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Original Article

Empagliflozin improves oxidative stress injury in the diabetic liver by regulating Nrf2 signaling

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Abstract: Objective: This study aimed to explore the therapeutic effect and molecular mechanism of Empagliflozin on diabetic hepatic oxidative stress injury. Methods: db/m mice were used as the control group, and db/db mice with spontaneous diabetes were randomly divided into a diabetes model group and an Empagliflozin group. The mice received continuous gavage treatment for 12 weeks. Serum was collected, and the liver tissues were fixed in 4% paraformaldehyde and frozen at -80°C. Results: Blood glucose, total cholesterol (TC), and triglycerides (TG) of diabetic mice were significantly increased, and the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also significantly elevated. After treatment with Empagliflozin, blood glucose, TC, and TG levels were significantly decreased, with ALT and AST also being reduced considerably. In the diabetic liver, the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were significantly decreased, and the level of malondialdehyde (MDA) was significantly increased. The expression of heme oxygenase-1 (HO-1), NAD(P)H quinone dehydrogenase 1 (NQO1), and nuclear factor erythroid 2-related factor 2 (Nrf2) were significantly decreased. Simultaneously, the expression of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) increased, and the deposition of collagen fibers was enhanced. After treatment with Empagliflozin, the liver cell structure of diabetic mice was partially restored. Conclusion: Empagliflozin can significantly improve the metabolic disorders of blood glucose and blood lipids in diabetes mellitus and play a therapeutic role in diabetic liver injury by reducing oxidative stress in the liver, inhibiting the inflammatory response, and improving liver fibrosis.

Keywords: Empagliflozin, diabetic liver injury, oxidative stress, inflammation, liver fibrosis

Introduction

The incidence of type 2 diabetes mellitus (T2DM) is increasing, and this disease is often accompanied by dysfunction of the liver, kidney, eye, nervous system, and cardiovascular system [1]. The liver is a crucial organ for glucose and lipid metabolism in the human body. When the liver fails to effectively clear the elevated blood sugar and excessive lipid accumulation, liver damage will occur [2, 3]. Liver damage caused by diabetes is known as diabetic liver injury (DLI), which is primarily manifested by abnormal liver function, non-alcoholic fatty liver disease (NAFLD), liver fibrosis, and even hepatocellular carcinoma. More than 70% of patients with type 2 diabetes have NAFLD [4, 5]. A prolonged hyperglycemic environment leads to lipid accumulation, liver steatosis, lipid peroxidation, and the release of pro-inflammatory factors, ultimately resulting in irreversible damage to liver tissue [6]. Oxidative stress (OS) induced by the formation of glycosylation products and reactive oxygen production triggered by hyperglycemia is an essential cause of diabetic liver injury [7]. In recent years, the incidence of DLI has increased significantly, and due to its complex pathogenesis, the therapeutic effects of drugs for this disease are limited. Therefore, it is of great significance to explore new therapeutic agents for the prevention and clinical treatment of DLI.

Sodium-glucose cotransporter 2 (SGLT2) is a high-capacity membrane transporter that facilitates glucose reabsorption in renal tubules,

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with an absorption rate of up to 97% [8]. In patients with diabetes, the increased expression of SGLT2 leads to enhanced reabsorption of glucose and sodium ions in the urine, resulting in elevated blood glucose levels [9]. Empagliflozin is an SGLT2 inhibitor that significantly reduces blood glucose concentration in diabetic patients by inhibiting glucose reabsorption and promoting glucose excretion [10]. Several clinical studies have shown that Empagliflozin can dramatically reduce the progression of chronic kidney disease [11], improve lipid metabolism, and decrease the occurrence of cardiovascular events [12, 13]. Primary research results have demonstrated that Empagliflozin can ameliorate diabetic nephropathy and diabetic cardiomyopathy by alleviating oxidative stress, inhibiting inflammation, and improving mitochondrial function [14]. However, it has not been reported whether Empagliflozin can improve diabetic liver injury (DLI) to any extent. In this study, db/db mice with spontaneous type 2 diabetes were treated with Empagliflozin intervention to observe the therapeutic effect of Empagliflozin on DLI and its underlying molecular mechanism.

Materials and methods

Treatment of animals and collection of specimens

Healthy male db/db and db/m mice, aged 6-8 weeks were purchased from Cavens Lab Animal Co., Ltd. in Changzhou, Jiangsu (animal license number: SCXK(Su) 2021-0013). After one week of acclimation, the db/m group mice were used as the control group. In contrast, the db/db mice were randomly divided into a diabetic group and an Empagliflozin group. There were 6 mice in each group. Empagliflozin (Shanghai Boehringer Ingelheim Pharmaceutical Co., LTD., National drug approval number J20171073) was dissolved in sterile normal saline to a working concentration of 10 mg/kg/day and administered orally once daily for 12 weeks. All mice were fed with 12 h light/dark cycle conditions, temperature (24±1)°C, humidity 50%-70%, and could eat and drink water freely. The control and diabetic groups received an equal volume of normal saline daily. After the last administration, the mice in each group were fasted overnight, and blood was collected from the orbital sinus, allowed to clot at room temperature for 2 hours, centrifuged at 3,000 rpm for 15 minutes, and the supernatant was aliquoted and stored at -80°C. The mice were then euthanized by cervical dislocation, and the abdominal cavity was quickly dissected. Liver tissue was harvested from the same region, fixed in 4% paraformaldehyde, and embedded in paraffin to prepare tissue sections. The remaining liver tissue was encapsulated and frozen at -80°C.

All animal experiments were approved by the Ethical Committee of Hebei University of Chinese Medicine and were conducted in accordance with all ethical standards (No. DWLL202203117).

Biochemical analysis

The changes in serum levels of blood glucose, alanine aminotransferase (ALT, solarbio, BC1550), aspartate aminotransferase (AST, solarbio, BC1560), triglycerides (TG, solarbio, BC0625), and total cholesterol (TC, solarbio, BC1985) were determined using an automated biochemical analyzer.

Liver tissue samples were weighed, and 0.9% saline was added to prepare a 10% tissue homogenate in an ice water bath. The homogenate was centrifuged at 3,000 rpm for 10 minutes, and the supernatant was collected to detect the changes in the levels of superoxide dismutase (SOD, A001-1), malondialdehyde (MDA, A003-1) and glutathione peroxidase (GSH-Px, A005) according to the instructions of the respective assay kits. SOD, MDA and GSH-Px kits were purchased from Nanjing Jiancheng Biotechnology Research Institute Co., Ltd.

H&E staining

Paraffin-embedded liver tissue sections were prepared by cutting the paraffin blocks at 60°C for 2 hours, followed by dewaxing in xylene and a graded alcohol series. The sections were then stained with Hematoxylin (solarbio, G1120) for 1-2 minutes and rinsed with tap water. Subsequently, they were briefly rinsed in 1% hydrochloric acid-ethanol, stained with Eosin solution (solarbio, G1100) for 1-2 minutes, and rinsed with tap water. After dehydration, the sections were mounted using a neutral mounting medium. The histopathological changes in the liver tissue sections were observed under a light microscope (Leica, DM750), and representative images were captured.

Table 1. The primers of target gene mRNA

Gene name	Forward Primer (5'-3')	Reverse primer (5'-3')
HO-1	GCCGAACACAAGAAGCTGGAGAG	GGCAAATCCTGCTACGAGCACT
NQO-1	CACTCTGGAGATGACACCTGAG	GTGTTCCTCTGTCAGCATCACC
β-actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT

performed, and the target gene expression levels were normalized to the housekeeping gene β-actin. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Masson staining

Paraffin-embedded liver tissue sections were prepared by cutting the blocks and then dewaxing in xylene and a graded alcohol series. The sections were then stained using a Masson's trichrome staining kit (solarbio, G1340), following the manufacturer's instructions. After dehydration, the sections were mounted with a neutral mounting medium. The extent of liver fibrosis in the tissue sections was evaluated under a light microscope, and representative images were captured.

Immunofluorescence (IF)

Paraffin-embedded tissue sections were first baked at 60°C for 1 hour. The sections were then dewaxed and subjected to antigen retrieval. After blocking with 5% bovine serum albumin (BSA), the tissues were incubated with a fluorescent-labeled rabbit anti-Nrf2 primary antibody (1:200 dilution, Proteintech) overnight at 4°C. The sections were then incubated with a fluorescent-labeled secondary antibody at 37°C for 15-30 minutes. Following PBS washes, the sections were mounted with a DAPI-containing medium and coverslipped. The immunofluorescent staining for Nrf2 was visualized and imaged using a Confocal Laser Scanning Microscope Systems (Leica, Wetzlar, Germany).

Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted from the liver tissue samples using TRIzol reagent. One microgram of the extracted RNA was then reverse-transcribed into cDNA using the M-MLV First Strand Kit (Invitrogen™ M-MLV Reverse Transcriptase Buffer, Product Code. 10512703). The mRNA expression levels of the target genes were quantified by quantitative PCR (qPCR) using gene-specific primer sets (as shown in **Table 1**) and SYBR Green qPCR SuperMix-UDG. The qPCR (Thermal Fisher, 7500) reactions were

Western blot

Equal amounts of liver tissue from each group were weighed and subjected to protein extraction using a commercial kit. The protein concentrations in the resulting supernatants were quantified.

Equal amounts of the total extracted proteins were then separated by agarose gel electrophoresis and transferred to PVDF membranes. The membranes were blocked with 5% skim milk and incubated overnight at 4°C with the following primary antibodies: mouse anti-HO-1 (1:500, Servicebio), rabbit anti-NQO1 (1:800, Servicebio), rabbit anti-Nrf2 (1:800, Proteintech), rabbit anti-IL-1β (1:1000, Servicebio), rabbit anti-TNF-α (1:250, Servicebio), and mouse anti-NF-κB p65 (1:500, Bioss). After washing, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hour. Protein expression was then detected using enhanced chemiluminescence (ECL). The protein band intensities were quantified using Image-Pro Plus software.

Statistical analysis

Data analyses were performed using GraphPad Prism 9 software. The results are presented as the mean ± Standard deviation (SD). Comparisons between the two groups were analyzed using Student's t-test, and comparisons among multiple groups were assessed by one-way analysis of variance (ANOVA). A *p*-value less than 0.05 was considered statistically significant.

Results

Empagliflozin improves blood glucose, blood lipids, and liver function

Compared to the control group, blood glucose levels were significantly elevated in the diabetic group ($P<0.01$, **Figure 1A**). However, treatment

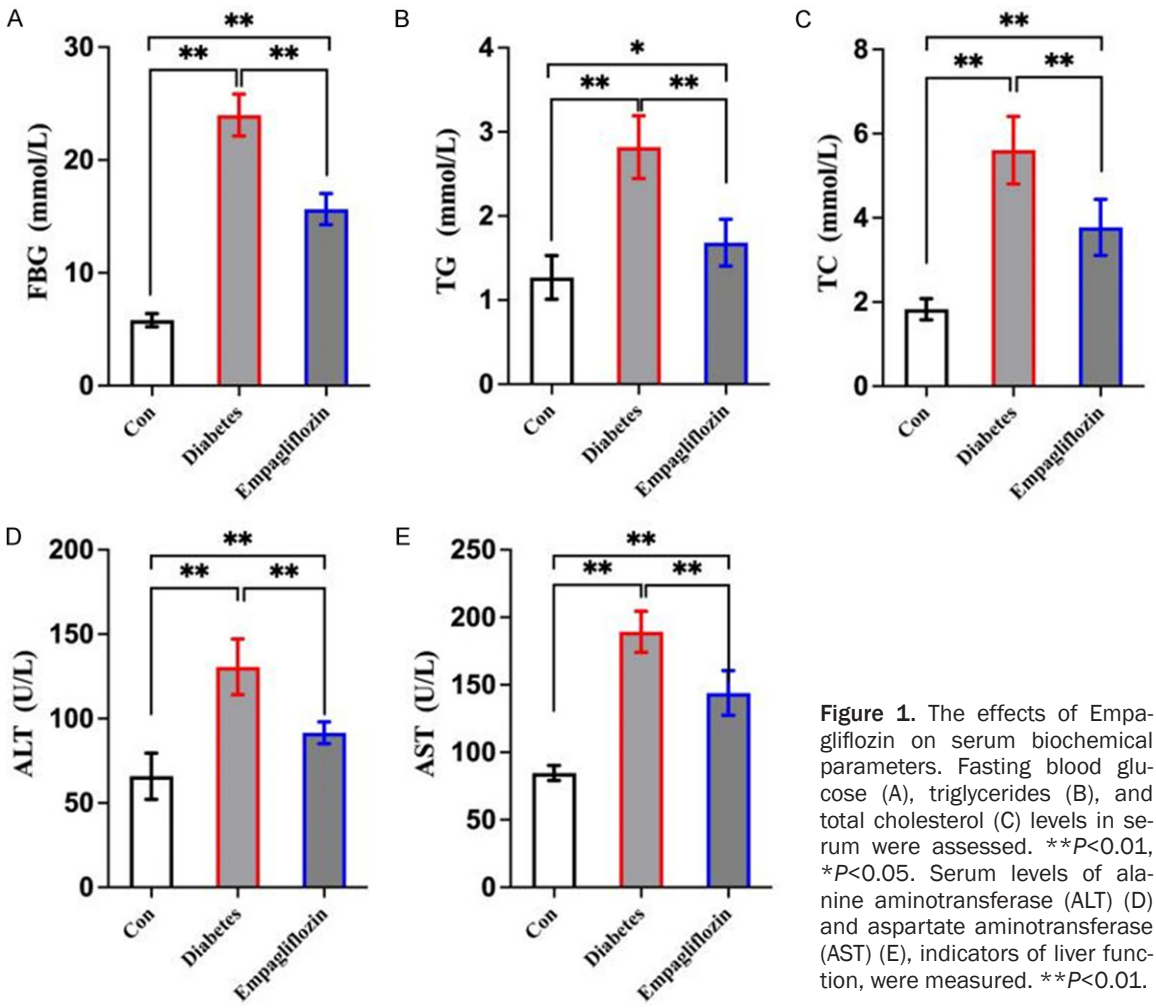

Figure 1. The effects of Empagliflozin on serum biochemical parameters. Fasting blood glucose (A), triglycerides (B), and total cholesterol (C) levels in serum were assessed. $^{**}P<0.01$, $^{*}P<0.05$. Serum levels of alanine aminotransferase (ALT) (D) and aspartate aminotransferase (AST) (E), indicators of liver function, were measured. $^{**}P<0.01$.

with empagliflozin significantly reduced blood glucose levels in the diabetic mice ($P<0.01$, **Figure 1A**). Relative to the control group, serum triglyceride (TG, $P<0.01$, **Figure 1B**) and total cholesterol (TC, $P<0.01$, **Figure 1C**) levels were markedly increased in the diabetic group. In contrast, empagliflozin treatment significantly decreased TG ($P<0.01$, **Figure 1B**) and TC ($P<0.01$, **Figure 1C**) levels in the diabetic mice. Serum alanine aminotransferase (ALT, $P<0.01$, **Figure 1D**) and aspartate aminotransferase (AST, $P<0.01$, **Figure 1E**) contents were also significantly elevated in the diabetic group compared to the control. Importantly, empagliflozin administration significantly reduced serum ALT ($P<0.01$, **Figure 1D**) and AST ($P<0.01$, **Figure 1E**) levels in the diabetic mice.

Empagliflozin improves pathological injury in liver tissue of diabetic mice

Hematoxylin and eosin (H&E) staining was performed to examine the liver histology (**Figure 2**). In the control group, the liver cells displayed a normal morphology, with the central vein as the central point and the hepatic plates arranged radially around it. In contrast, the hepatocytes in the diabetic group exhibited loose cytoplasm, water degeneration, irregular cell morphology, and disordered hepatic plate arrangement. Notably, empagliflozin treatment significantly ameliorated hepatocyte steatosis and partially restored the normal hepatic plate arrangement in the diabetic mice.

Empagliflozin ameliorates oxidative damage in the diabetic liver

Compared to the control group, the activities of the antioxidant enzymes superoxide dismutase (SOD, $P<0.01$, **Figure 3A**) and glutathione peroxidase (GSH-Px, $P<0.01$, **Figure 3B**) were significantly reduced in the liver homogenates of the diabetic group, while the level of the oxidative stress marker malondialdehyde (MDA) was markedly elevated ($P<0.01$, **Figure 3C**). In con-

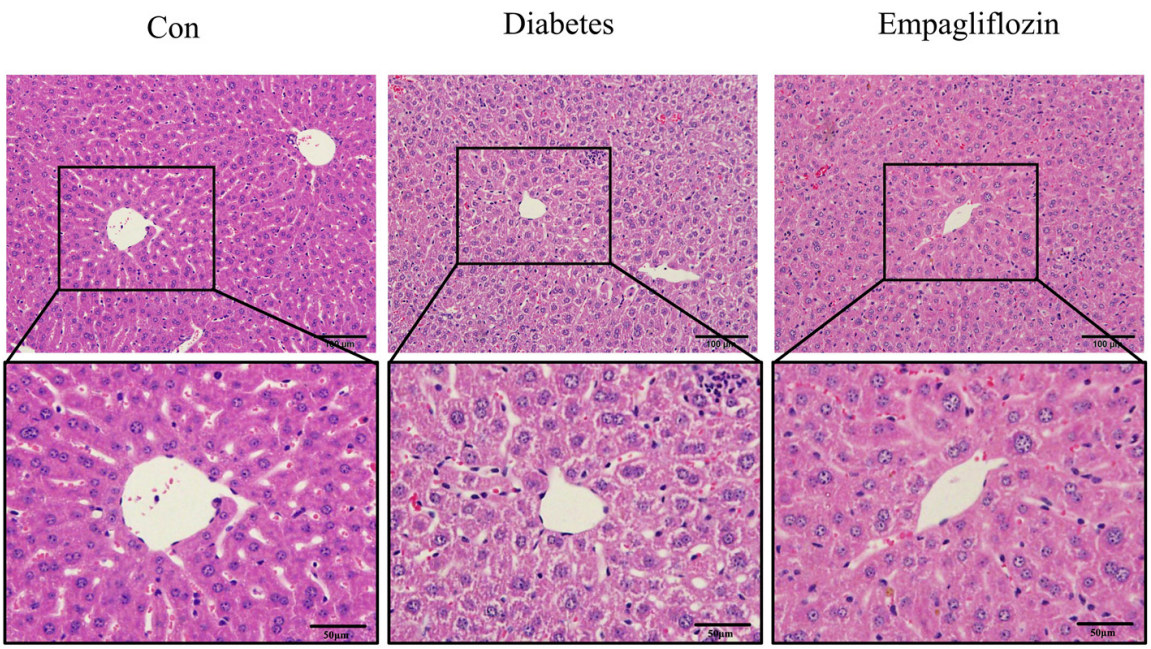

Figure 2. The impact of Empagliflozin on the pathological alterations associated with diabetic liver tissue injury.

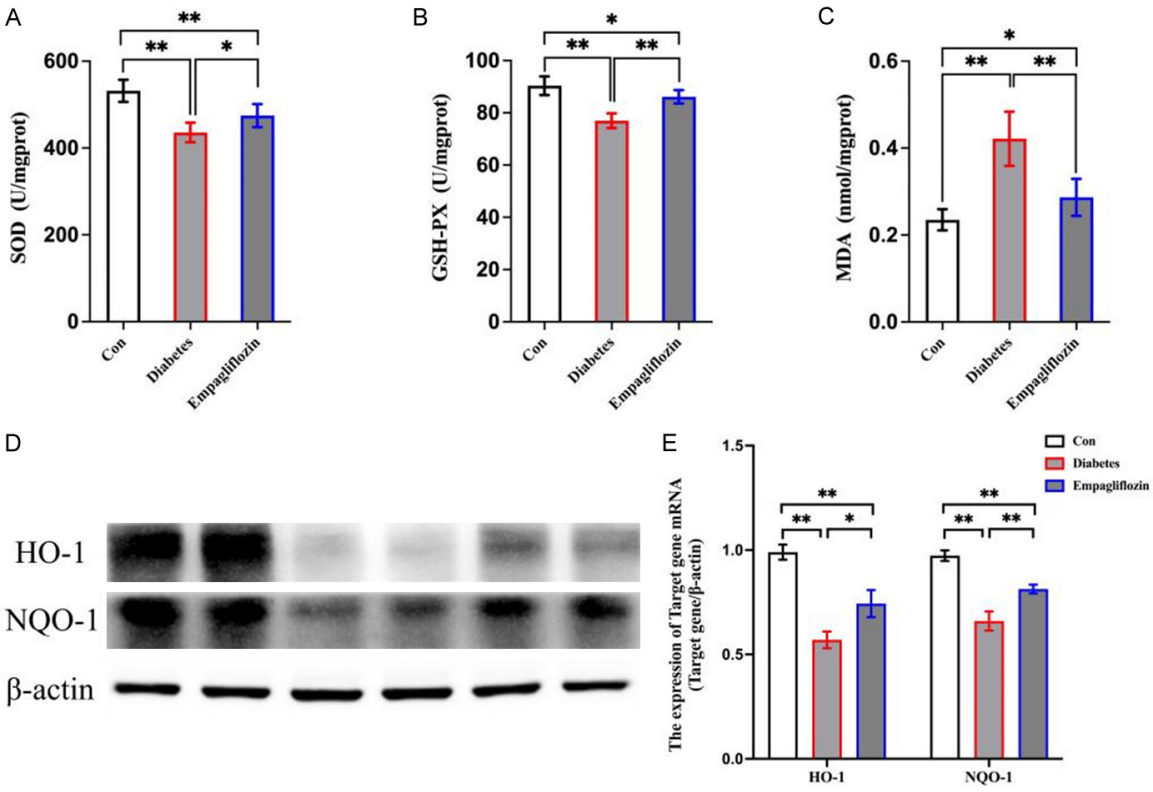

Figure 3. The ameliorative effects of Empagliflozin on oxidative stress-induced liver injury in diabetes. The levels of SOD (A), GSH-Px (B), and MDA (C) were measured in liver tissue homogenates (A-C). ***P*<0.01, **P*<0.05. Western blot analysis was conducted to evaluate the protein expressions of HO-1 and NQO-1 in liver tissues from each group (D). The HO-1 and NQO-1 mRNA expression levels in liver tissues from each group were assessed using qRT-PCR (E). ***P*<0.01, **P*<0.05.

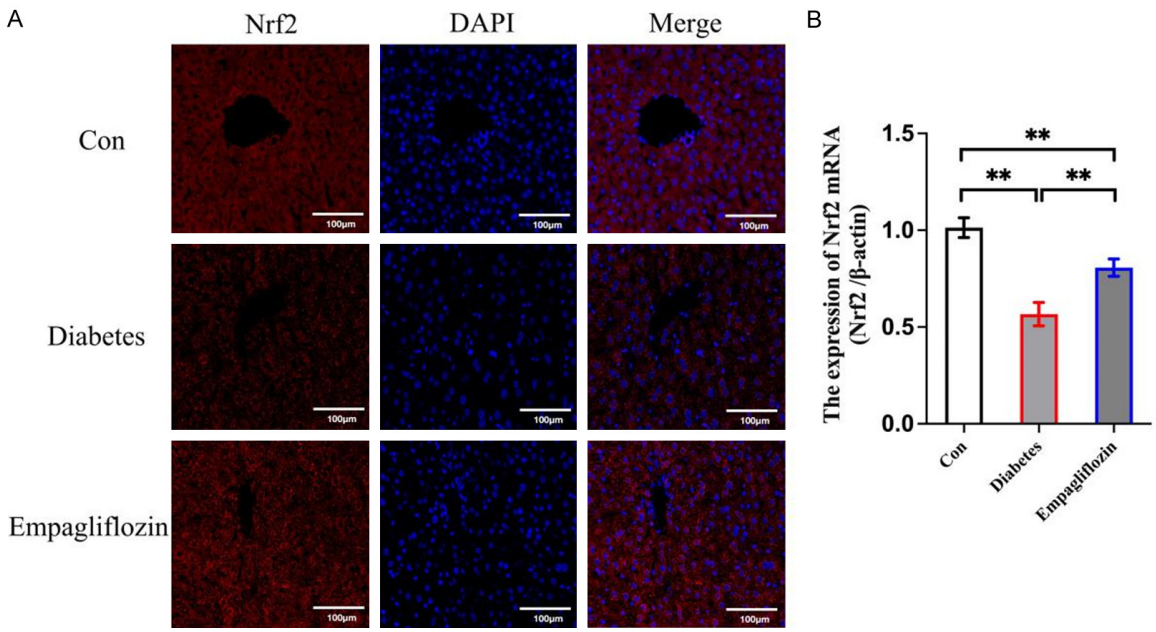

Figure 4. The effect of Empagliflozin on the Nrf2 signaling pathway. Immunofluorescence was employed to assess the expression of Nrf2 protein in liver tissues across all experimental groups. Red fluorescence indicates the presence of Nrf2 protein, while blue fluorescence, stained with DAPI, marks the nuclei (A). qRT-PCR detected the expressions of Nrf2 mRNA in each group liver tissue (B). $^{**}P<0.01$.

trast, empagliflozin treatment significantly increased SOD (P<0.01, **Figure 3A**) and GSH-Px (*P*<0.01, **Figure 3B**) activities and decreased MDA content (*P*<0.01, **Figure 3C**) in the diabetic liver.

Heme oxygenase-1 (HO-1) and NAD(P)H: quinone oxidoreductase 1 (NQO-1) are necessary antioxidant enzymes. Compared to the control group, the protein and mRNA expression levels of HO-1 and NQO-1 were decreased in the diabetic group. However, empagliflozin administration significantly upregulated the protein and mRNA expression of these antioxidant enzymes in the diabetic liver (**Figure 3D**, **3E**). These results indicate that empagliflozin ameliorates oxidative damage in the diabetic liver by enhancing the activities of antioxidant enzymes and increasing the expression of key antioxidant proteins.

Empagliflozin significantly enhances the expression of the Nrf2 protein

Compared with the control group, the expression of Nrf2 protein was significantly decreased in diabetic group. The expression of the Nrf2 protein with Empagliflozin treatment increased more than in the diabetic group (**Figure 4A**). The Nrf2 mRNA expression trend was consistent with the protein level (**Figure 4B**).

Empagliflozin inhibits inflammation in diabetic-induced liver injury (DIL)

The protein levels of IL-1β and TNF-α in liver tissues from each group were evaluated (**Figure 5A**). The results demonstrated that the expressions of TNF-α (*P*<0.01, **Figure 5B**) and IL-1β (*P*<0.01, **Figure 5C**) were significantly elevated in the diabetic group compared to the control group. Notably, treatment with empagliflozin resulted in a significant reduction in the expression of TNF-α (*P*<0.01, **Figure 5B**) and IL-1β (*P*<0.01, **Figure 5C**) in the liver tissues of diabetic mice, relative to the untreated diabetic group.

Empagliflozin reduces collagen fiber deposition associated with DIL

Masson staining revealed minimal collagen fiber deposition in the liver tissue of control mice. In contrast, significant collagen fiber accumulation was observed in the hepatic sinusoids surrounding the central vein and between hepatocytes in diabetic mice. Notably, the extent of collagen fiber deposition in the

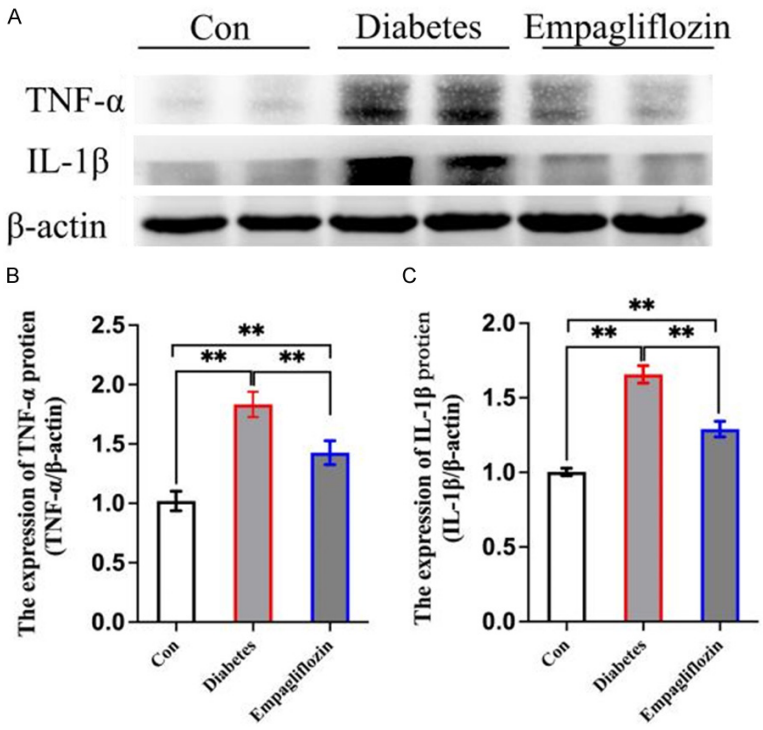

Figure 5. Empagliflozin inhibits inflammation in DIL. Western blot analysis was performed to assess the expression levels of TNF-α and IL-1β (A). TNF-α (B) and IL-1β (C) expressions were quantitatively analyzed using integrated density measurements. $^{**}P<0.01$.

liver of the Empagliflozin-treated group was significantly reduced compared to that observed in the diabetic group (**Figure 6**).

Discussion

The pathogenesis of diabetic liver injury remains incompletely understood. Increasing evidence suggests that the onset and progression of type 2 diabetes mellitus (T2DM)-induced non-alcoholic fatty liver disease (NAFLD) are associated with a complex interplay of multiple factors [15]. Recent studies have highlighted the pivotal role of oxidative stress in this process [16]. Currently, SGLT2 inhibitors are widely employed in the clinical management of diabetes and cardiovascular diseases [17, 18]. Empagliflozin binds to SGLT-2 receptor subunits, forming a complex that enhances the potential difference across the membrane of renal tubular epithelial cells, thereby reducing glucose reabsorption, inhibiting renal oxidative stress, and providing renal protection [19, 20]. However, there is a lack of reports regarding the potential of Empagliflozin to mitigate liver oxidative stress induced by hyperglycemia. Consequently, this study aimed to establish a diabetes model to investigate the occurrence of diabetic liver injury and evaluate the therapeutic effects of Empagliflozin on diabetic liver injury (DIL).

Hyperglycemia results in decreased levels of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in the liver, accompanied by increased production of reactive oxygen species, lipid accumulation, and peroxidation, leading to elevated levels of malondialdehyde (MDA) [21, 22]. To further elucidate the mechanism underlying the therapeutic effects of Empagliflozin on diabetic liver injury (DIL), we subsequently assessed oxidative stress in liver tissue. The results demonstrated a significant reduction in the levels of antioxidant enzymes SOD and GSH-Px in the livers of diabetic mice, alongside a marked increase in MDA levels, indicating oxidative stress-induced liver damage. Following treatment with Empagliflozin, SOD and GSH-Px levels were significantly elevated, while MDA levels were notably decreased, suggesting that Empagliflozin can effectively ameliorate oxidative stress-mediated damage in the diabetic liver.

Recent studies have highlighted that nuclear factor erythroid 2-related factor 2 (Nrf2) not only promotes the transcription of downstream antioxidant enzymes, including heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1), by binding to antioxidant response elements but also regulates the biosynthesis of glutathione (GSH), thereby enhancing the body's overall antioxidant capacity and playing a critical role in mitigating oxidative damage [23-26]. Subsequently, we examined proteins associated with the Nrf2 signaling pathway. The results revealed a significant reduction in the levels of HO-1, NQO1, and Nrf2 proteins in the livers of diabetic mice. Conversely, treatment with Empagliflozin resulted

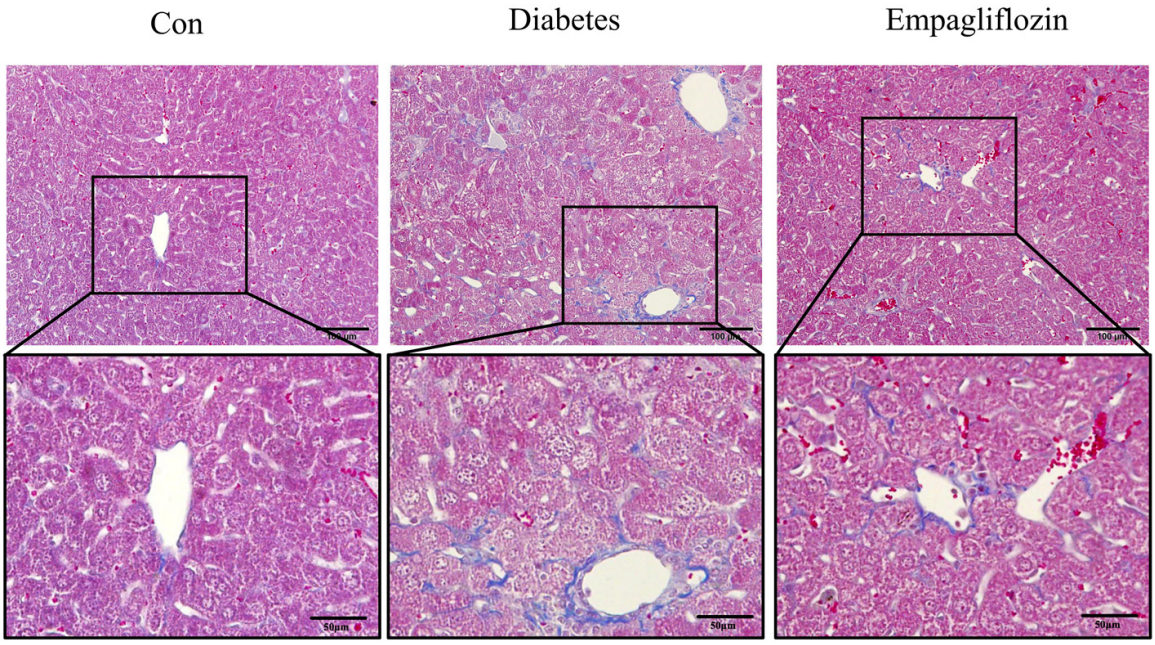

Figure 6. The inhibitory effect of Empagliflozin on diabetic liver fibrosis.

in varying degrees of upregulation of HO-1, NQO1, and Nrf2, suggesting that Empagliflozin exerts its antioxidant effects by modulating the Nrf2 signaling pathway.

Oxidative stress (OS) is closely linked to inflammation, and the elevation of oxidative stress markers alongside inflammatory factors is a critical indicator of the onset and progression of liver fibrosis [27, 28]. The results of this study demonstrated that the expression levels of interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) were significantly elevated in the livers of diabetic mice. In contrast, treatment with Empagliflozin markedly reduced the expression of both cytokines, suggesting that Empagliflozin can effectively inhibit the inflammatory response associated with diabetic liver injury (DIL).

When hepatocytes are subjected to external stressors, oxidative stress intensifies, leading to increased inflammation and necrosis of hepatocytes. Concurrently, heightened lipid peroxidation exacerbates oxidative stress, resulting in excessive deposition and impaired extracellular matrix degradation, ultimately contributing to liver fibrosis. To further investigate this phenomenon, we assessed collagen deposition in the liver tissue of mice across the experimental groups. The results indicated a significant increase in collagen fiber deposition in the livers of diabetic mice, whereas treatment with Empagliflozin led to a marked reduction in collagen accumulation, suggesting that Empagliflozin can substantially ameliorate diabetic liver fibrosis.

In summary, Empagliflozin exhibits a significant protective effect against diabetic liver injury, potentially through mechanisms that involve reducing oxidative stress, inhibiting inflammatory responses, and improving liver fibrosis. These findings provide a novel theoretical foundation for the expanded clinical application of Empagliflozin in managing diabetic liver complications.

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Disclosure of conflict of interest

None.

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