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Liposomal Silybin Improves Glucose and Lipid Metabolisms in Type 2 Diabetes Mellitus Complicated with Non-Alcoholic Fatty Liver Disease via AMPK/TGF-β1/Smad Signaling

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Improving hepatic glucose and lipid metabolisms is an important strategy to treat type 2 diabetes mellitus complicated with non-alcoholic fatty liver disease (T2DM-NAFLD). Silybin (SLB) has the potential hepatoprotection, while its oral bioavailability is poor. This study aims to investigate the functional role and mechanism of liposomal SLB in modulating glucose/lipid metabolism in T2DM-NAFLD. SLB was prepared by thin film dispersion method and characterized using dynamic light scattering, scanning electron microscope, high performance liquid chromatography and zeta potential analyzer. A rat model of T2DM-NAFLD was used to determine the role of liposomal SLB in regulating glycolipid metabolism and hepatic damage. Rat primary hepatocytes were used to demonstrate the hepatoprotection mechanism of liposomal SLB. The encapsulation efficiency was more than 80%, which showed the average particle size of 119.76 nm. Also, the average Zeta potential was −4.76 mV. These liposomes were spherical. In rats with T2DM-NAFLD, liposomal SLB alleviated insulin resistance and lipid metabolism, thereby improving hepatic lipid accumulation, inflammation and fibrosis. Besides, liposomal SLB elevated AMPK phosphorylation, and decreased collagen I/III, α-smooth muscle actin (α-SMA), transforming growth factor-β1 (TGF-β1) and the phosphorylation of Smad2/3. In hepatocyte model, compound C partially reversed the effects of liposomal SLB on cell viability, glycolipid metabolism and AMPK/TGF-β1/Smad pathway activation. Liposomal SLB ameliorates hepatic glucose and lipid metabolisms in T2DM-NAFLD via activating AMPK/TGF-β1/Smad pathway, providing an efficient strategy for treating T2DM-NAFLD.

Keywords: diabetes mellitus; glycolipid metabolism; liposomes; non-alcoholic fatty liver disease; silybin
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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a metabolic hepatic damage affecting a quarter of the global population (Cotter and Rinella 2020). NAFLD increases the risk of cirrhosis and hepatocellular carcinoma when steatosis progresses to non-alcoholic steatohepatitis (Abdelmalek 2021). Insulin resistance in diabetes mellitus (DM) evokes metabolic alterations in lipotoxicity, fat accumulation, oxidative stress and mitochondrial dysfunction in the liver, which is the core event linking DM with NAFLD (Tanase et al. 2020). A 2-fold increase in the risk of NAFLD is observed in patients with T2DM (Ballestri et al. 2016; Tanase et al. 2020). There is still no pharmacological ther-

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apy for type 2 diabetes mellitus complicated with non-alcoholic fatty liver disease (T2DM-NAFLD) due to its complex pathogenesis (Manka et al. 2021). Inhibiting insulin resistance may provide the potential insight in the improvement of hepatic lipid metabolism and subsequent liver tissue impairment during T2DM-NAFLD.

Silybin (SLB), the major component of silymarin extracted from *Silybum marianum*, is a flavonolignan consisting of a taxifolin and a phenylpropanoid unit (Bijak 2017). SLB acts as the protective role of metabolic homeostasis. For example, SLB contributes to resisting oxidant injury via resulting in reactive oxygen species (ROS) reduction and glutathione (GSH) elevation (Rajnochova Svobodova et al. 2018). Importantly, SLB protects the liver from metabolic disorder, oxidative stress and endothelial dysfunction in NAFLD (Federico et al. 2019). SLB can mitigate high serum lipid and promote insulin-sensitizing (Bouderba et al. 2014), suggesting its possible capability to block DM-induced metabolic and histopathological changes in the liver. Also, SLB has determined to inhibit collagen deposition and extracellular matrix (ECM) formation during NAFLD (Anfuso et al. 2019). Thus, SLB has the potential of T2DM-NAFLD therapy. However, SLB is hardly soluble in water and poorly absorbed in the intestine, which leads to the low bioavailability during SLB administration (Pignatelli et al. 2019). Liposome loading may overcome these weaknesses due to their biocompatibility and biodegradability. Owing to the phospholipid bilayer structure, liposomes can load both hydrophilic and hydrophobic drugs to effectively delivery these drugs into the body (Abu Lila and Ishida 2017). To improve the bioavailability of SLB, a delivery carrier based on liposome was constructed to encapsulate SLB.

TGF-β1/Smad pathway plays the core pathogenic role in NAFLD. In the occurrence and development of NAFLD, the pathological condition such as DM results in inflammation infiltration in the liver that contributes to the production and release of TGF-β1 (Nair and Nath 2020). Then, TGF-β1 induces the activation in Smad family to trigger pro-fibrotic genes expressed in cells, which is the basic of fibroblast proliferation and ECM formation at the molecular level (Hu et al. 2018). Also, TGF-β1/Smad pathway regulates the expressions of β-oxidation-related and lipogenesis-related genes to mediate lipid accumulation in hepatocytes (Yang et al. 2014). Due to the two mechanism, TGF-β1/Smad pathway is the crucial link of lipid dysregulation and hepatic fibrosis in NAFLD. Importantly, its activation is monitored by adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) via protein-protein interaction. AMPKα1, a subunit of AMPK can block the promoter of TGF-β1, which inhibits Smad-dependent transcription and fibrosis (Mishra et al. 2008; Li et al. 2016). AMPK has been determined to the significant target of DM-induced NAFLD. AMPK activation triggers lipid metabolism reprogram that inhibits inflammation and fibrosis in the impaired liver (Garcia et al. 2019). There is the possible link between SLB and AMPK/TGF-β1/Smad pathway. In the rat model of T2DM-NAFLD, there were the significant changes in AMPK/TGF-β1/Smad pathway due to SLB treatment. It was observed that SLB enhanced AMPK phosphorylation in the liver of rats. Therefore, we wonder whether SLB protects the liver from impaired lipid metabolism and fiber deposition via activating AMPK to inhibit TGF-β1/Smad pathway.

This study aims to solve two problems of SLB. On the one hand, we wonder if liposome can promote the delivery of SLB into organism to overcome its weak bioavailability. On the other hand, we aim to explore the therapeutic role of SLB in DM-NAFLD. There is an intriguing hypothesis that SLB targets AMPK/TGF-β1/Smad pathway to improve lipid metabolism in the impaired hepatocytes, thereby contributing to overcoming insulin resistance and impaired hepatic function. This study will provide a novel treatment for T2DM-NAFLD.

Materials and Methods

Animals and cell culture

Male Sprague-Dawley rats (6 weeks old; Vital River, Beijing, China) weighted 120-130 g were housed in a room at 21 ± 1°C [mean ± standard deviation (SD)] and under 50% ± 5% humidity and freely accessed to diet and drinking in a cage with 12-h cycle of light and darkness. After 1 week of adaptive feeding, the rats were used for indicated experiments. The procedures involving animals was approved by the Animal Ethics Committee of The First Hospital of Hunan University of Chinese Medicine.

Rat hepatocytes were purchased from Gibco (Grand Island, NY, USA). In an incubator with 5% CO_2 at 37°C, these cells were cultured in William's E medium (Gibco) containing Collagen I (Thermo Fisher Scientific, Waltham, MA, USA), Primary Hepatocyte Maintenance Supplements (Thermo Fisher Scientific) and Reduced Growth Factor Basement Membrane Matrix (Thermo Fisher Scientific).

SLB-liposome construction and characterization

SLB, phospholipids and cholesterol (1:20:2, w/w/w) were dissolved in 100% ethanol solution for 20 min at 35°C. Through a 1 mL syringe, the mixed solution was added in water with stirring at 35°C. The solution was stirred for 30 min at 35°C to remove ethanal. To collect SLB-liposome, the solution was filtered with 0.45 μm and 0.22 μm microporous membranes, successively. The average particle size and the encapsulation were determined using dynamic light scattering and high-performance liquid chromatography (HPLC), respectively. Also, scanning electron microscope (SEM) was used to observe liposome morphology. The Zeta potential was measured by the Zeta potential analyzer.

Animal experiments

Thirty rats were divided into 5 groups including control, model, metformin (MET), free SLB and liposomal

SLB groups (n = 6). To construct the *in vivo* model of T2DM-NAFLD, rats were fed with high-fat diet (HFD) for 4 weeks after a 1-week adaption and then intraperitoneally injected with 36 mg/kg 1% streptozotocin (STZ) diluted in 0.1 M citric acid buffer. Three days after injection, T2DM was identified when the serum glucose level of rats was greater than or equal to 11.1 mmol/L. Rats in model, MET, free SLB and liposomal SLB were performed with HFD and STZ injection whereas control group was fed with normal diet and injected with equal volume of 0.1 M citric acid buffer. For treatment, rats in MET were orally administrated with 100 mg/kg MET once a day. Rats in free SLB were intragastrically with free SLB for 70 mg/kg/day. Also, rats in liposomal SLB group received SLB-liposome intragastrically in which the content of SLB was equivalent to 70 mg/kg/day. After 4 weeks of administration, rats were euthanized on the last day of the experiment, and the blood and liver samples of the rats were collected for subsequent experiments.

Oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance test (IPITT)

OGTT and IPITT were used to determine the role of liposomal SLB in insulin resistance. After a 24-h fasting, intragastric administration of glucose solution at 2 g/kg was performed in rats. IPITT was carried out 48 h after OGTT. Insulin at the dose of 2 units/kg was administered intraperitoneally in each rat. Thirty, 60, 90 and 120 min after insulin administration, blood sample in the caudal vein were collected for blood glucose measurement. Glucose concentration was determined using Glucose Assay Kit (A154-1-1, Jiancheng Bioengineering Institute, Nanjing, China).

Serum biochemical analysis

Serum sample was collected from each rat, and commercial kits (Jiancheng Bioengineering Institute) were used to measure the levels of high-density lipoprotein cholesterol (HDL-C; A112-1-1), total cholesterol (TC; A111-1-1), triglyceride (TG; A110-1-1), alanine aminotransferase (ALT; C009-2-1) and aspartate aminotransferase (AST; C010-2-1) based on the manufacturer's protocol.

Histopathology

To perform histopathological staining, liver tissues were collected and subjected to a series of procedures. Fresh tissues were initially fixed in 10% neutral formalin for 24 h, followed by dehydration using a series of ethanol concentrations ranging from 30% to 100%. The tissues were then incubated in xylene for 2 h, with the solution being replaced hourly. Subsequently, the tissues were embedded in paraffin and sectioned to a thickness of 3 μm. These sections were then stained using various dyes, including hematoxylin and eosin (H&E), Oil Red O, Sirius red, and Masson Dye. For H&E, the NAFLD activity score was the sum of the three features of steatosis, lobular inflammation, and hepatocellular, following the scoring system outlined in a previous study (Zhu et al. 2022). The severity of liver damage is positively correlated with the scores. ImageJ was used to quantify the Oil Red O, Sirius red and Masson's staining positive regions.

ELISA

Serum samples of rats were collected to determine the levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6. TNF-α (E-EL-R2856c, Elabscience, Wuhan, China), IL-1β (E-EL-R0012c, Elabscience) and IL-6 (E-EL-R0015c, Elabscience) ELISA kits were used to measure the three pro-inflammatory cytokines.

Western blot

The protein extracts from hepatocyte or tissue lysate using radioimmunoprecipitation (RIPA) buffer (Beyotime, Shanghai, China) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA, USA) after the concentration measure of BCA kit (Abcam, Cambridge, MA, USA). Protein blots were transferred from gels to membranes using a trans-blot system (Bio-Rad), followed by the blocking by 5% skim milk for 1 h at room temperature. Membranes were incubated with primary antibodies diluted in 1 × Tris-HCl buffer containing tween 20 overnight at 4°C. Subsequently, these membranes were incubated with secondary antibody (ab6721, 1:10,000, Abcam) for 1 h at 37°C. Blots on membranes were cultured in enhanced chemiluminescence (Abcam) for 5 min, protected from light. The visualization of blots was performed under an X-ray imaging system (Bio-Rad). GAPDH was used for the internal reference. Primary antibodies were listed as follows: phospho (p)-AMPK (AF3422, 1:1,000, Affinity Biosciences, Jiangsu, China), AMPK (AF6422, 1:1,000, Affinity Biosciences), TGF-β1 (AF1027, 1:1,000, Affinity Biosciences), Smad2 (AF6449, 1:500, Affinity Biosciences), Smad3 (AF6362, 1:1,000, Affinity Biosciences), p-Smad2 (AF3449, 1:500, Affinity Biosciences), p-Smad3 (AF8315, 1:2,000, Affinity Biosciences), Collagen I (AF7001, 1:500, Affinity Biosciences), Collagen III (AF5457, 1:1,000, Affinity Biosciences), α-SMA (AF1032, 1:800, Affinity Biosciences), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AF7021, 1:10,000, Affinity Biosciences).

Cell experiment

Hepatocytes were divided into 5 groups: control, model, free SLB, SLB-liposome and SLB-liposome + Compound C. To mimic hepatic injury in T2DM-NAFLD *in vitro*, cells were stimulated with 25 mM glucose plus 125 μM palmitic acid (PA) for 24 h. Cells in control group were cultured in normal medium. Cells in free SLB and SLB-liposome were treated with free SLB (60 μM) and SLB-liposome (60 μM) for 24 h, respectively. Cells in SLB-liposome + Compound C group were treated with Compound C (10 μM) for 2 h prior to SLB-liposome incubation.

Cell counting kit-8 (CCK-8)

CCK-8 kit (C0037, Beyotime) was used to detect the cell viability. Five thousand cells were cultured in 96-well plates at 37℃ and 5% CO_2 for 24 h. To detect the cell viability, 10 μL CCK-8 reagent was added in each well. After a 1-hour incubation with CCK-8 at 37℃, the absorbance of each well was read under the microplate reader.

Glucose consumption assay

Glucose Assay Kit (A154-1-1, Jiancheng Bioengineering Institute) was used to detect cellular glucose uptake in hepatocytes. Cells were seeded into 96-well plates (2×10^5 cells/well) and cultured to 80% confluence. After treatment, cells were incubated with 1 μM insulin for 20 min at 37℃. Cells lysates were used to measure the absorbance at 505 nm with a microplate.

Statistical analysis

Experiments in the present study were repeated 3 times to ensure the reliability of data. We used SPSS software to analyze the difference in data. Graphic data were processed in GraphPad 9.0. One way ANOVA followed by Tukey's multiple comparisons test was used for the comparison among the experiment groups. When $p < 0.05$, there was a significant difference among the groups at 95% confidence interval.

Results

Characterization of prepared SLB-liposome

SLB was loaded in the liposome to overcome the low bioavailability. Based on HPLC, the encapsulation efficiency was more than 80% (Fig. 1A), which showed the average particle size of 119.76 nm (Fig. 1B). Also, the average Zeta potential was −4.76 mV (Fig. 1C). These liposomes were spherical according to SEM (Fig. 1D).

Liposomal SLB alleviated insulin resistance in T2DM-NAFLD rats

The model of T2DM-NAFLD was established in rats to investigate the therapeutic role of liposomal SLB. MET was used for the positive control. The body weight of rats with T2DM-NAFLD was significant increased after liposomal SLB treatments (Fig. 2A). Meanwhile, liposomal SLB reduced the increased fasting blood glucose due to T2DM-NAFLD (Fig. 2B). Although T2DM-NAFLD resulted in insulin resistance in rats, liposomal SLB decreased sugar tolerance and increased the sensitivity to insulin (Fig. 2C, D). Importantly, the therapeutic role of liposomal SLB was more effective than that of SLB (Fig. 2A-D). Taken together, liposomal SLB could improve insulin resistance in T2DM-NAFLD.

Liposomal SLB improved lipid metabolism and liver function in T2DM-NAFLD rats

We wondered the effect of liposomal SLB on lipid metabolism and liver function during T2DM-NAFLD. In the serum of rats with DM-NAFLD, HDL-C (Fig. 3A) was decreased whereas TC (Fig. 3B) and TG (Fig. 3C) were increased. Liposomal SLB significantly improved the disorder in serum lipid (Fig. 3A-C). Also, liposomal SLB inhibited the protease activity of ALT (Fig. 3D) and AST (Fig. 3E) that were enhanced in the serum of rats with T2DM-NAFLD. The histopathological changes in liver were studied by H&E, Oil Red O, Sirius red and Masson staining. In the liver, T2DM-NAFLD caused lipid accumulation, fiber deposition, inflammation infiltration and the impairment of hepatic lobular structure (Fig. 3F). Liposomal SLB rescued these histopathological impairments resulted by T2DM-NAFLD (Fig. 3F). Based on ELISA, we measured the levels of pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α in the serum to show the anti-inflammatory role of liposomal SLB. Obviously, liposomal SLB reduced the T2DM-NAFLD-elevated cytokines in the serum (Fig. 3G). Notably, the protective role of liposomal SLB in lipid metabolism and liver function in T2DM-NAFLD rats was more significant than that of free SLB.

Liposomal SLB activated AMPK/TGF-β1/Smad signaling in T2DM-NAFLD rats

AMPK/TGF-β1/Smad pathway functions as the key role in energy homeostasis and tissue fibrosis. We wondered if liposomal SLB could affect the activation of AMPK/TGF-β1/Smad pathway to play the therapeutic role in T2DM-NAFLD. Liver tissues of rats were collected to determine the changes in AMPK/TGF-β1/Smad pathway via measuring protein expression. In addition to TGF-β1 downregulation, AMPK phosphorylation was enhanced and Smad 2/3 was inactivated in response to liposomal SLB treatment (Fig. 4A, B). Moreover, T2DM-NAFLD showed an increase of ECM proteins including Collagen I, Collagen III, and α-SMA that contributed to the collagen deposition during liver fibrosis, which was significantly reversed by liposomal SLB (Fig. 4A, B). Liposomal SLB played the more significant role in AMPK/TGF-β1/Smad pathway as compared to free SLB in T2DM-NAFLD rats.

Liposomal SLB improved glycolipid metabolism dysfunction via AMPK/TGF-β1/Smad pathway in rat hepatocytes

To investigate whether liposomal SLB regulated lipid metabolism via AMPK/TGF-1/Smad pathway, rat hepatocytes were stimulated by high glucose (HG) plus PA. Compound C was unitized to inhibit AMPK protein in hepatocytes. In HG-PA stimulated hepatocytes, the cell viability (Fig. 5A) and glucose consumption (Fig. 5B) were elevated by liposomal SLB, respectively, which was offset by AMPK inhibitor. Also, liposomal SLB modulated AMPK/TGF-β1/Smad pathway in hepatocytes. HG-PA administration inactivated AMPK and promoted the activation of Smad2 and Smad3 except the upregulated expressions of TGF-β1, Collagen I, Collagen III and α-SMA (Fig. 5C, D). Liposomal SLB enhanced AMPK phosphorylation

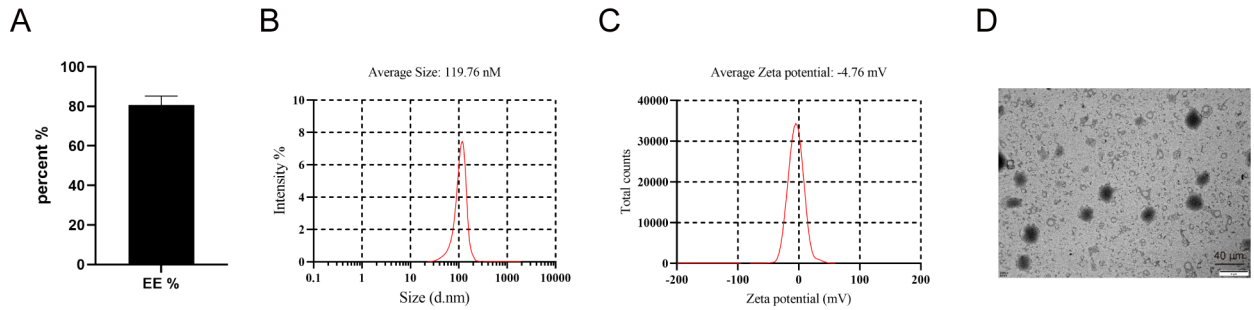

Fig. 1. Characterization of prepared Silybin (SLB)-liposome.
(A) Encapsulation efficacy (EE) by high performance liquid chromatography. (B) Average particle size by dynamic light scattering. (C) Average Zeta potential. (D) Liposome morphology by electron microscope.

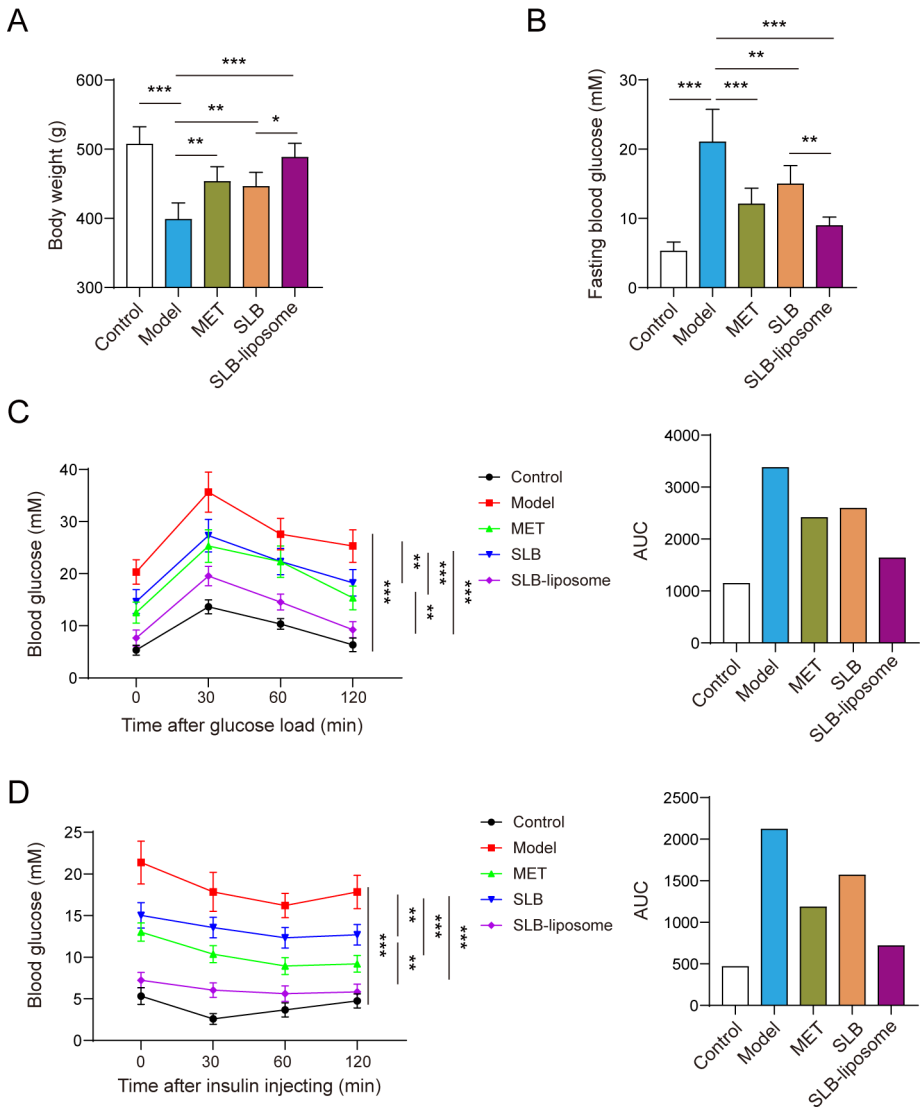

Fig. 2. Liposomal SLB alleviated insulin resistance in rats with type 2 diabetes mellitus complicated with non-alcoholic fatty liver disease (T2DM-NAFLD).
(A) Body weight. (B) Fasting blood glucose. (C) Oral glucose tolerance test (OGTT). (D) Insulin tolerance test (IPITT). Data are shown as mean ± SD. AUC, area under curve. *p < 0.05, **p < 0.01, ***p < 0.001.

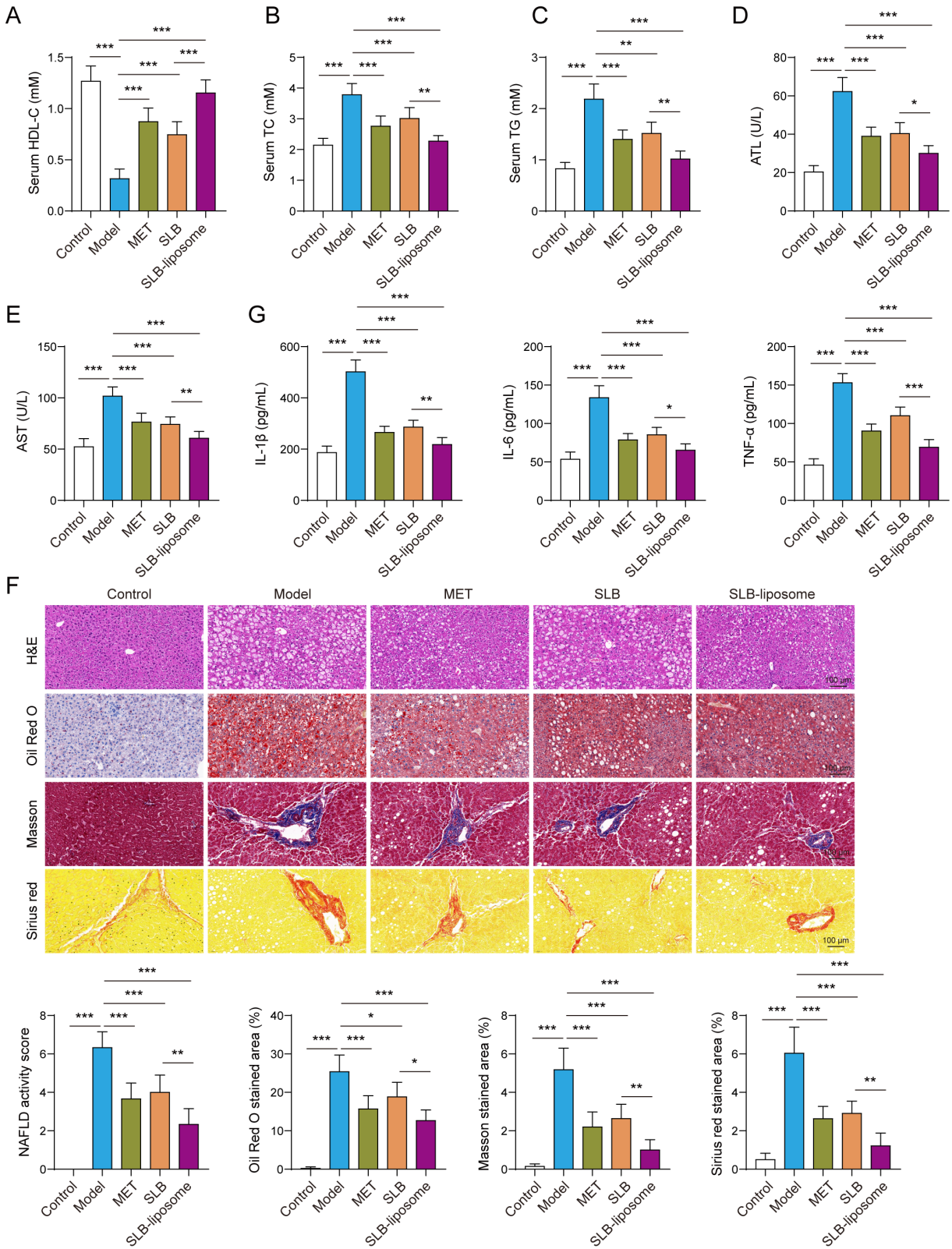

Fig. 3. Liposomal SLB improved lipid metabolism and liver function in T2DM-NAFLD rats.
(A-C) High density lipoprotein cholesterol (HDL-C; A), total cholesterol (TG; B) and triglycerides (TC; C) in the serum by commercial kits. (D, E) Alanine aminotransferase (ALT; D) and aspartate aminotransferase (AST; E) in the serum. (F) Histopathological changes of liver and NAFLD activity score based on histopathological staining. (G) TNF-α, IL-1β and IL-6 in the serum by ELISA. Data are shown as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

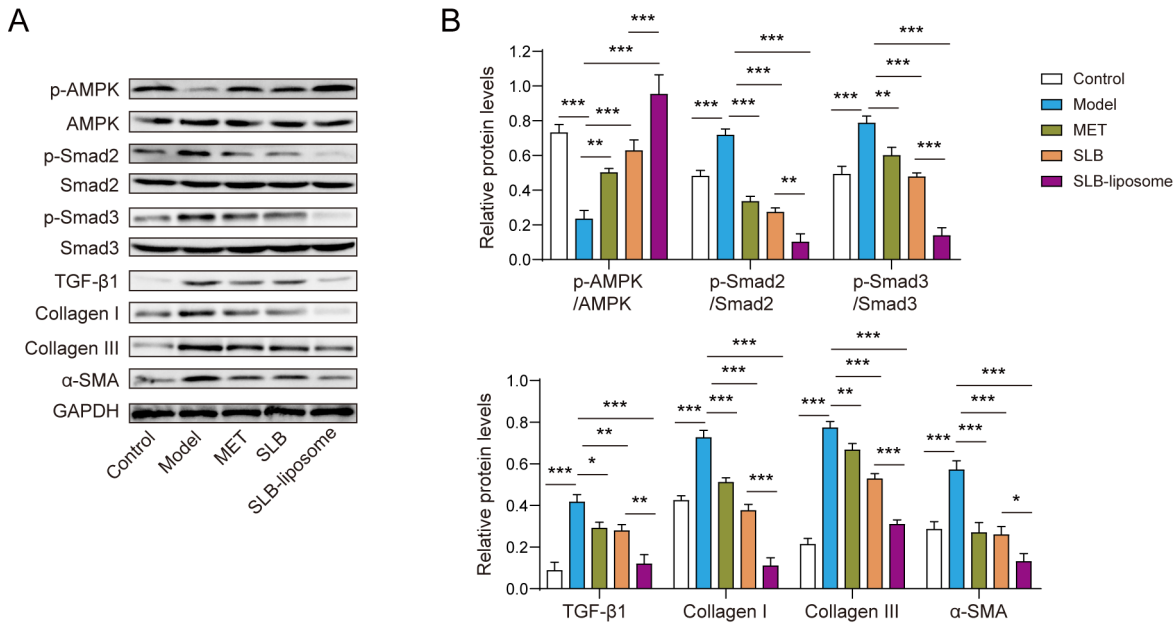

Fig. 4. Liposomal SLB activated AMPK/TGF-β1/Smad signaling in T2DM-NAFLD rats. (A) Protein blots of p-AMPK, AMPK, TGF-β1, Smad2/3, p- Smad2/3, Collagen I/III and α-SMA. (B) Histogram of protein expression levels. Data are shown as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

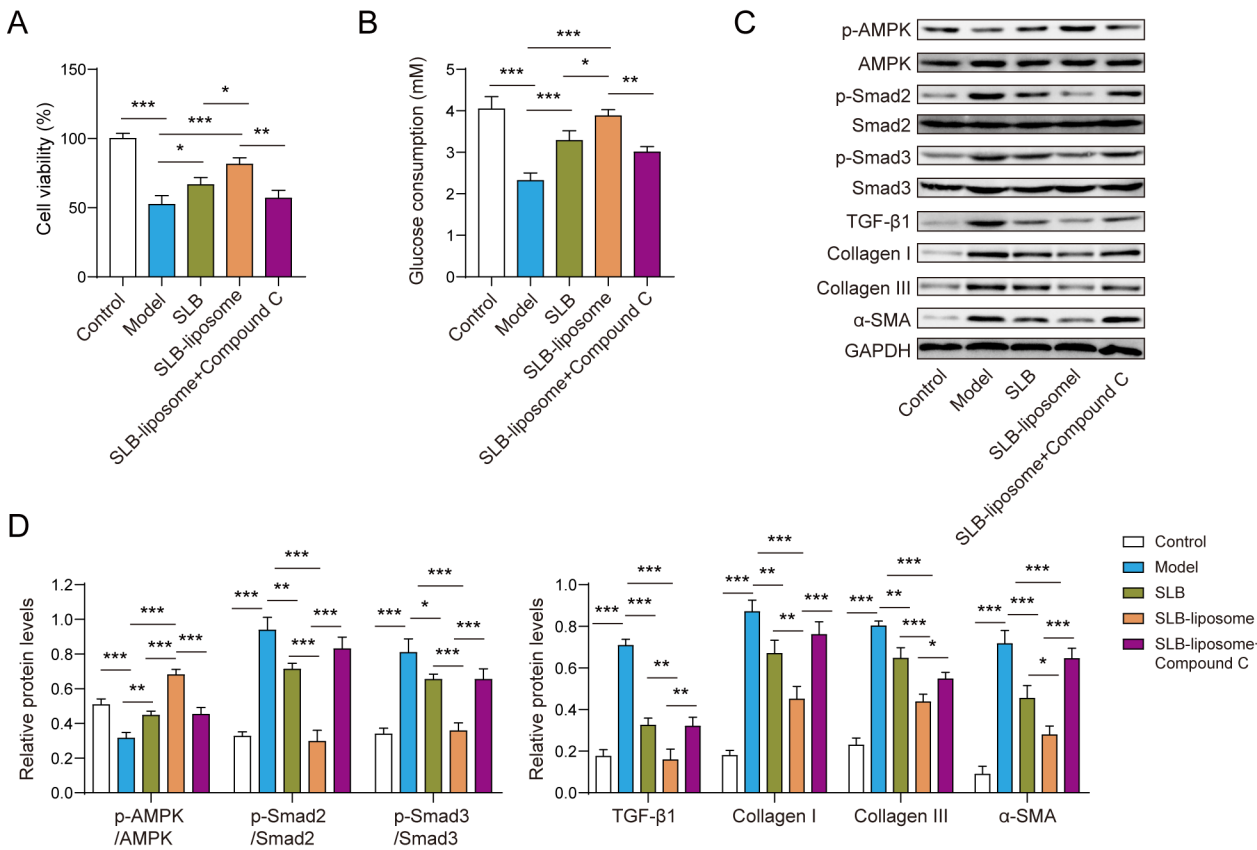

Fig. 5. Liposomal SLB improved glycolipid metabolism dysfunction via AMPK/TGF-β1/Smad pathway in rat hepatocytes. (A) Cell viability by CCK-8. (B) Glucose consumption in hepatocytes by Glucose Assay Kit. (C) Protein blots of p-AMPK, AMPK, TGF-β1, Smad2/3, p- Smad2/3, Collagen I/III and α-SMA. (D) Histogram of protein expression levels. To mimic hepatic injury in T2DM-NAFLD *in vitro*, cells were stimulated with 25 mM glucose plus 125 μM palmitic acid (PA) for 24 h. Data are shown as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

and declined phosphorylated Smad proteins (Fig. 5C, D). Further, the treatment decreased expressions of TGF-β1, Collagen I, Collagen III and α-SMA (Fig. 5C, D). Collectively, liposomal SLB may improve lipid and glucose metabolisms through AMPK/TGF-β1/Smad pathway.

Discussion

T2DM-NAFLD causes poor outcome and decreased quality of life. Although SLB can significantly protect hepatic tissue from DM, the low bioavailability limits the therapeutic efficiency of SLB. SLB-liposome was constructed to enhance the therapeutic role during DM-NAFLD. Firstly, SLB was loaded onto liposomes to increase the efficiency of drug delivery. Then, it was found that liposomal SLB had the more significant effects on insulin resistance, lipid metabolism and liver function compared to free SLB. Finally, liposomal SLB was found to activate AMPK and then inhibit TGF-β1/Smad pathway that is the core role in hepatocyte survival, glycolipid metabolism and hepatic fibrosis.

SLB is a potential hepatoprotective agent that can repress steatosis, oxidation and inflammation infiltration in NAFLD process at the pathophysiological level (Zhang et al. 2021). However, SLB, similar to other flavonolignan, has the poor bioavailability due to low solubility in water. Liposomal encapsulation contributes to increasing water-insoluble molecule bioavailability owing to uniform particle size, well-organized morphology and good sustained delivery (Gopi and Balakrishnan 2021). Gheybi et al. (2021) formed nanoliposomes to incorporate SLB. They found that liposomes could enhance the uptake of SLB in cancer cells, thereby leading to the decrease of self-replication and diffusion in cells. We constructed SLB-liposome with excellent stability, dispersion and uniformity. Liposomal SLB with high bioavailability has the potential of improving T2DM-NAFLD.

Liposomal SLB was found to significantly mitigate histological and physiological damages in T2DM-NAFLD. SLB can improve T2DM-NAFLD progression. On the one hand, SLB is widely known for its hepatoprotection such as anti-inflammation, anti-oxidant, anti-fibrotic and pro-regeneration. On other hands, SLB can modulate insulin resistance and hyperlipidemia to maintain metabolic homeostasis (Abenavoli et al. 2018). We established a rat model of T2DM-NAFLD characterized by insulin resistance, dysregulation in lipid metabolism and hepatic fibrosis. Obviously, liposomal SLB inhibits diabetes symptoms, particularly insulin resistance and high blood fat. Insulin resistance is the common T2DM-induced cause of NAFLD. Also, inflammation infiltration and hepatic fibrosis in the model were mitigated by liposomal SLB treatment. Insulin resistance increases free fatty acids in the liver and leads to the subsequent lipotoxicity such as inflammation, mitochondrial dysfunction and oxidative stress, which ultimately results in cell death and hepatic fibrosis (Marusic et al. 2021). Thus, liposomal SLB can inhibit insulin resistance to ameliorate inflammation, hepatocyte death and fibrosis progression in the model of T2DM-NAFLD. Importantly, the protection of liposomal SLB was better than that of free SLB, suggesting that liposome loading was necessary to enhance the therapeutic role of SLB in T2DM-NAFLD.

AMPK/TGF-β1/Smad pathway is the target of liposomal SLB. AMPK/TGF-β1/Smad pathway mediates metabolic dysregulation and fibrosis progression in T2DM-NAFLD. Metabolic disorder and tissue fibrosis in T2DM-NAFLD are closely related to AMPK/TGF-β1/Smad pathway. AMPK can inhibit TGF-β1/Smad pathway to modulate adipogenic gene and collagen expression in the liver, which develops lipid accumulation and hepatic fibrosis (Yang et al. 2014; Xu et al. 2016). Also, AMPK/TGF-β1/Smad pathway can affect insulin pathway (Budi et al. 2019). SLB has been determined to protect the liver from mitochondrial damage and oxidative stress via AMPK pathway during NAFLD progression (Salomone et al. 2017). Based on that, we further demonstrated that SLB could trigger AMPK activation to improve T2DM-NAFLD damage associated with TGF-β1/Smad pathway. There were significant changes in AMPK/TGF-β1/Smad pathway during SLB treatment, indicating that this pathway might mediate the metabolic action of SLB. SLB treatment could activate AMPK to decrease the phosphorylation of TGF-β1 and Smad in the model of T2DM-NAFLD. In hepatocytes undergoing HG-PA stimulation, SLB modulated the activation of AMPK/TGF-β1/Smad pathway to rescue hepatocytes. Thus, AMPK/TGF-β1/Smad pathway mediates the therapeutic mechanism of SLB in the modulation of glycolipid metabolism and hepatic fibrosis.

In general, liposome-loading system can enhance the bioavailability of SLB. Importantly, liposomal SLB protects the liver from lipotoxicity and subsequent fibrosis injury via AMPK/TGF-β1/Smad pathway in T2DM-NAFLD. This finding provides a potential therapeutic method of T2DM-NAFLD based on liposomal SLB. Also, the interaction between SLB and AMPK/TGF-β1/Smad pathway is first revealed. The mechanism of SLB provides the intriguing and potential targets to elevate the curative effect during T2DM-NAFLD.

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Author Contributions

Xinhua Xia guaranteed the integrity of the entire study. Jialuo Cai designed the study and literature research. Jialuo

Cai defined the intellectual content. Yilin Zhu and Xiaoping Li performed experiment. Guiming Deng and Yuanshan Han collected the data. Feiyun Yuan and Gangqiang Yi analyzed the data. Xinhua Xia wrote the main manuscript and prepared figures. All authors reviewed the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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