformin treatment compared with the model mice. Additionally, the Oil Red O staining further revealed that fat droplets accumulated in the hepatocyte, while GLP and metformin treatment significantly decreased the lipid droplets accumulation (Fig. 4).

GLP inhibited lipogenesis and promoted fatty acids β-oxidation in the liver tissue in *db/db* mice

The lipogenesis signals of SREBP1c, FAS,

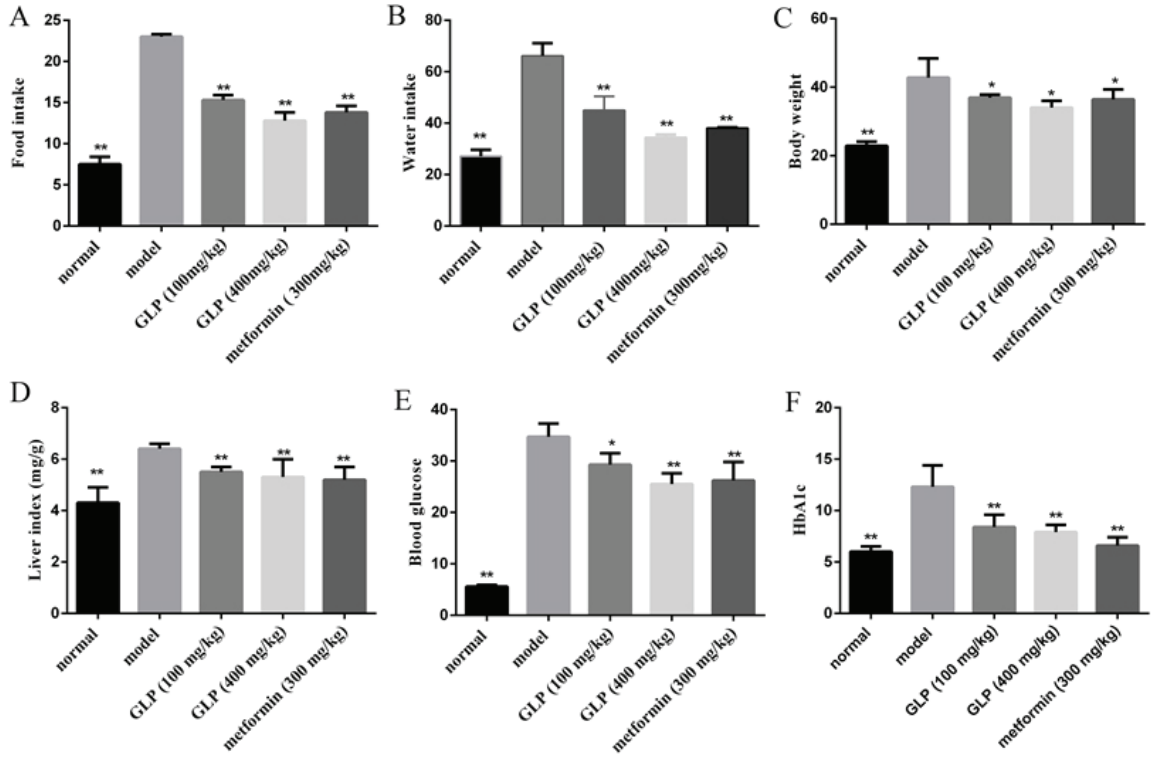

Figure 1. GLP alleviated dietary patterns, body weight, blood glucose, and HbA1c levels in *db/db* mice. (**A**) food intake, (**B**) water intake, (**C**) body weight, (**D**) liver index, (**E**) blood glucose, and (**F**) HbA1c levels of *db/db* mice after eight weeks of treatment. Data were expressed as mean ± standard deviation (SD). *indicates a significant difference compared to the model group ($^{*}p < 0.05$ and $^{**}p < 0.01$).

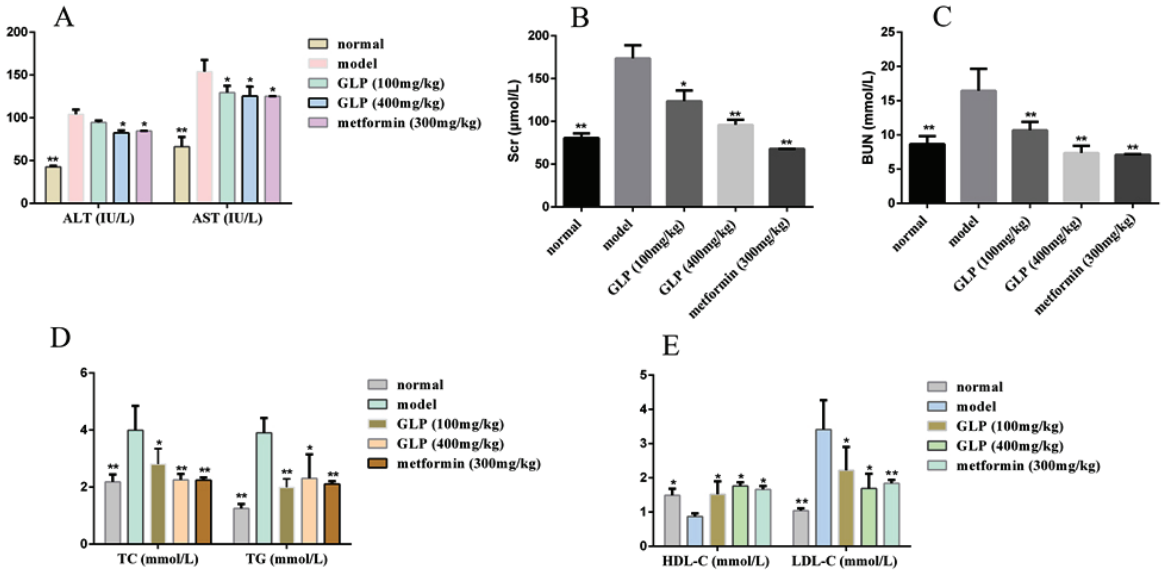

Figure 2. GLP alleviated hepatic lipid metabolism disorder in *db/db* mice. The levels of (**A**) ALT, AST, (B) Scr, (**C**) BUN, (D) TG, TC, (**E**) LDL-C, HDL-C in the serum of *db/db* mice with hepatic steatosis. Data were expressed as mean ± standard deviation (SD). *indicates a significant difference compared to the model group ($^{*}p < 0.05$ and $^{**}p+ < 0.01$).

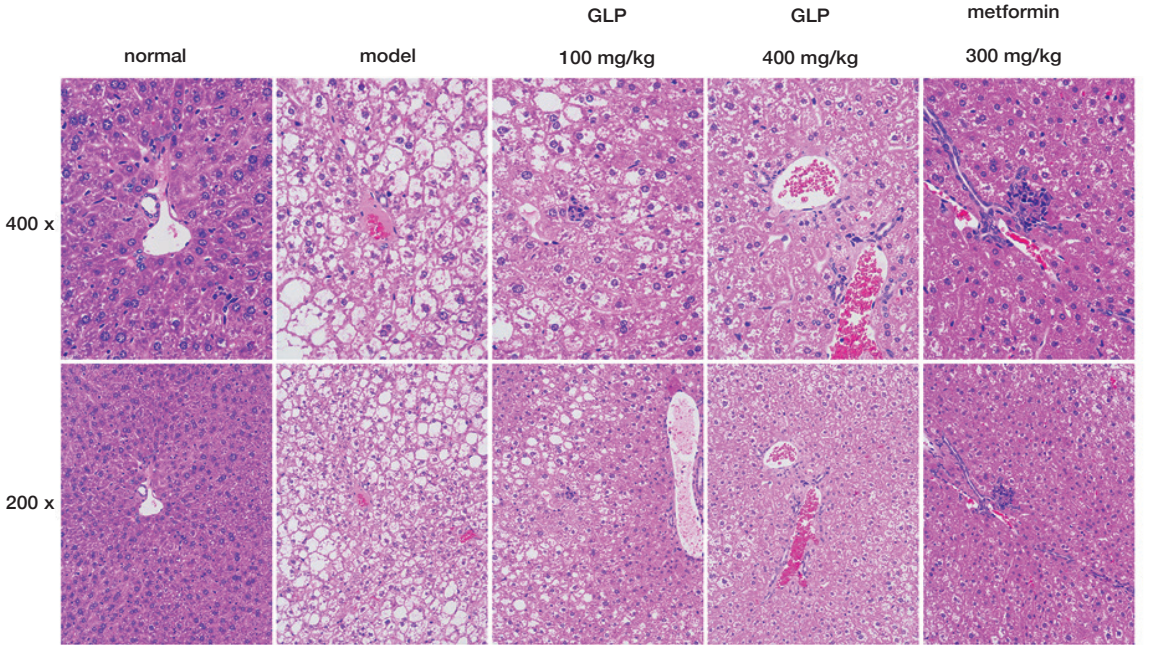

Figure 3. GLP alleviated the hepatic injury *db/db* mice. Paraffin-embedded liver tissues were stained with H&E (200× and 400×) to show the changes of pathogenesis in *db/db* mice.

SCD1, DGAT1, and PPAR-γ were highly expressed in the model mice, while, GLP and metformin treatment attenuated the expression of SREBP1c, FAS, SCD1, DGAT1, and PPAR-γ compared with the model mice (Figs. 5A-5B). Besides, the therapeutic effects of GLP (400 mg/kg) were superior to GLP (100 mg/kg). Conversely, the fatty acids β-oxidation and ketogenic metabolic regulators PPAR-α, Acox1, Cpt1, and Hmgcs2 were significantly down-regulated in model mice, while, GLP and metformin treatment were significantly increased the mRNA expression of these genes compared with the model mice (Fig. 5C). Additionally, GLP and metformin treatment increased the protein expression levels of phosphorylated protein AMPK and PPAR-α in model mice (Figs.

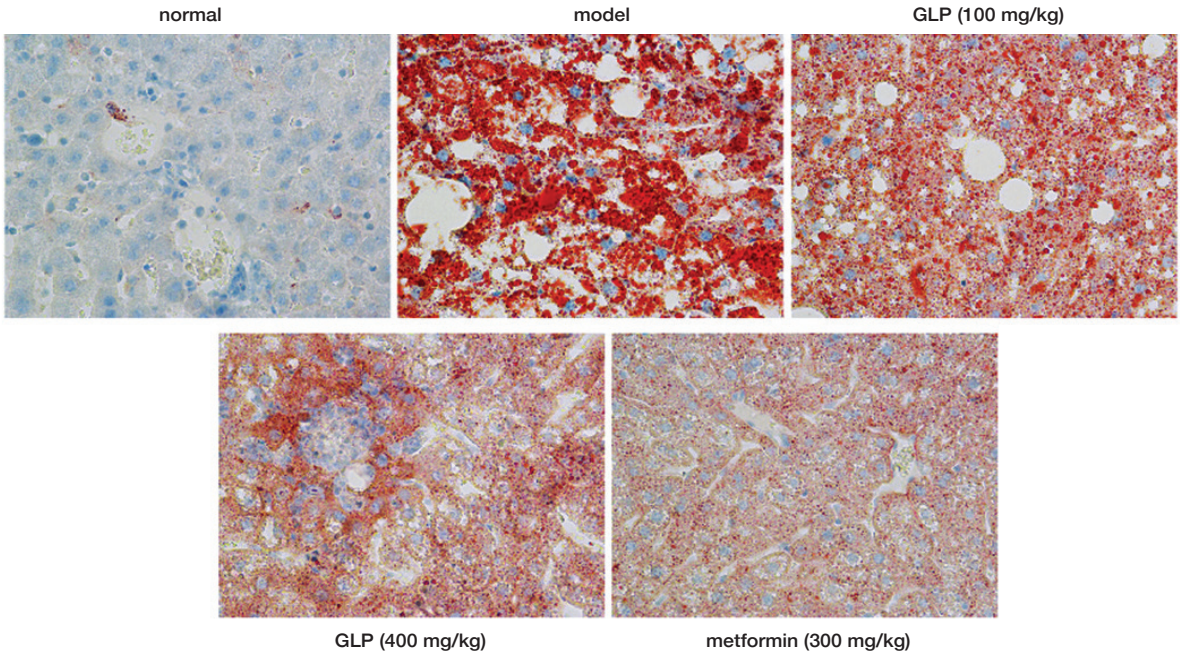

Figure 4. GLP alleviated the accumulation of hepatic lipid droplets in *db/db* mice. Paraffin-embedded liver tissues were stained with Oil Red O (400×) to demonstrate the accumulation of hepatic lipid droplets in *db/db* mice.

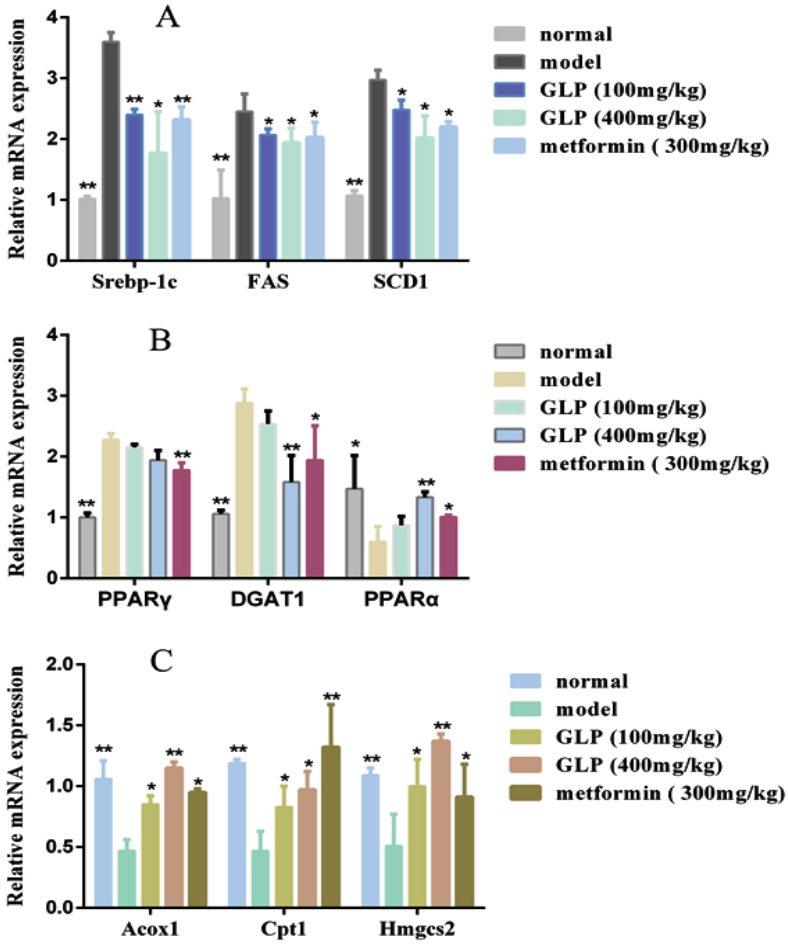

Figure 5. Effect of GLP on the mRNA expression levels of lipid metabolism-related genes in liver tissues of *db/db* mice. GLP was significantly decreased the mRNA expression levels of (**A**) SREBP1c, FAS, SCD1, (**B**) PPAR-γ, DGAT1, PPAR-α, and also increased the (**C**) Acox1, Cpt1, and Hmges2 levels in liver tissues of *db/db* mice with hepatic steatosis. Data were expressed as mean ± standard deviation (SD). *indicates a significant difference compared to the model group ($^{*}p$ <0.05 and $^{**}p$ < 0.01).

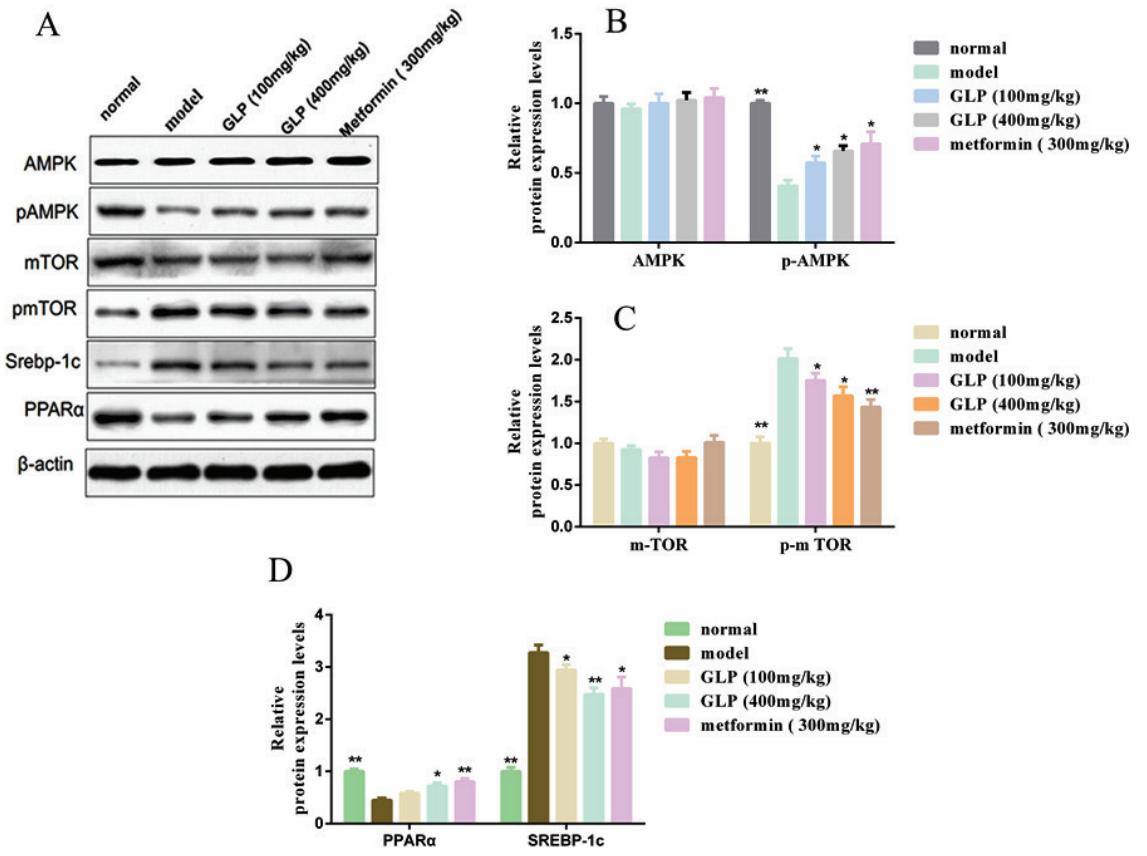

Figure 6. Effect of GLP on the protein expression levels of the AMPK pathway of liver tissues in *db/db* mice. (**A**) Representative protein bands of AMPK, pAMPK, mTOR, pmTOR, Srebp-1c, PPARα, and β-actin. Statistical analysis of (**B**) AMPK, pAMPK, (**C**) mTOR, pmTOR, (D) Srebp-1c, PPAR-α in liver tissues of *db/db* mice with hepatic steatosis. Data were expressed as mean ± standard deviation (SD). *indicates a significant difference compared to the model group (*p <0.05 and **p < 0.01).

6A, 6C), whilst, the protein expression levels of phosphorylated mTOR and SREBP1c were decreased after GLP and metformin treatment (Figs. 6B, 6C).

DISCUSSION

In this study, we found that GLP significantly balanced the metabolic disorder of *db/db* mice with hepatic steatosis. GLP not only promoted fatty acid oxidation and ketogenic metabolism but also protected against lipotoxicity that induced hepatocyte injury. As the evidence showed that the decrease of biochemical parameters of glucose and lipid metabolism, along with increasing the secretion of HDL-C, our results were consistent with the activity of degraded polysaccharides (GLP_{UD}) from *Ganoderma lucidum* and mexican *G. lucidum* in anti-hypolipidemic [19,20]. In addition, the mechanism of fatty acid, triglyceride, and cholesterol metabolism was closely associated with sterol regulatory elements and lipid metabolic enzymes of lipid synthesis and lipolysis [21-23]. Correspondingly, we also found that GLP significantly attenuated the expression levels of SREBP1c that was involved in the de novo lipogenesis. Besides, Ahmed *et al.* [24] found that the de novo synthesis of fatty acids activity was suppressed through down-regulating the expression of FAS, and Jiang *et al.* [25] reported that the activity of synthesizing monounsaturated fatty acids was also inhibited through down-regulating mRNA expression level of SCD1. In addition, DGAT1 contributed to TG synthesis and promoted the activity of PPAR-γ in facilitating lipid esterification into pre-formed fatty acids [26-28]. In the present study, we have found that GLP and metformin enhanced the fatty acid ω-oxidation, peroxisomal, and mitochondrial β-oxidation activities through up-regulating the expression of PPAR-α and ACOX1. Furthermore, the levels of Cpt1 that activities of converting fatty acyl-CoAs into fatty acyl carnitine derivatives [29] and HMGCS2 that regulate mitochondrial fatty

acid oxidation [30] were also markedly upregulated with GLP or metformin treatment in the mice model. Previous studies have shown that phosphorylated AMPK and mTOR were involved in lipid metabolism activities [31,32].

The energy sensor "AMP-activated protein kinase, AMPK" is an important regulator of hepatic lipid metabolism [33]. Studies had revealed that overexpression of AMPK promoted fatty acid oxidation [34,35]. Indeed, we have also found the expression of phosphorylated AMPK was inhibited in model mice, and GLP and metformin treatment increased expression of the phosphorylated AMPK. Conversely, the phosphorylated mTOR facilitated the lipid esterification through its downstream signal PPAR-γ [36]. Consistently, Our results indicate that GLP and metformin suppressed the activity of phosphorylated mTOR. Therefore, our results suggest that the GLP ameliorated hepatic metabolic disorder by facilitating the fatty acid oxidation metabolism and inhibiting the lipogenesis activity in *db/db* mice with hepatic steatosis were potentially through activating the AMPK pathway to inhibit its downstream signals, such as SREBP1c, FAS, SCD1, Cpt1, *etc.* However, the therapeutic effects in lipid metabolism of GLP had also been identified in adipocytes of fat tissues [37]. And, GLP was identified to enhance insulin sensitivity in the T2D mice model [38] and showed prebiotic effects in obesity [39]. Hence, further studies are needed to investigate whether the above-mentioned effects of GLP were involved in shaping the hepatic metabolism disorder in *db/db* mice.

CONCLUSION

GLP might significantly improve glucose and hepatic lipid metabolism disorder by facilitating the fatty acid oxidation and inhibiting the lipogenesis activity via the AMPK signaling pathway in *db/db* mice with hepatic steatosis, which may be a promising therapeutic strategy of hepatic steatosis in diabetes and obesity.

Author contribution. Lanxiang He wrote the manuscript, Liqiang Ji, Jia Wu and Rong Yao analyzed the data, Minghua Xie, Yifang Chen and Ren Nu do the experiments, Lanxiang He and Haiyan Hua designed the study.

Data availability. The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

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