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Glycyrrhizin improves inflammation and apoptosis *via* suppressing HMGB1 and PI3K/mTOR pathway in lipopolysaccharide-induced acute liver injury

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Abstract. – OBJECTIVE: Acute liver injury (ALI) is mainly characterized by the symptom of metabolic disorders, homeostasis unbalance, and loss of liver function. There are no effective treatment methods at present stage except the liver transplantation. Effective treatment for early ALI is of great significance for the treatment of liver injury thereof. Glycyrrhizin (GL) is a promising inhibitor of the high-mobility group box-1 gene (HMGB1) which is expressed much higher in an inflammatory injury. However, it is not clear whether GL improves ALI via the inhibition of HMGB1. The present study is to probe the function and mechanism of glycyrrhizin on acute liver injury.

MATERIALS AND METHODS: The expression of HMGB1 and inflammation in liver macrophages were analyzed. Lipopolysaccharide (LPS) was used in stimulating the macrophages to activate inflammatory response and recombined human HMGB1 was used to resist the function of GL to explore whether GL acted via the target of HMGB1. Then, LPS injection was utilized to induce ALI in mice, and then we evaluated GL treatment in ALI model.

RESULTS: The results showed that the expressions of HMGB1 and inflammatory factors were markedly increased in LPS-activated liver macrophages. GL inhibited the progress of macrophages inflammation by restraining HMGB1, and the administration of GL could reverse the effects of LPS-induced ALI in mice. Moreover, PI3K/mTOR pathway was significantly suppressed by GL application.

CONCLUSIONS: These results suggest that GL prevents inflammation in liver macrophages via inhibition of HMGB1. GL restrains inflammation and cell apoptosis by inhibiting HMGB1 via PI3K/mTOR signaling pathway in ALI. GL may become a novel drug for the therapy of ALI in the future.

Key Words:
Liver macrophages, Acute liver injury, Glycyrrhizin, HMGB1, Inflammation.

Introduction

Acute liver injury (ALI) may be induced by multiple factors, including infection, drugs, ischemia, metabolic disorders, and autoimmune[1,2]. Mild ALI can be gradually healed or transformed into chronic liver damage, which, in turn, causes liver fibrosis, cirrhosis of the liver or liver cancer[3]. Severe acute injury can progress to acute liver failure with high mortality, but there is no effective treatment except liver transplantation currently for this situation[4]. Therefore, effective treatment and prevention in the early stage of ALI are of great significance. The high-mobility box-1 gene (HMGB1) produces a protein that, as a cytokine, can be released from the nucleus to the cytoplasm and even the extracellular space under the stimulation of inflammation, mediating the inflammatory response of various injury models[5,6]. HMGB1 stimulates macrophages to release tumor necrosis factor (TNF) and other products. On the other hand, monocytes release HMGB1 and activate inflammatory stimuli. HMGB1 can also be released in the case of injury or necrosis, which is a major irritant to inflammation caused by necrosis[7,8]. Recently, HMGB1 was highly expressed in lipopolysaccharide induced ALI model in mice. However, the mechanism of action of HMGB1 in ALI process is still unclear. The upregulation of HMGB1 is directly related to the pathogenesis of various human diseases[9,10]. It is thought that the control of inflammation can reduce the damage of inflammatory mediators to tissues. As a result, it is considered as a promising therapeutic target. To inhibit HMGB1 activity, natural triterpenoid glycyrrhizin (GL), as an effective and direct HMGB1 inhibitor, has been routinely used in some *in vitro* and *in vivo* models[11]. Based on these preliminary

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data, the purpose of this project was to explore the GL application in progress of anti-ALI inflammation and the potential mechanism affecting HMGB1.This study provides a new target for the treatment of acute liver disease.

Materials and Methods

Cells Culture and Drug Treatment

Human liver macrophages (HLMs) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium and Ham's F-12 medium (DMEM/F12, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). The macrophages were seeded in six-well plates at 1×10^5 cells per well. Then, the activated macrophages model was established by lipopolysaccharide (LPS, 100 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) stimulus for 24 h. After the cells attached to the culture plate, the optimized concentration was determined based on different concentrations of glycyrrhizin (100 μM, Selleck, Houston, TX, USA). Recombined human HMGB1 (rh-HMGB1, 0.25 μg/mL, Sigma-Aldrich, St. Louis, MO, USA) was used as an antagonist to counteract the function of glycyrrhizin.

Mice

Six- to eight-week-old C57/B6J male mice (20-25 g) obtained from Fudan University, were bred and maintained at the Fudan University Animal Center. Animals were provided with available food and water. Five mice were housed in a cage, suitable temperature and humidity (22°C-26°C, 50%-60%), and 12 h light/dark cycle. This investigation was approved by the Animal Ethics Institutional Committee of Fudan University, and all researches were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the Fudan University.

Acute Liver Injury Model

The animals were randomly divided into three groups, namely control group (n=8), ALI group (n=8), and GL group (n=8). Briefly, mice were treated with 8 mg/kg LPS to established acute liver injury model through intraperitoneal injection. Then, GL was introduced to mice at the dose of 10 mg/kg *via* oral gavage, and control group was injected with equal volume normal saline.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from macrophages with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) directed by the manufacturer's protocol. Reverse transcription was conducted to synthesize complementary deoxyribose nucleic acid (cDNA) using PrimeScript™ RT Master Mix (Applied Biosystems, Foster City, CA, USA). RT-PCR was conducted to quantify iNOS, COX-2, HMGB1, and U6 mRNA expression levels. Then RT-PCR was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). 18S rRNA was used for normalization. Relative mRNA expression levels were quantified by the $2^{-\Delta\Delta Ct}$ methods.

Primer sequences are listed as follows: iNOS: Forward (5'>3'), TTCAGTATCACAACCTCAGCAAG Reverse (5'>3'), TGGACCTGCAAGTTAAAATCCC, COX-2: Forward (5'>3'), CTGTTCCAACCCATGTCAAAACC Reverse (5'>3'), GTACAGTTTTCACCGTAGAATCCA, HMGB1: Forward (5'>3'), TATGGCAAAAGCGGACAAGG Reverse (5'>3'), CTTCGCAACATCACCAATGGA, 18S rRNA: Forward (5'>3'), GTTGGTTTTCGGAACTGAGGC, Reverse (5'>3'), GTCGGCATCGTTTATGGTCG.

Western Blot (WB) Analysis

Liver tissue and human liver macrophages (HLMs) were harvested in lysis buffer. Proteins were isolated using a Total Protein Extraction Kit (Keygen, Nanjing, China) according to the manufacturer's instructions. Next, protein concentrations were measured with the enhanced bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, Shanghai, China). After separation, transferring and blocking with 5% skim milk for 1 h at room temperature, proteins were incubated overnight with anti-HMGB1 (Abcam, Cambridge, MA, USA, 1:1000), anti-caspase-3/8 (Abcam, Cambridge, MA, USA, 1:1000), anti-Bax (Abcam, Cambridge, MA, USA, 1:1000), anti-Bcl-2 (Abcam, Cambridge, MA, USA, 1:1000), anti-PI3K (Millipore, Billerica, MA, USA, 1:1000), anti-mTOR (Millipore, Billerica, MA, USA, 1:2000), anti-p-PI3K (Abcam, Cambridge, MA, USA, 1:1000), anti-p-mTOR (Abcam, Cambridge, MA, USA, 1:1000), anti-COX-2 (Abcam, Cambridge, MA, USA, 1:1000), anti-iNOS (Abcam, Cambridge, MA, USA, 1:1000), and anti-β-actin (Cell Signaling Technology, Danvers, MA, USA, 1:2000). After washing with Tris-Buffered Saline and Tween-20 (TBST), the membrane was

incubated with secondary antibody (Abcam, Cambridge, MA, USA, 1:2000) for 1 h at room temperature. Protein bands were visualized and detected using an enhanced chemiluminescence (ECL) system.

Flow Cytometry Analysis

The apoptosis was determined using the 70-AP101-30 Annexin V-FITC/PI Apoptosis Kit (MultiSciences, Hangzhou, China) based on the manufacturer's procedures. Liver cells were incubated with Annexin V-FITC and propidium iodide (PI) away from light for 30 min, whereas apoptotic cells and viable cells were sorted using a fluorescence-activated cell sorting flow cytometer.

Histologic Staining

Hepatic tissues were fixated with 4% paraformaldehyde and dehydration was obtained through different concentration alcohol. Then tissues were embedded into paraffin and cut into sections (4 μm). Hematoxylin-eosin staining was conducted using Hematoxylin and Eosin Staining Kit (Beyotime, Shanghai, China) following manufacturer's protocol.

Enzyme Linked Immunosorbent Assay (ELISA)

Serum was taken from mice. The cell medium and serum were centrifuged for 10 min and then the supernatant was collected. Standard product was added in a 96-well plate with different concentration successively. Then, the colorant was seeded into each well and the samples were incubated in avoided light for 15 min. The termination solution was added to terminate the reaction and the absorbance (OD value) of each well was measured sequentially at 450 nm.

MDA Detection Assay

Malonaldehyde (MDA) detection was conducted using a Micro Malondialdehyde Assay Kit (BC0025, Solarbio, Beijing, China). Briefly, the sample was subjected to condensation reaction with thiobarbituric acid (TBA), and then the absorbance at 532 nm and 600 nm of the product was measured, respectively. Differential was calculated for MDA content.

GSH/GSSG Ratio, SOD and Catalase Detection

GSH/GSSG Ratio, SOD and catalase detection was performed using GSH/GSSG Ratio Detection Assay Kit (Abcam, Cambridge, MA, USA), SOD Activity Assay Kit (Abcam, Cambridge, MA, USA), and Catalase Activity Assay Kit (Abcam, Cambridge, MA, USA), respectively following manufacturer's protocol.

Statistical Analysis

Data were displayed as the means ± standard deviations. Comparison between the two groups was performed using Student's *t*-test, whereas comparisons among multiple groups were done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Data were collected and assessed using GraphPad Prism (Version X, La Jolla, CA, USA). When $p<0.05$, differences were considered statistically significant.

Results

GL Attenuated LPS-Induced Inflammation in HLMs

First, whether GL could influence human liver macrophages (HLMs) inflammation after LPS activation was explored. HLMs with LPS and GL treatment were extracted into protein and RNA to detect inflammation-relative protein. The results showed that LPS stimulation induced abundant inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and HMGB1 both in protein and RNA levels; however, GL treatment decreased iNOS, COX-2 and HMGB1 expression in protein and RNA (Figure 1A and 1B). Then, the cell free supernatant was measured pro-inflammatory factors level using ELISA, exhibiting that LPS administration elevated tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and interleukin 6 (IL-6) expression, but it was found that GL could restrain the excessive release of inflammatory mediators (Figure 1C). Therefore, GL treatment alleviated LPS-induced inflammation in HLMs.

GL Anti-Inflammation Effect Obtained Through Inhibiting PI3K/mTOR Pathway Via HMGB1 Suppression

GL and HMGB1 correlation in inflammatory macrophage was further investigated. Rh-HMGB1 was utilized to reverse GL-induced HMGB1 decrease. Then, the iNOS and COX-2 expression in protein level was continuously detected, finding that rh-HMGB1 could neutralize HMGB1 inhibition *via* GL treatment and reversed GL anti- in-

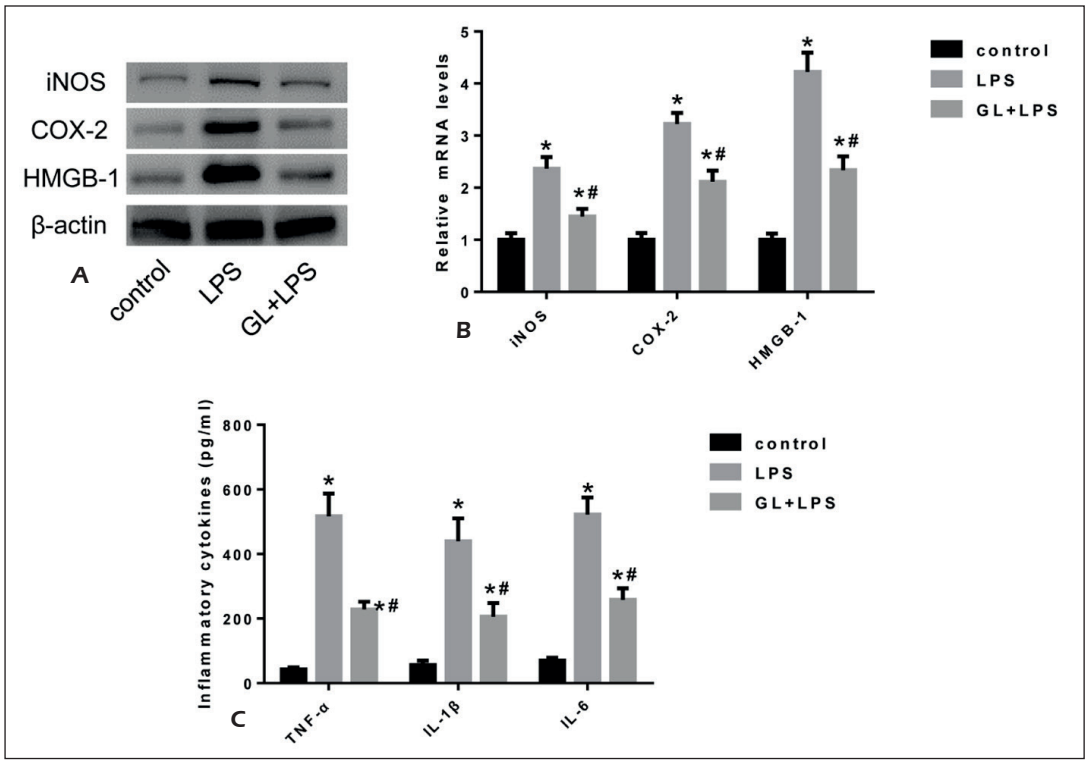

Figure 1. GL attenuates LPS-induced inflammation in HLMs. **A**, Representative Western blotting of iNOS, COX-2, HMGB1 in the HLM with GL and LPS treatment. **B**, Representative RNA levels of iNOS, COX-2, HMGB1 in the HLM with GL and LPS treatment. **C**, Representative ELISA detection of TNF-α, IL-1β and IL-6 in the HLM with GL and LPS treatment. "*" means *vs.* control group with statistical significance. "#" means *vs.* LPS group with statistical significance.

flammation effect (Figure 2A). PI3K/mTOR pathway is a critical signaling pathway that involves multiple biological events, including inflammation, metabolism, and proliferation. Hence, phosphorylated-PI3K (p-PI3K) and phosphorylated-mTOR (p-mTOR) were measured *via* Western blotting, and it was showed that GL application reduced p-PI3K and p- mTOR levels but it did not affect PI3K and mTOR expression. Besides, rh-HMGB1 intervening could enhance p-PI3K and p-mTOR following GL administration (Figure 2B). Therefore, it can be concluded that GL inhibited macrophage inflammation *via* negatively regulating HMGB1 to suppressing PI3K/mTOR pathway.

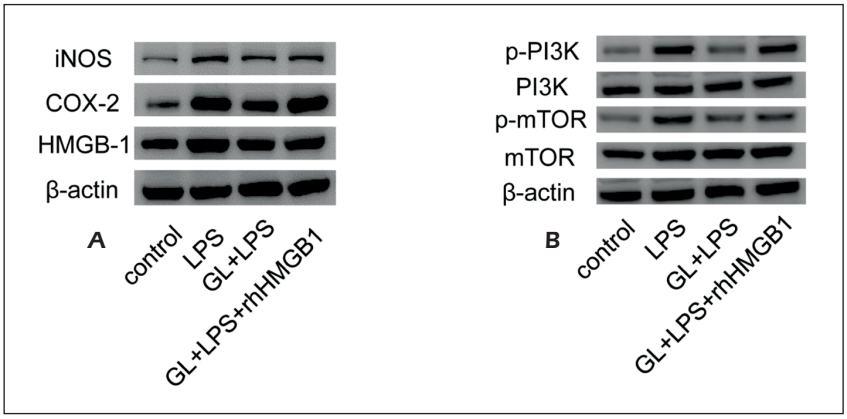

Figure 2. GL anti-inflammation effect gets through inhibiting PI3K/mTOR pathway via HMGB1 suppression. **A**, Representative Western blotting of iNOS, COX-2, HMGB1 in the control, LPS, GL+LPS and GL+LPS+rh-HMGB1 group. **B**, Representative Western blotting of p-PI3K, p-mTOR, PI3K and mTOR in the control, LPS, GL+LPS and GL+LPS+rh-HMGB1 group.

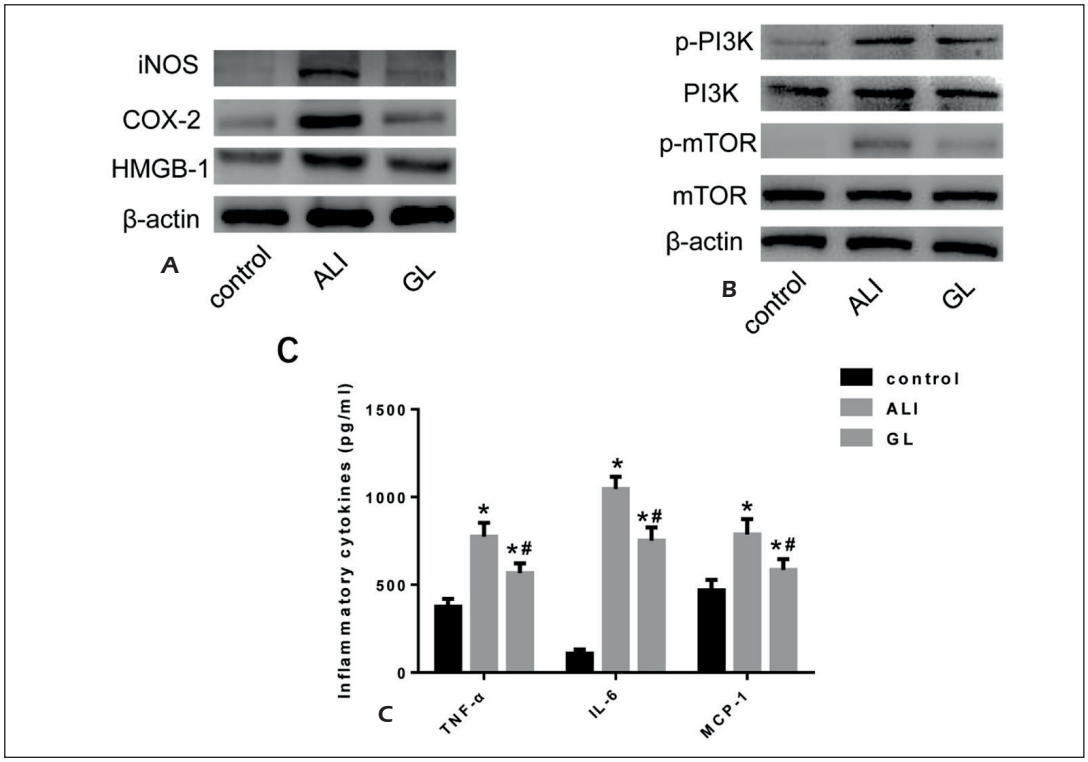

Figure 3. GL ameliorates inflammatory response following ALI in mice. **A**, Representative Western blotting of iNOS, COX-2, HMGB1 in the control, ALI and GL group. **B**, Representative Western blotting of p-PI3K, p-mTOR, PI3K and mTOR in the control, ALI and GL1 group. **C**, Representative ELISA detection of TNF-α, IL-6 and MCP-1 in the control, ALI and GL group. "*" means *vs.* control group with statistical significance. "#" means *vs.* ALI group with statistical significance.

GL Ameliorates Inflammatory Response Following ALI in Mice

To verify the curative effect of GL to ALI, ALI model was established in mice and anti-inflammation function was first evaluated. One day after the treatment of LPS-induced ALI with GL, liver tissue proteins and serum were extracted to determine the inflammatory indicators. The results showed that compared with the ALI group, GL treatment effectively alleviated the accumulation of a variety of inflammatory markers, such as iNOS, COX-2, and HMGB1 (Figure 3A). Furthermore, it was reconfirmed in mice that GL inhibited the phosphorylation of PI3K and mTOR, but had no effect on the total PI3K and mTOR expressions (Figure 3B). Besides, ELISA assay of mouse serum found that the utilization of GL reduced serum levels of TNF-α, IL-6, and monocyte chemo-attractant protein-1 (MCP-1) compared with those in ALI group, indicating that the anti-inflammatory function of GL inhibits inflammatory chemotaxis in peripheral blood (Figure 3C). Therefore, GL ameliorates inflammatory response in ALI by regulating PI3K and mTOR pathway.

GL Suppresses Oxidative Stress and Apoptosis in ALI

The aggravation of inflammation after liver injury mediates the occurrence of oxidative stress and apoptosis. It was hypothesized whether GL could regulate oxidative stress and apoptosis while being anti-inflammatory. MDA assay showed that GL treatment significantly reduced the level of MDA (Figure 4A), and it was found in the antioxidant substance that the expressions of reduced glutathione (GSH)/oxidized glutathione (GSSH) ratio, superoxide dismutase (SOD) and catalase were significantly increased after GL application compared with ALI group (Figure 4B-4D). In addition, the measure of apoptosis-related factors showed that the treatment of GL significantly reduced the levels of caspase-3 and caspase-8 along with decreased Bax and increased Bcl-2, indicating that GL inhibited the occurrence of inflammation-mediated apoptosis (Figure 4E). Moreover, flow cytometry showed that LPS-mediated ALI significantly increased the amount of apoptotic cells in liver tissues, while the number of apoptotic cells decreased significantly after GL treatment

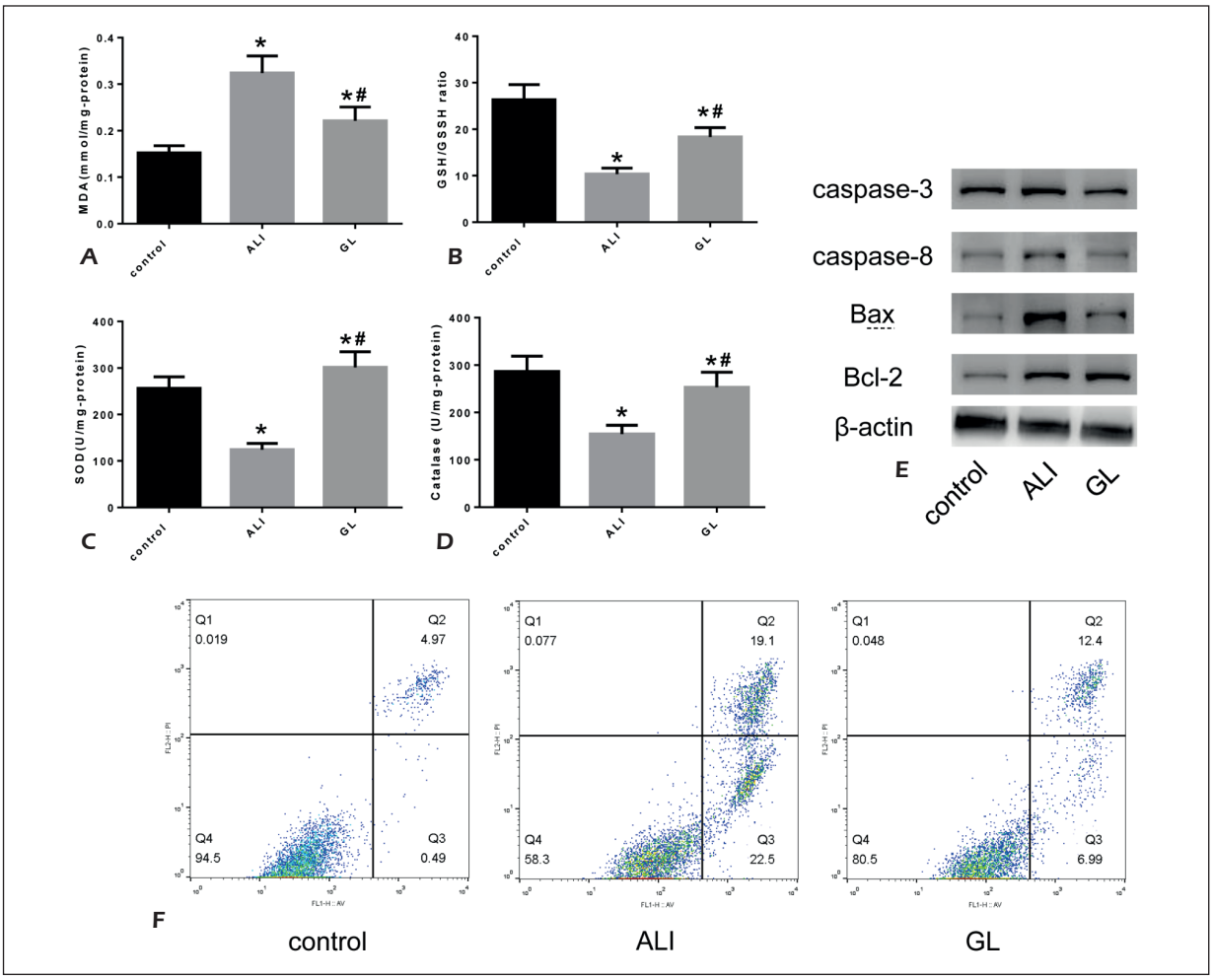

Figure 4. GL suppresses oxidative stress and apoptosis in ALI. **A**, Representative MDA detection in the control, ALI and GL group. **B**, Representative GSH/GSSH ratio in the control, ALI and GL group. **C**, Representative SOD detection in the control, ALI and GL group. **D**, Representative catalase detection in the control, ALI and GL group. **E**, Representative Western blotting of caspase-3, caspase-8, Bax, Bcl-2 in the control, ALI and GL group. F, Representative flow cytometry of apoptosis in the control, ALI and GL group. "*" means *vs.* control group with statistical significance. "#" means *vs.* ALI group with statistical significance.

(Figure 4F). Hence, GL improves anti-oxidative stress and anti-apoptosis capacity of liver tissue following ALI.

GL Improves Hepatic Organization Structure and Function in ALI

The effects of GL on hepatic organization structure and function were assessed following ALI. HE staining showed that in normal liver tissue, the cell morphology was intact, the nucleus was prominent, and there was no inflammation or necrosis in the section. However, LPS caused diffuse injury and necrosis of liver cells, resulting in hemorrhage, swelling, necrosis, and inflammatory cell infiltration. Under the intervention of GL, LPS-mediated changes in liver pathomorphology were significantly improved (Figure 5A). In addition, LPS treatment increased serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) activity and total bilirubin (TBIL) level, but GL treatment decreased serum ALT, AST activity and TBIL concentration caused by LPS injection (Figure 5B and 5C). Therefore, GL ameliorates hepatic organization structure and function in ALI.

Discussion

The liver contains a large number of macrophages, accounting for 20% to 40% of the total number of cells[12]. These macrophages can clear bacterial and microbial products from the intes-

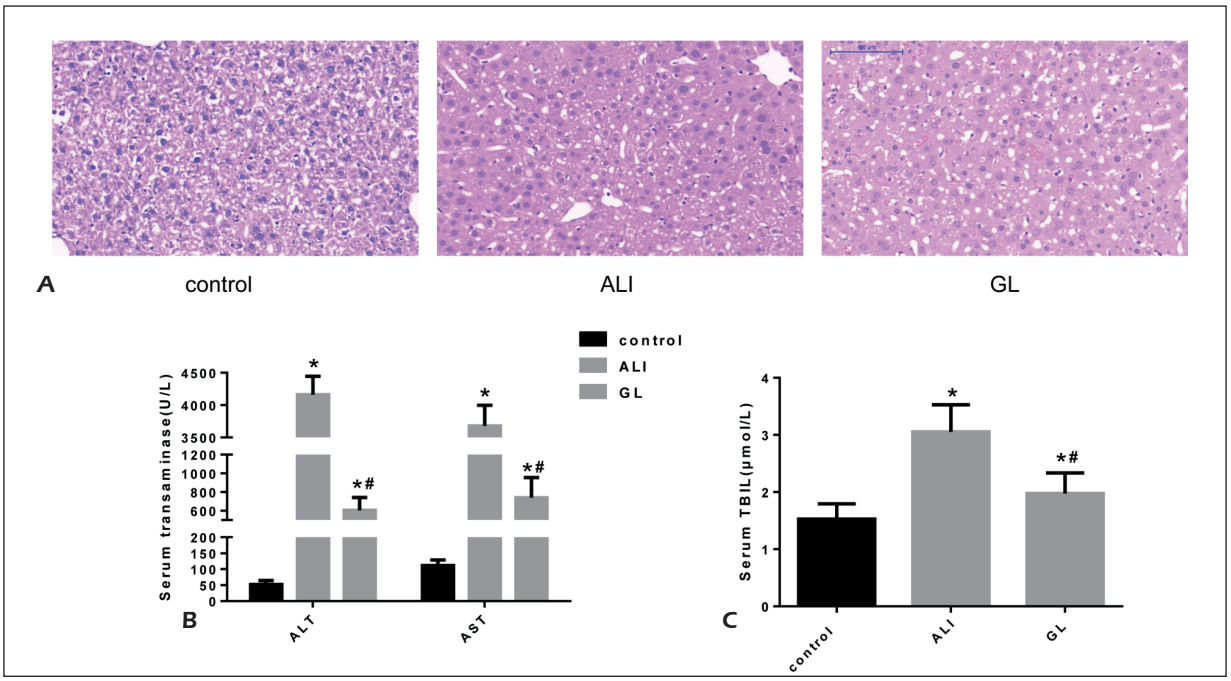

Figure 5. GL improves hepatic organization structure and function in ALI. **A**, Representative HE staining of hepatic tissue in the control, ALI and GL group (200X). **B**, Representative serum ALT and AST in the control, ALI and GL group. **C**, Representative serum TBIL in the control, ALI and GL group. "*" means *vs.* control group with statistical significance. "#" means *vs.* ALI group with statistical significance.

tinal tract to the liver *via* the portal vein, engulf and degrade exogenous substances directly, and participate in the formation of an adaptive immune response in the liver[13]. Kupffer cells (KCs), as the innate macrophages in the liver, account for 80-90% of the macrophages in the body tissues[14]. In LPS-induced ALI, LPS can directly act on KCs to induce liver inflammation, and stimulate KCs to secrete abundant cytokines, leading to secondary injury of liver cells[15]. On the other hand, chemokines released from KCs can recruit peripheral granulocytes and monocytes to the liver, thus aggravating liver injury[16]. Some studies[17-19] have found that GL, as an effective HMGB1 inhibitor, has a significant improvement effect in a variety of inflammation-mediated diseases, including epilepsy, disc degeneration, and arthritis. Therefore, it was hypothesized that GL in ALI may also play a role in improving damage effects during inflammation by inhibiting HMGB1 and inflammation-related signaling pathway. In *vitro* study, inflammatory response in HLMs was first introduced *via* LPS stimulation and pretreated with GL, and it was found that the expressions of inflammatory proteins iNOS and COX-2 in macrophages were significantly declined. Similarly, the inhibitory effect of GL on HMGB1 was further confirmed, and GL was found to down-regulate the expression of PI3K/mTOR pathway by inhibiting HMGB1. Several reports[20-22] have proved that PI3K and mTOR proteins are involved in the mediation of inflammatory response. This research also proved that the overexpression of PI3K/mTOR pathway after inflammatory response and GL to inflammatory response inhibition of ALI is realized *via* down-regulating PI3K/mTOR pathway phosphorylation by inhibiting HMGB1. Hence, the concentrations of pro-inflammatory factors and chemokines such as TNF-α, IL-1β, and IL-6 released from GL treated HLMs were significantly reduced. Besides, LPS-induced ALI disease model was established in mice, and GL treatment was applied to evaluate the efficacy. *In vivo*, it was showed that GL, as a HMGB1 inhibitor, down-regulated PI3K/mTOR pathway activation and played an anti-inflammatory role. Of note, GL was discovered to block the inflammation-mediated elevation of TNF-α, IL-1β, and MCP-1in peripheral blood, suggesting that GL also reduces the infiltration of peripheral inflammatory cells into liver tissue. MDA is one of the products of lipid oxidation. The oxidative stress of cells will lead to lipid oxidation,

which is gradually decomposed into a series of compounds including MDA. The results exhibited that LPS could significantly increase the level of MDA in liver, while GL could significantly reduce the content of MDA in the liver. In addition, LPS induced antioxidant enzymes SOD and catalase activity as well as GSH level decreasing significantly in liver, but GL could effectively relieve the change of antioxidant enzymes in liver caused by LPS and restore the content of GSH in liver tissue and prevent the depletion of GSH. This explained that GL could increase antioxidant enzyme activity and keep the content of intrahepatic GSH in LPS stimulating oxidative stress in liver. LPS stimulates KCs to secrete large amounts of TNF-α, which directly mediates apoptosis by binding to TNF-α receptor 1, and TNF-α activates caspase-8 in liver cells, leading to Bax oligomerization and insertion into mitochondria, initiating apoptosis of the mitochondrial pathway. Therefore, a large number of apoptotic cells can be observed in animal models. However, GL could effectively decrease the level of TNF-α and down-regulate the expression of caspase3/8. In addition, GL antagonized the pro-apoptotic effect of Bax by increasing the level of Bcl2. Therefore, it was found that the apoptosis level in liver tissue was significantly improved following GL treatment in ALI, thereby ultimately improving liver function and protecting liver tissue structure. Therefore, GL finally lowered the serum content of ALT, ST and TBIL after ALI and reduced the area of tissue damage caused by inflammation in liver tissue, thus improving liver function and protecting liver tissue structure. To sum up, the present study proved GL mediated liver protection in LPS-induced ALI, GL got by inhibiting HMGB1 and negatively regulating PI3K/mTOR pathway to play an antagonism of inflammatory infiltration, which triggered by macrophages in the liver. Moreover, GL mitigated the levels of oxidative stress and cell apoptosis in liver tissue, so as to improve hepatic function and prevent excessive damage to hepatic structure.

Conclusions

These results suggest that GL prevented inflammation in liver macrophages *via* inhibition of HMGB1. GL restrained inflammation and cell apoptosis by inhibition of HMGB1 *via* PI3K/mTOR signaling pathway in ALI. GL may become a novel drug for the therapy of ALI in the future.

Conflict of Interests

The authors declare that they haves no conflict of interests.

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