Sodium glucose cotransporter-2-inhibitor dapagliflozin improves nonalcoholic fatty liver disease by ameliorating dipeptidyl-peptidase-4 protein expression in diabetic mice

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Abstract

Introduction: Nonalcoholic fatty liver disease (NAFLD) is the leading cause of chronic liver disease worldwide. It can progress from simple steatosis to nonalcoholic steatohepatitis and may even develop into liver fibrosis, hepatocirrhosis, or hepatocellular carcinoma, but there is no effective treatment.
Material and methods: Wild-type (wt) and diabetic (db/db) mouse NAFLD-induced models were used to investigate the hepatoprotective effects and potential mechanisms of dapagliflozin (a new oral hypoglycaemic drug) on type 2 diabetes mellitus (T2DM) complicated with NAFLD, and to establish wt and db/db mouse NAFLD-induced and dapagliflozin treatment models.
Results: Dapagliflozin reduces blood glucose, glycosylated haemoglobin, blood lipids, and serum transaminase levels in db/db mice and improves T2DM-related liver injury accompanied by NAFLD; the mechanism may be related to the decrease in dipeptidyl-peptidase-4 (DPP4) protein expression and improvement in liver enzymes. Further mechanism-related studies by our team revealed that dapagliflozin can also downregulate the expression of DPP4 proteins in the liver and reduce serum soluble DPP4 enzyme levels, thereby improving the hepatic steatosis and insulin resistance of NAFLD.
Conclusion: Dapagliflozin may be an effective drug for the treatment of T2DM-induced NAFLD and NAFLD, providing a reliable laboratory basis and new treatment methods for the clinical treatment of NAFLD.

Key words: *nonalcoholic fatty liver disease; type 2 diabetes; sodium glucose cotransporter-2 inhibitor; dapagliflozin; dipeptidyl-peptidase 4*

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the main complication of type 2 diabetes mellitus (T2DM)-related liver disease and is increasingly attracting attention. More pronounced insulin resistance and hyperglycaemia can be observed in patients with T2DM combined with NAFLD, who also have a higher incidence rate of cardiovascular and cerebrovascular disease [1–3]. Insulin resistance is the main pathogenesis of T2DM, and it can also cause excessive lipid deposition in liver cells, causing oxidative stress and the lipid peroxidation of hepatocytes. Moreover, inflammatory and oxidative stress induced by lipid accumulation in the liver eventually accelerates hepatocyte necrosis and fibrosis [4]. NAFLD may become the main cause of end-stage liver disease, hepatocellular carcinoma, and liver transplantation [5], placing a heavy economic burden on individuals, families, and society. Currently, because of the complex pathogenesis of NAFLD, there is no effective treatment method, with existing therapies mainly relying on exercise and diet control. Therefore, new drugs or therapeutic targets for this disease are urgently required.

Sodium glucose cotransporter-2 (SGLT2) is one of the sodium glucose cotransporter families; it is mainly present in the renal proximal tubules of the kidney, and it mediates the reabsorption of glucose. The representative drug of SGLT2 inhibitors (SGLT2i) is dapagliflozin, a new class of hypoglycaemic drug, which has an action mechanism that reduces blood glucose by inhibiting the reabsorption of glucose by the proximal convoluted tubules of the kidney, thus allowing excess glucose to be excreted from urine. Studies have shown that SGLT2i not only reduces blood glucose but also has a protective effect on liver function [6]. However, the specific mechanism has yet to be determined. The pathogenesis of NAFLD is complex but is primarily focused on insulin resistance and inflammation, with changes in liver enzymes in blood being a key indicator for determining abnormalities in

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liver function. Recent studies have demonstrated that changes in liver enzymes in NAFLD are associated with dipeptidyl peptidase-4 (DPP4).

DPP4 is a type-II transmembrane protease with serine catalytic activity on the cell surface. It is secreted by hepatocytes and induces adipose tissue inflammation and insulin resistance. In this study, SGLT2i dapagliflozin was used to treat a diabetic (db/db) mouse NAFLD model to observe its effect on NAFLD and to further explore whether its mechanism is related to the expression of DPP4 protein.

Material and methods

Animal studies

Male db/db mice (average age: 6 weeks; body weight: 55 ± 10 g; n = 21) and male wild-type (wt) mice (average age: 6 weeks; body weight: 26 ± 1 g; n = 20) were selected for this study, which was conducted at the National Experimental Centre of Nanjing University. The mice were kept at room temperature (20°C ± 1°C) in a 12-h light and 12-h dark cycle and provided with routine mouse food (Beijing Oxerie Feed, Beijing, China) and clean water. Before the start of the experiment, the mice were fed adaptively for one week, fasted for 12 hours, and given purified drinking water to observe their natural condition. After adaptation, the mice were fed normally for 12 weeks, and at the end of week 12, one db/db mouse was randomly selected for liver pathology to determine the success of the NAFLD modelling. In weeks 4, 8, and 12, after formal feeding, body weight was measured and tail venous blood was taken from the mice to measure levels of fasting glucose, glycated haemoglobin, lipids, liver enzymes, and serum DPP4 enzymes.

After the NAFLD model had successfully been built, the wt mice were randomly divided into a wt group (untreated wild group, n = 10) and a wt + SGLT2i group (treated wild group, n = 10), and the NAFLD model was randomly divided into a db/db group (untreated wild group, n = 10), NAFLD group (n = 10) and a db/db + SGLT2i group (treated NAFLD group, n = 10). The treatment group was treated with dapagliflozin (10 mg/tablet) dissolved in normal saline, which was intragastrically administered (1 mg/kg) once a day for 8 weeks. Body weight, fasting blood glucose levels, glycosylated haemoglobin levels, blood lipid levels, liver function, serum DPP4 enzymes, and other biochemical indexes were measured at weeks 16 and 20. At the end of week 20, liver tissue was extracted for pathological analysis, immunohistochemistry, and a Western blot analysis of related proteins.

db/db mice — NAFLD model

db/db mice are spontaneous type 2 diabetic mice caused by a leptin receptor gene defect on chromosome 4, which was discovered by the Jackson Laboratory in 1966. Bulimia and obesity appear from 4 weeks of age, and obvious characteristics such as hyperglycaemia, hyperlipids, and insulin resistance appear with increasing age. The progression is very similar to that of people with type 2 diabetes.

db/db mice belong to the group of obese spontaneous diabetic mice. Similar to ob/ob animals, db/db mice are hyperphagic, obese, and insulin resistant, and they spontaneously develop liver steatosis under normal dietary conditions. After 12 weeks of normal diet feeding, the abdominal fat content of db/db mice was significantly increased, the liver was significantly increased, and the liver weight was significantly higher than that of wild-type mice. The pathological changes of liver were observed by HE staining, which indicated that the liver tissue of db/db mice showed obvious pathological changes at the age of 19 weeks, mainly manifested as hypertrophy of liver cells in band II and band III, and loose or empty cytoplasm, suggesting that the NAFLD model was successfully established. With regard to the liver phenotype, a clinically relevant mouse model of NAFLD should therefore display the typical histopathological features of non-alcoholic steatohepatitis (i.e. steatosis, inflammation, and ballooning) together with a certain degree of liver fibrosis. The study was conducted according to the guidelines of the Declaration of Helsinki, and it was approved by the Institutional Animal Protection and Use Committee of Beihua University. The mice were treated in strict accordance with the *Guidelines for the Protection and Use of Medical Laboratory Animals* (Ministry of Health of the People's Republic of China, 2011) and the *Guidelines for Ethical Standards for Laboratory Animals of Beihua University* (protocol code MRCProject: JJKH20200055KJ, approved on 1 January 2020).

Mouse body weight, blood collection, and detection of related indicators

The body weights of all 5 groups of mice were measured and their tail venous blood collected. The collected blood samples were then analysed in the Laboratory Animal Center of Beihua University using an automatic biochemical analyser (China Leadman Company, Beijing, China) and the corresponding commercial assay kits.

Histology analysis

The liver tissues of mice in each group were collected, fixed with 40 g/L of paraformaldehyde for 24 h, embedded in paraffin (Beyotime Institute of Biotechnology, Haimen, China), cut into slices 3–4 μm thick, and stained with haematoxylin (1 g of haematoxylin dissolved in 100 mL of distilled water) and eosin (Beyotime Institute, Nanjing, China).

Liver function measurement and biochemical analysis

Blood glucose, glycosylated haemoglobin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase (γ-GT), non-high-density lipoprotein (non-HDL), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were detected using an automatic biochemical analyser at the Affiliated Hospital of Beihua University (Jilin, China).

Exendin-4 experiment

The wt and db/db mice were fed in the prescribed environment for 16–18 weeks. After the success of the NAFLD model had been established, 5 nmol/kg of exendin-4 (intestinal insulin analogues) was intraperitoneally injected twice a day (at 9 a.m. and 5 p.m.) for 7 consecutive days. Fasting blood glucose levels and plasma DPP4 enzyme activity were measured at a specified time.

Immunohistochemical staining

The liver tissue was fixed with 40 g/L of paraformaldehyde for 24 h, embedded in paraffin, sliced into thicknesses of 3–4 μm, and sealed at room temperature for 30 min after dewaxing. Phosphate buffered saline (PBS) was preincubated with 2% bovine serum albumin for 30 min to block any nonspecific reactions, and the recombinant anti-DPP4 antibody (Shanghai Abcam, Shanghai, China) was incubated overnight at a dilution of 1 : 2000 (4°C). The sections were washed with PBS, and the bound antibodies were detected using an SP1 kit (Beijing Zhongshan Jinqiao Biotechnology, Beijing, China). The immunoreactive products were observed in 0.05% 3,3-diaminobenzidine and 0.03% H_2O_2. Slices were counterstained with haematoxylin, dehydrated, cleared, counted, and observed under an Olympus BX51 microscope (Olympus, Tokyo, Japan). During the control staining, sections were incubated with goat anti-mouse immunoglobulin (IgG; ZB-2305; Beijing Zhongshan Jinqiao Biotechnology).

Western blot analysis

The total protein in the liver tissue was extracted using a high-efficiency radioimmunoprecipitation lysis buffer. The protein concentration was determined through a colorimetric analysis using a spectrophotometer and ThermoFisher Science Entifi kit. Protein lysates (30–50 μg) were dissolved using 12% SDS-polyacrylamide gel electrophoresis (Beyotime Institute of Biotechnology) and then transferred to the immobilized transfer membrane (Milipol, Boston, MA, United States). The sample was then shaken with 5% skimmed milk powder in buffer solution at room temperature using a closed decolouring shaker for 1 h and then with anti-recombinant anti-DPP4 antibody (EPR18215, ab187048; Abcam, Shanghai) and mouse anti-β-actin monoclonal antibody (CAT. no.Ab6276 1: 1000; Abcam, Hong Kong). After being incubated overnight at 4°C, a goat anti-rabbit IgG horseradish peroxidase labelled secondary antibody (Beijing Zhongshan Jinqiao Biotechnology) was incubated at a dilution of 1: 10,000 for 1.5 h at room temperature, and then a goat anti-mouse IgG horseradish peroxidase-labelled secondary antibody (Beijing Zhongshan Jinqiao Biotechnology) was incubated at room temperature for 1.5 h. The immunoreaction bands were developed using a BeyoECL PLus high-sensitivity enhanced chemiluminescence kit (Biyuntian Biotechnology, Shanghai, China). The representative bands were measured and analysed using a Tanon GIS gel imager (China Tanon Technology, Shanghai,China). Protein levels were normalized to β-actin, and the ratios were expressed as the mean ± standard error (SEM) of 3 independent experiments.

Statistical analysis

The results were expressed as SEM or single value and median. Each experiment was conducted at least 3 times. Comparison of data and histopathological scores: Student's t-test was used to measure the difference between the 2 groups, and the Kruskal-Wallis H nonparametric test and multiple comparison test were used to evaluate the difference between multiple groups. Statistical significance was set at $p < 0.05$ and $p < 0.001$. GraphPad Prism 9.2.0 (Redmond, Washington, United States) and SPSS 19.0 software (IBM, Armonk, NY, United States) were used for the statistical and data analysis.

Results

Dapagliflozin improves blood sugar, glycosylated haemoglobin, and dyslipidaemia levels in diabetic mice with nonalcoholic fatty liver disease

To study the internal protective mechanism of dapagliflozin on a T2DM-induced NAFLD liver, a db/db mouse NAFLD model was selected to form the basis of the experiment. In this study, our team studied the effects of dapagliflozin on body weight, blood glucose, and blood lipids. As illustrated in Figure 1A, the study data demonstrate that compared with db/db mice, dapagliflozin reduced the body weight of mice to a certain extent, although the difference was not statistically significant. Dapagliflozin is a new type of oral hypoglycaemic drug, and db/db mice treated with dapagliflozin exhibit improved blood glucose and glycated haemoglobin (Fig. 1B, C). Studies have demonstrated that excessive lipid deposition in hepatocytes and hepatocyte steatosis are the most important features of NAFLD. In the present study, serum non-HDL (TG, LDL-C, and TC) levels were higher in db/db mice with NAFLD, and dapagliflozin also signifi-
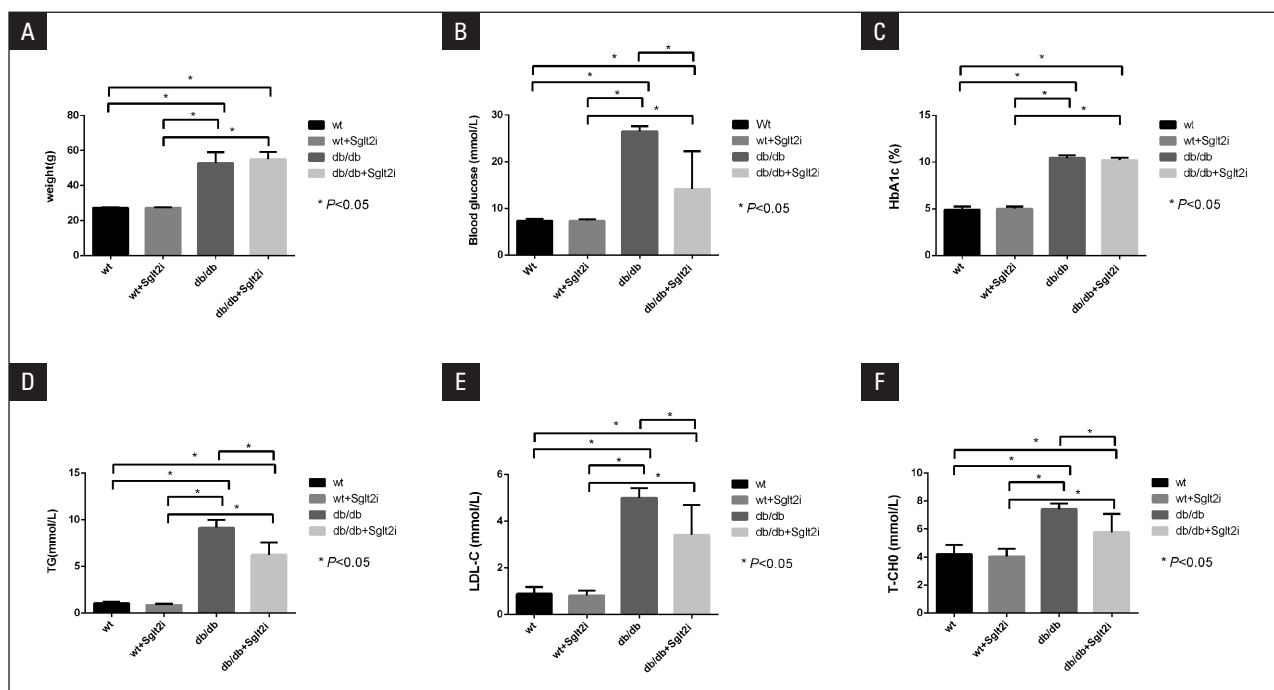
Figure 1. *Effect of dapagliflozin on blood glucose and blood lipids in diabetic mice.* **A.** *Body weight;* **B.** *Blood glucose;* **C.** *Glycosylated haemoglobin (HbA$_{1c}$), respectively, in different groups.* **D.** *Triglycerides (TG);* **E.** *Low-density lipoprotein cholesterol (LDL-C);* **F.** *Total cholesterol (TC) levels, respectively, in different groups. Each bar represents the mean ± standard error for groups of 10.* *p < 0.05, **p < 0.001 denotes statistically significant differences*

cantly improved serum TG, LDL-C, and TC. Consistent with the liver histopathological examination, liver TG (p < 0.001), TC (p = 0.002), and LDL-C (p = 0.001) levels were significantly increased in db/db mice (Fig. 1D–F), and these levels were significantly downregulated following dapagliflozin treatment, indicating that dapagliflozin improves hepatocyte lipid accumulation in db/db mice with NAFLD.

Dapagliflozin improves liver injury and lipid accumulation in diabetic mice with nonalcoholic fatty liver disease

The pathogenesis of T2DM with NAFLD involves multiple pathological processes, with the main pathological changes characterized by macrovesicular or predominantly macrovesicular hepatocellular steatosis. The decrease in liver weight and liver index because of dapagliflozin treatment also prevented the development of T2DM with liver enlargement (Fig. 2A–C). Changes in serum liver enzymes can reflect the severity of liver injury to some extent. Another protective effect of dapagliflozin on T2DM with NAFLD was that it reduced serum ALT, γ-GT, and AST levels (Fig. 2D–F), although the reduction in serum AST levels was not statistically significant.

In addition, as presented in Figure 2G, liver histopathology revealed that compared with wt mice, a large amount of predominantly macrovesicular hepatocellular steatosis and a very severe hepatocellular inflammatory response were visible in db/db mice. On the contrary, following 8 weeks of dapagliflozin treatment, hepatic steatosis in mice with NAFLD was significantly improved and the volume of fat vacuoles was much smaller. Therefore, these experimental results support the hypothesis that dapagliflozin can ameliorate T2DM with NAFLD-induced liver injury and excessive lipid accumulation in hepatocytes.

Dipeptidyl peptidase-4 involvement in dapagliflozin liver injury protection mechanism in diabetic mice with nonalcoholic fatty liver disease

Recent preliminary studies have revealed that soluble DPP4 (sDPP4) is secreted by hepatocytes, inducing adipose tissue inflammation and insulin resistance; sDPP4 is therefore considered an NAFLD biomarker [7]. Liver puncture biopsy is the gold standard for confirming the diagnosis of NAFLD. Combined with changes in liver enzymes, sDPP4 is considered to be the main early warning signal of NAFLD (liver injury). Recent studies have found that hepatic enzyme changes in NAFLD are associated with DPP4. After verifying the protective effects of dapagliflozin on blood glucose, dyslipidaemia, and hepatocyte steatosis in db/db mice

with NAFLD, our team further investigated the underlying mechanism of dapagliflozin on hepatoprotection complicated by T2DM-induced NAFLD. As presented in Figure 3B, serum DPP4 levels were substantially increased in db/db mice (p < 0.001) compared with serum liver enzyme levels (mainly serum ALT), which were also greatly increased (p < 0.001), which is consistent with the results illustrated in Figure 2D. Our team then further verified the changes in DPP4 protein levels in liver tissues using immunohistochemistry (Fig. 3A) and Western blot assay (Fig. 3C). After the dapagliflozin treatment, the expression levels of DPP4 proteins in liver tissue were significantly downregulated compared with those presented in Figure 3A and C, indicating that dapagliflozin improves hepatocyte DPP4 protein expression levels in db/db mice with NAFLD, thereby ameliorating hepatocyte steatosis and insulin resistance in NAFLD.

Discussion

Nonalcoholic fatty liver disease is an important chronic liver disease that is becoming increasingly common globally and is the main indication of end-stage liver disease and liver transplantation. In the next decade, the burden of NAFLD will further increase because the prevalence of metabolic-related liver diseases such as T2DM is increasing, and there are insufficient approved effective drugs to treat the progression of the disease. Therefore, our team selected db/db mice as the basis of our research. An increasing amount of evidence indicates that the development and progress of T2DM and NAFLD have the same aetiology, which includes hyperglycaemia, oxidative stress, cell inflammatory factors, and insulin resistance [8, 9]. Our research group demonstrated the protective effect of dapagliflozin against diabetes and diabetic complications by administering a dose of 1 mg/kg/d to db/db mice [10, 11]. SGLT2i plays a key role in glucose reabsorption in the proximal tubule of the kidney as a highly selective low-affinity inhibitor of the SGLT2 protein, causing 90% of the glucose reabsorbed in the kidney to be excreted from the body. Controlling blood sugar can improve high blood sugar, which causes toxic damage and inflammation in the liver. In the present study, concomitant with dapagliflozin treatment, blood glucose and glycosylated haemoglobin in db/db mice were improved (Fig. 2B, C) but not weight loss, which may be the result of significant differences within the db/db group and between dapagliflozin treatment groups.

Hepatic steatosis is another major histopathologic finding in T2DM-induced NAFLD mice [12, 13]. Our study revealed that dapagliflozin significantly reversed dyslipidaemia and hepatic steatosis in db/db mice.
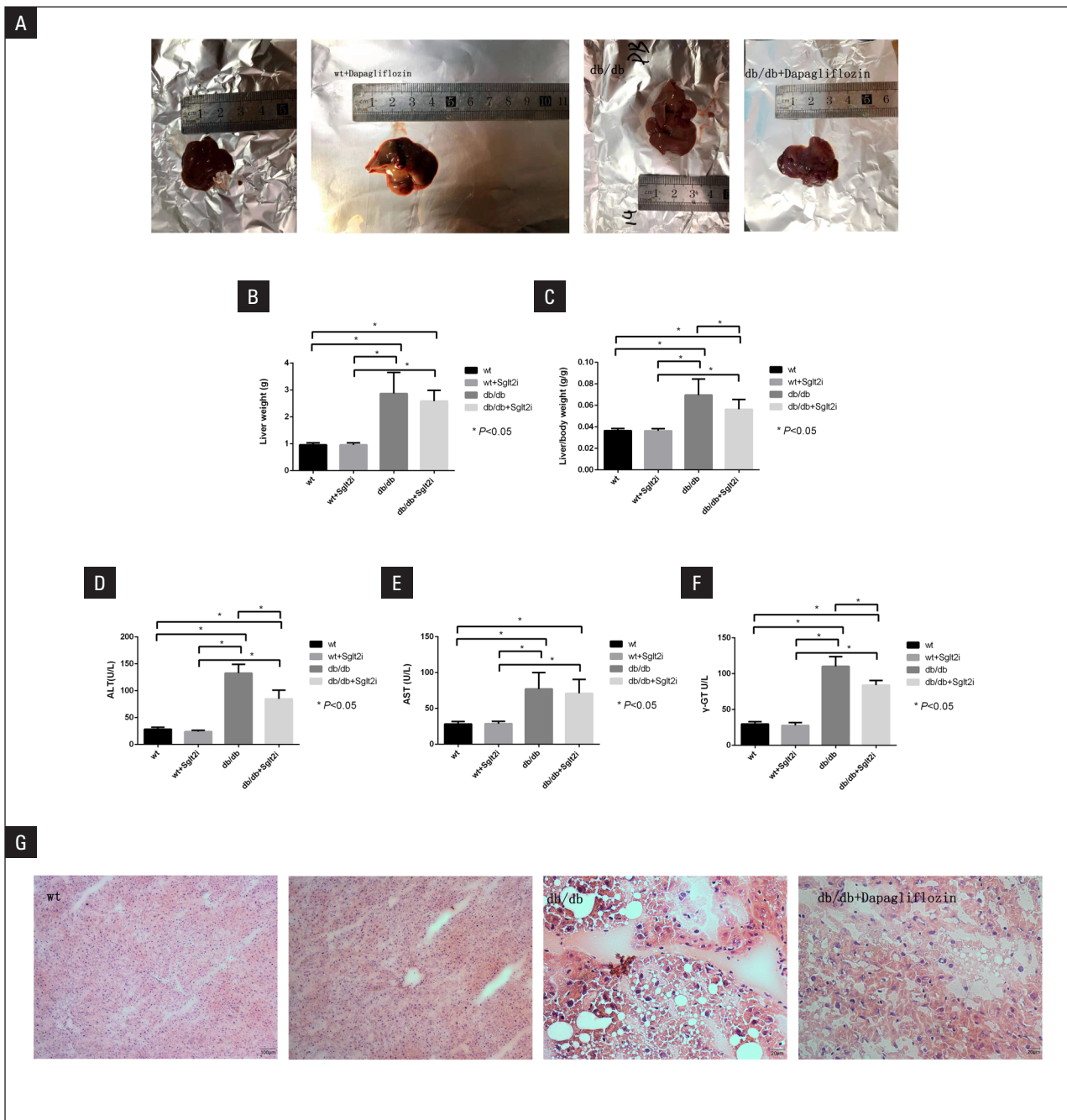
Figure 2. *Effect of dapagliflozin on liver histology, liver injury, and lipid accumulation in diabetic mice.* **A.** *Liver size in different groups;* **B.** *Liver weight;* **C.** *Liver index;* **D.** *alanine aminotransferase (ALT);* **E.** *Aspartate transaminase (AST);* **F.** *γ-glutamyl transpeptidase (γ-GT levels) in different groups;* **G.** *Haematoxylin-eosin staining in different groups. Each bar represents the mean ± standard error for groups of 10. *p < 0.05, **p < 0.001 denotes statistically significant differences*

Compared with the wt group, the blood lipids of the db/db mice with NAFLD treated with dapagliflozin improved (Fig. 2D–F; p < 0.05); in particular, TG, TC, and LDL-C levels changed significantly, but HDL-C levels did not change significantly, which is consistent with the pathological changes in liver tissue (Fig. 2A, G; p < 0.05). Thus, SGLT2-inhibitor dapagliflozin can alleviate the liver-related pathological changes of NAFLD, which suggests that dapagliflozin has a liver protective effect on db/db mice with NAFLD. Our study suggests that hyperglycaemia and an abnormal lipid metabolism are involved in the development of T2DM-induced NAFLD, and these pathological changes are restored with dapagliflozin treatment, which plays a role in liver protection.

Changes in serum liver enzymes are the main indicators of liver injury in NAFLD, and recent studies have found that these changes are associated with
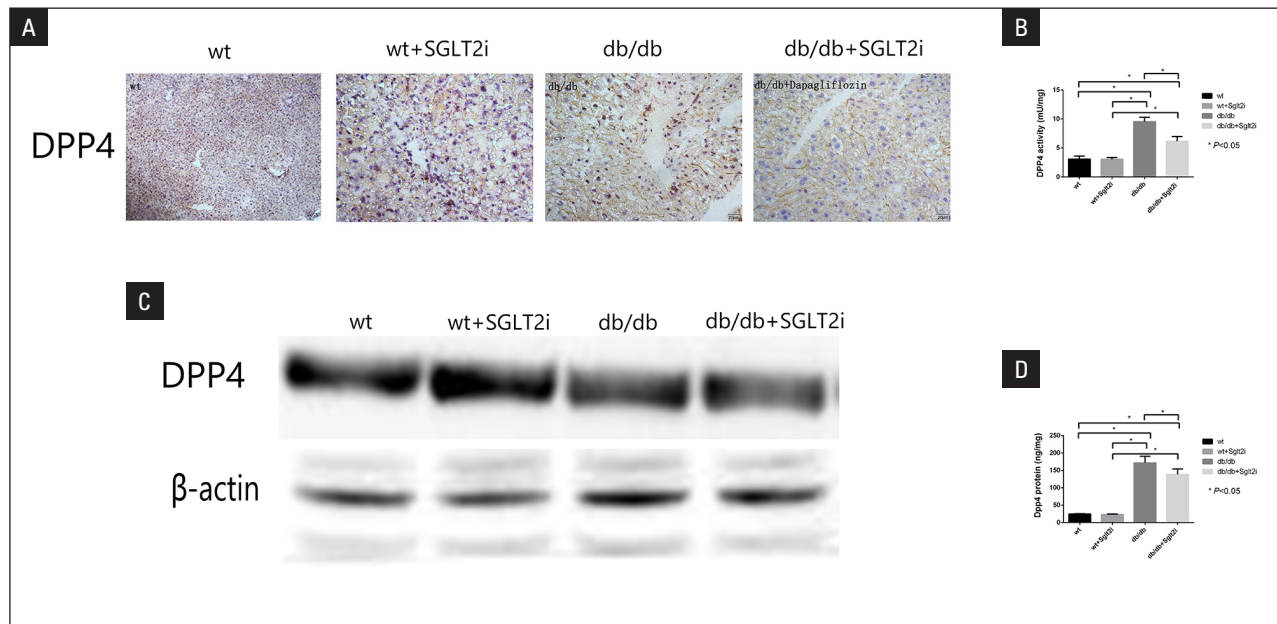
Figure 3. *Effect of dapagliflozin on the expression of dipeptidyl-peptidase-4 (DPP4) proteins in the liver of diabetic mice.* **A.** *Immunohistochemical analysis;* **B.** *levels of serum DPP4 in different groups;* **C.** *Western blot analysis;* **D.** *DPP4 protein expression. Each bar represents the mean ± standard error for groups of 10. *p < 0.05, **p < 0.001 denotes statistically significant differences*

DPP4. Our team further conducted molecular studies to examine the potential protective mechanism of dapagliflozin against changes in DPP4 (also known as CD26) expression and serum liver enzymes in a T2DM-induced NAFLD liver. CD26 is expressed on the surface of a variety of cells, including endothelial cells, epithelial cells, hepatocytes, and T lymphocytes [14]. Recent studies have shown that obesity can stimulate hepatocytes to produce and secrete soluble DPP4, possibly through caveolin-1, to promote adipose tissue inflammation and insulin resistance, suggesting a possible interaction of DPP4 in liver and adipose tissue [15]. Another study revealed that the upregulation of hepatic DPP4 expression was associated with NAFLD [16, 17]. In addition, transgenic mice overexpressing DPP4 in the liver exhibit increased hepatic and adipose tissue inflammation and insulin resistance [18]. In recent years, studies have demonstrated that serum sDPP4 is positively correlated with liver enzymes (ALT and gamma-glutamyl transferase) in patients with NAFLD [19, 20]; the higher the expression of DPP4 protein in hepatocytes, the more obvious NAFLD and nonalcoholic steatohepatitis are [21, 22]. This is consistent with our findings. In the present study, treating db/db mice with NAFLD with dapagliflozin reduced the expression of hepatic DPP4 proteins, alleviated serum sDPP4 enzyme levels, and improved the level of serum liver enzymes (in particular, serum ALT and γ-GT levels were restored, although AST levels were nonsignificant; Fig. 2D–F), thereby ameliorating

NAFLD-associated liver injury. Of course, the effect of SGLT2i on NAFLD is not caused by a single mechanism, and many specific mechanisms must be further explored. An in-depth study of SGLT2-inhibitor dapagliflozin could provide more laboratory evidence for the clinical treatment of T2DM with NAFLD.

Conclusions

Our preliminary study demonstrated the favourable effect of dapagliflozin in the treatment of T2DM-induced NAFLD. In particular, dapagliflozin significantly lowered blood sugar levels and improved liver damage. In summary, our experimental studies have demonstrated that dapagliflozin can significantly reduce hepatic steatosis, ameliorate liver damage (recovery of transaminase), improve blood glucose, dyslipidaemia, and liver lipid accumulation, and provide useful laboratory support for the clinical treatment of T2DM-induced NAFLD.

Competing interests
The authors declare that they have no competing interests.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and it was approved by the Institutional Animal Protection and Use Committee of Beihua University. The mice were treated in strict accordance with the "Guidelines for the Protection and Use of Medical Laboratory Animals" (Ministry of Health of the People's Republic of China, 2011) and the Guidelines for Ethical Standards for Laboratory Animals of Beihua University (protocol code MRCProject: JJKH20200055KJ, approved on 1 January 2020).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

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