Original Research

Protective effects of glutamine on lipopolysaccharide/D-galactosamine-induced fulminant hepatitis in mice

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Impact Statement

Fulminant hepatitis is a serious clinical syndrome with high morbidity and mortality rates. Glutamine is the most abundant free amino acid in mammals. Accumulating evidence indicates that glutamine supplementation provides protection against inflammation-related disorders. The current results showed that glutamine supplementation alleviated lipopolysaccharide (LPS)/D-galactosamine (D-Gal)-induced fulminant liver injury, suppressed the release of inflammatory factors, and reduced apoptosis. Importantly, the therapeutic administration of glutamine post-LPS/D-Gal also provided appreciable protection against fulminant hepatitis. These results indicate that glutamine may have potential value as a pharmacological intervention for fulminant hepatitis.

Abstract

Fulminant hepatitis remains a critical health problem owing to its high mortality rate and the lack of effective therapies. An increasing number of studies have shown that glutamine supplementation provides protective benefits in inflammation-related disorders, but the pharmacological significance of glutamine in lipopolysaccharide (LPS)/D-galactosamine (D-Gal)-induced fulminant hepatitis remains unclear. In the present study, the potential effects of glutamine on LPS/D-Gal-induced fulminant hepatitis were investigated. Pretreatment with glutamine decreased plasma activities of alanine and aspartate aminotransferases, and ameliorated hepatic morphological abnormalities in LPS/D-Gal-exposed mice. Glutamine pretreatment also inhibited LPS/D-Gal-induced tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) production. In addition, glutamine pretreatment decreased the level of cleaved cysteinyl aspartate–specific proteinase 3 (caspase-3), suppressed the activities of caspase-3, caspase-8, and caspase-9, and reduced the number of cells positive for TdT-mediated dUTP nick-end labeling in LPS/D-Gal-challenged mice. Interestingly, post-treatment with glutamine also provided protective benefits against LPS/D-Gal-induced acute liver injury, although these effects were less robust than those of glutamine pre-treatment. Thus, glutamine may have potential value as a pharmacological intervention in fulminant hepatitis.

Keywords: Glutamine, fulminant hepatitis, inflammation, apoptosis, lipopolysaccharide, D-galactosamine

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Introduction

Fulminant hepatitis remains a critical health problem due to its high mortality, which is caused by several factors, including viruses, bacteria, drugs, alcohol, and so on.[1] It is widely accepted that uncontrolled inflammation and excessive apoptosis are the main underlying mechanisms.[2–4] Lipopolysaccharide (LPS), a key pathogenic component of Gram-negative bacteria, is a crucial pro-inflammatory factor that triggers the release of inflammatory cytokines and initiates an inflammatory response.[5,6] LPS can lead to acute liver injury with severe inflammation and massive hepatocyte apoptosis in D-galactosamine (D-Gal)-sensitized mice, which has been extensively used for the experimental investigation of fulminant hepatitis.[7–9]

Glutamine is the most abundant free amino acid in mammals.[10,11] A growing body of evidence indicates that glutamine provides protective benefits against inflammation-related disorders.[12,13] It has been reported that glutamine supplementation suppresses the production of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) and attenuates the degree of colon injury in dextran sodium sulfate (DSS)-induced colitis.[14] In addition, glutamine supplementation was shown to inhibit inflammatory responses, relieve organ injury, and improve the survival rate of mice with systemic inflammation.[12,15–17] Therefore, glutamine may

be a promising reagent for the treatment of inflammation-related disorders, but it remains unclear whether glutamine supplementation also results in beneficial outcomes in fulminant hepatitis.

To investigate the potential effects of glutamine in fulminant hepatitis, an LPS/D-Gal-induced acute liver injury model was employed in the current study. Glutamine was injected into mice with LPS/D-Gal-induced acute liver injury and the degree of liver injury, production of inflammatory cytokines, and induction of hepatocyte apoptosis were evaluated.

Materials and methods

Materials and regents

Regents were obtained as follows: LPS, D-Gal were obtained from Sigma-Aldrich (St. Louis, MO, USA). Glutamine was obtained from Adamas-beta (Shanghai, China). The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay kits were produced by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The ELISA kits for mouse TNF-α (EMC102a) and mouse interleukin-6 (IL-6) (EMC004) were from Neo Bioscience Technology Company (Shenzhen, China). Assay kits for caspases 3 (C1116), 8 (C1152) and 9 (C1158) were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). Antibodies against cleaved caspase-3, the HRP-conjugated anti-rabbit IgG, and the HRP-conjugated anti-mouse IgG were provided by Cell Signaling Technology (Ann Arbor, MI, USA). Antibody against β-actin were purchased from 4A BIOTECH (Beijing, China). The BCA protein assay kit was purchased from Pierce Biotechnology (Rockford, IL, USA). The enhanced chemiluminescence (ECL) reagent was obtained from Advansta (Menlo Park, CA, USA).

Animals

Male C57BL/6J mice (18–22 g) with an average age of 6–8 weeks were provided by the Experimental Animal Center of Chongqing Medical University (Chongqing, China). The animals were housed under controlled temperatures (22°C–25°C), relative humidity (50% ± 5%), and light conditions (12 h:12 h light/dark cycle), and were allowed ad libitum access to water and diet. The Ethics Committee of Chongqing Medical University approved all the mouse experiments.

Experimental protocol

To induce fulminant hepatitis, the mice were intraperitoneally injected with LPS (10 μg/kg, dissolved in normal saline) combined with D-Gal (700 mg/kg, dissolved in normal saline). Mice were randomly divided into the following four groups ($n = 8$ per group): (1) the control group: mice were treated with the same volume of solvent; (2) the glutamine group: mice were injected intraperitoneally with glutamine (50 mg/kg, dissolved in normal saline); (3) the LPS/D-Gal group: mice received intraperitoneal injection of LPS combined with D-Gal; and (4) the glutamine + LPS/D-Gal group: glutamine was intraperitoneally injected 30 min before LPS/D-Gal exposure. The mice were anesthetized and sacrificed 1.5 h after the LPS/D-Gal challenge. Blood samples were collected and added to tubes, followed by centrifugation to collect plasma to measure the level of TNF-α. In addition, another 32 mice underwent the same procedure as described above, and were sacrificed 6 h after LPS/D-Gal injection. Blood and liver tissues were collected and stored at −80°C for protein extraction, histopathological analysis, and other tests.

Since pretreatment is not practical in clinical practice, an additional 24 mice were selected to investigate the therapeutic effect of glutamine post-treatment on acute liver injury. Mice were randomly divided into three groups ($n = 8$ per group): (1) the control group: mice were treated with the same volume of solvent; (2) the LPS/D-Gal group: mice were intraperitoneally injected with LPS and D-Gal; and (3) the glutamine + LPS/D-Gal group: glutamine was injected 1.5 h after LPS/D-Gal exposure. Finally, the mice were anesthetized and killed 6 h after LPS/D-Gal injection. Blood and liver tissues were collected and stored at −80°C.

Histological analysis

Liver tissues of mice were fixed in 4% paraformaldehyde and embedded in paraffin. The samples were then sectioned (4 μm) and stained with hematoxylin and eosin (H&E) to evaluate pathological changes in the liver. Finally, the sections were examined under a light microscope (Olympus, Japan).

Evaluation of plasma AST and ALT

Blood samples were separated by centrifugation to obtain plasma samples. The biochemical indices in plasma, including ALT and AST, were determined according to the manufacturer's instructions to determine the degree of liver injury. First, plasma samples and an ALT (or AST) matrix solution were added to 96-well plates and incubated for 30 min at 37°C. Second, 2-4-dinitrophenylhydrazine was added to each well and incubated for 20 min at 37°C. Finally, NaOH solution was added, and the levels of ALT or AST were calculated according to the absorbance measured at 490 nm based on the standard curve.

Analysis of inflammatory cytokines

TNF-α levels were determined 1.5 h after LPS/D-Gal injection, whereas IL-6 levels were determined 6 h after LPS/D-Gal injection. Both values were determined using ELISA kits according to the manufacturer's instructions. TNF-α and IL-6 levels were calculated using a standard curve.

Determination of caspase activity

To evaluate the activities of caspases 3, 8 and 9, liver tissue was homogenized in lysis buffer, and then centrifuged at 4°C for 15 min at 16,000 × g. Appropriate amounts of supernatant were transferred to a clean test tube, and the protein concentration of the supernatant was determined using the Bradford protein determination kit. According to the instructions of the caspase activity assay kit, 40 μL detection buffer, 50 μL supernatant, and 10 μL of substrate for caspase (AC-DevD-ρNA for caspase-3, AC-IETD-ρNA for caspase-8, and AC-LEhd-ρNA for caspase-9) were mixed.

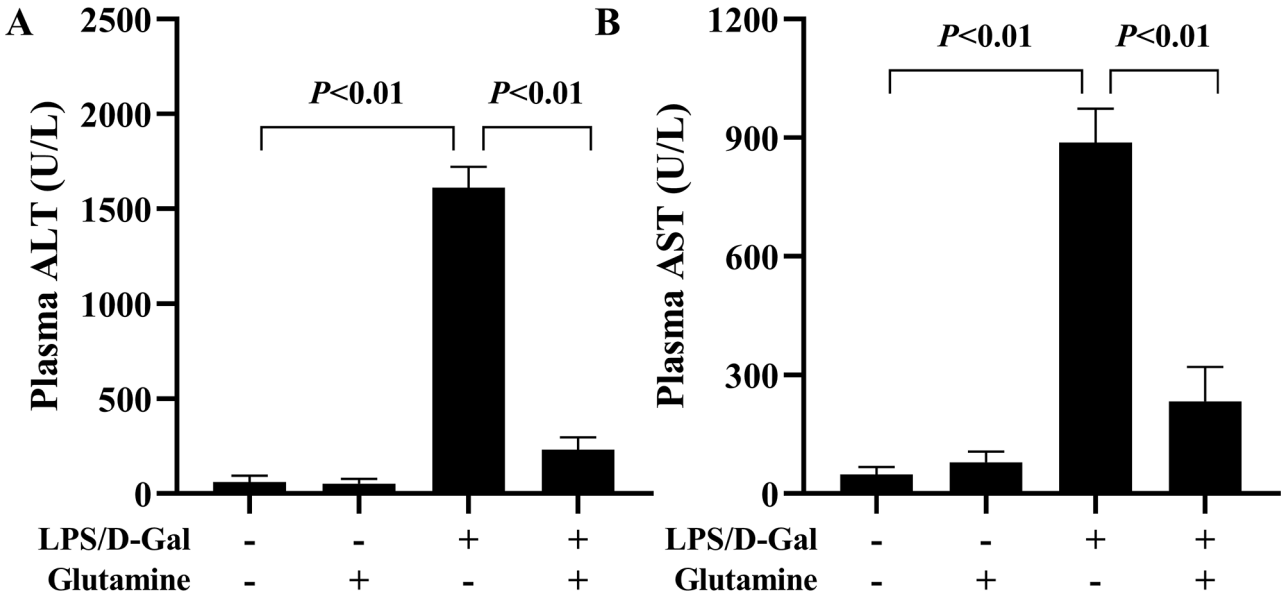

Figure 1. Pretreatment with glutamine reverses LPS/D-Gal-induced elevation of plasma transaminase. Glutamine was administered 30 min before the LPS/D-Gal challenge. Plasma was collected 6 h after LPS/D-Gal injection to determine alanine aminotransferase (ALT, A) and aspartate aminotransferase (AST, B) activities. Values are means ± SD, $n = 8$ per group.

After incubation at 37ºC for 1.5 h, absorbance was measured at 405 nm. One unit (U) of caspase activity is defined as the amount of caspase cleaving 1 nmol of colorimetric substrate within 1 h at 37ºC when the substrate is saturated. Caspase activities were expressed as units per mg total protein.

Western blot analysis

Total protein was prepared from the liver tissues. Protein concentration was determined using the BCA reagent. Protein was separated using SDS-PAGE and then transferred to nitrocellulose membrane. After blocking with 5% non-fat milk in TBST (TBS + 0.1% Tween-20) for 2 h, the blots were incubated overnight with a primary antibody against cleaved caspase-3, followed by three washes in TBST for 10 min each. The membranes were then incubated with the secondary antibody for 2 h at room temperature, followed by three washes in TBST for 10 min each. Finally, Ab binding was visualized with ECL reagents and the ChemiDoc Touch Imaging System (Bio-Rad), and the intensity of the bands was quantified using the ImageJ software.

TUNEL assay

Paraffin-embedded liver tissue sections were prepared for the TUNEL assay, which was performed by using the In Situ Cell Death Detection Kit (Roche), according to the manufacturer's instructions. A dark-brown precipitate in the nuclei was produced in terminal transferase reactions, which represent apoptotic cells. Images were observed under a light microscope (Olympus).

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 8.0.1). All data are presented as means ± SD. Statistical significance between multiple groups was analyzed using one-way analysis of variance (ANOVA) with Tukey's post hoc test. Statistical significance was set at a p-value < 0.05.

Results

Pretreatment with glutamine alleviates LPS/D-Gal-induced liver injury

Damaged hepatocytes release ALT and AST into the circulation. Thus, elevated plasma transaminase activities indicate the degree of liver injury.[18] In this study, compared to the control group, plasma ALT and AST activities significantly increased after LPS/D-Gal challenge. However, glutamine pretreatment suppressed LPS/D-Gal-induced elevation of plasma ALT and AST (Figure 1). LPS/D-Gal exposure also produced disorganized hepatic lobules, hepatocyte necrosis and inflammatory cell infiltration, but glutamine pretreatment attenuated these hallmarks of ultrastructural damage (Figure 2).

Pretreatment with glutamine decreases the level of pro-inflammatory cytokines

TNF-α and IL-6 are the major pro-inflammatory cytokines involved in LPS/D-Gal-induced liver injury.[19,20] LPS/D-Gal injection sharply increased plasma TNF-α and IL-6 concentrations at 1.5 and 6 h post-injection, respectively, which was suppressed by glutamine pretreatment (Figure 3).

Pretreatment with glutamine suppresses hepatocytes apoptosis

LPS/D-Gal exposure induces massive apoptosis of hepatocytes,[21] which is mediated by the activation of caspase cascades and cleavage of a large number of functional proteins.[22] To confirm whether glutamine modulates

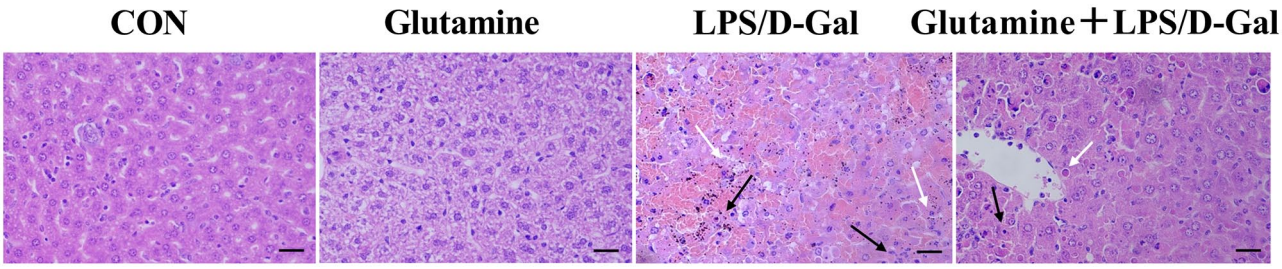

Figure 2. Pretreatment with glutamine alleviates LPS/D-Gal-induced liver histological abnormalities. Liver samples were collected 6 h after LPS/D-Gal exposure and stained with hematoxylin and eosin. White arrows indicate hepatocyte necrosis; black arrows indicate inflammatory cell infiltration. Scale bar = 50 μm.

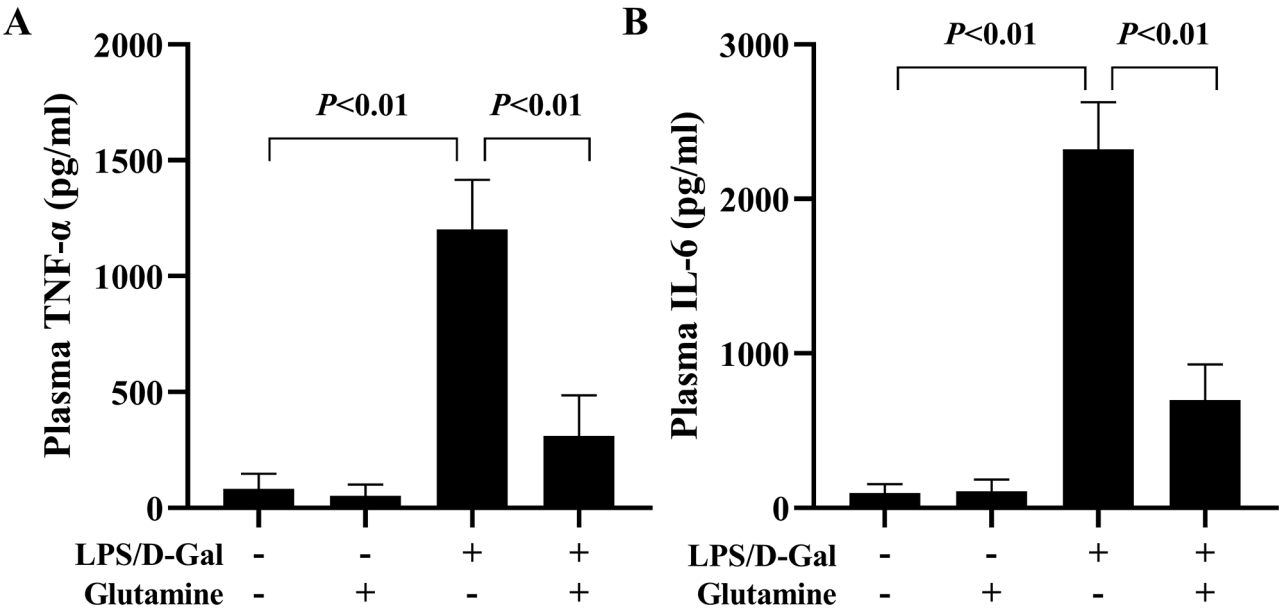

Figure 3. Pretreatment with glutamine decreases plasma concentrations of pro-inflammatory cytokines in LPS/D-Gal-induced liver injury. Plasma samples were collected 1.5 h after LPS/D-Gal exposure to measure TNF-α (A) and 6 h after LPS/D-Gal exposure to measure IL-6 levels (B). Values are means ± SD, $n = 8$ per group.

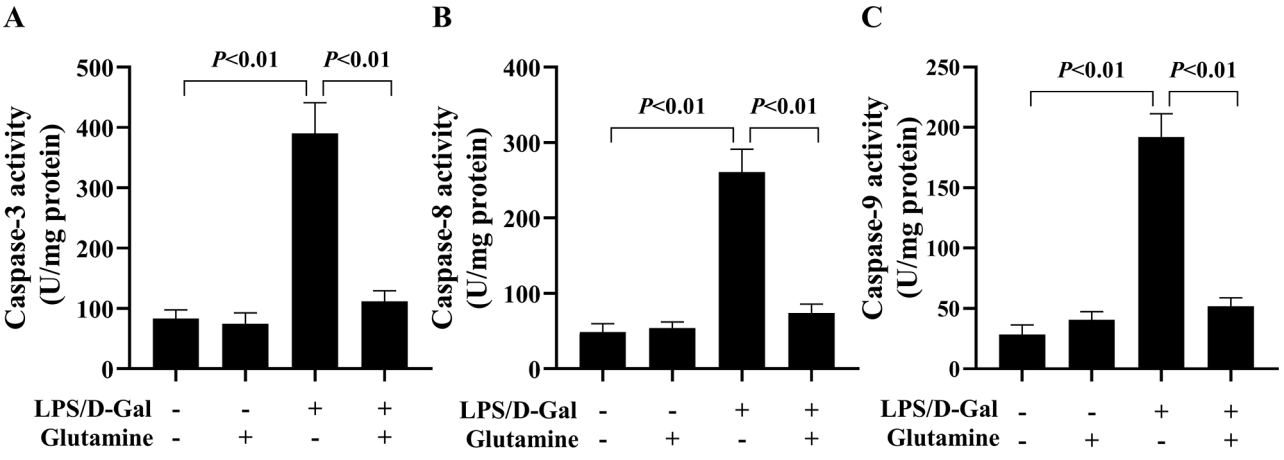

Figure 4. Pretreatment with glutamine suppresses LPS/D-Gal-induced activation of caspases. Liver samples were collected 6 h after LPS/D-Gal exposure. The activity of (A) caspase-3, (B) caspase-8, and (C) caspase-9 in liver tissue were determined. Values are means ± SD, $n = 8$.

LPS/D-Gal-induced hepatocyte apoptosis, the activities of caspases 3, 8 and 9 were measured. Pretreatment with glutamine suppressed activation of all three caspases in the liver of LPS/D-Gal-exposed mice (Figure 4). In addition, western blot analysis showed that pretreatment with glutamine attenuated procaspase-3 cleavage (Figure 5) and reduced the number of TUNEL-positive cells (Figure 6) in the LPS/D-Gal-challenged mice.

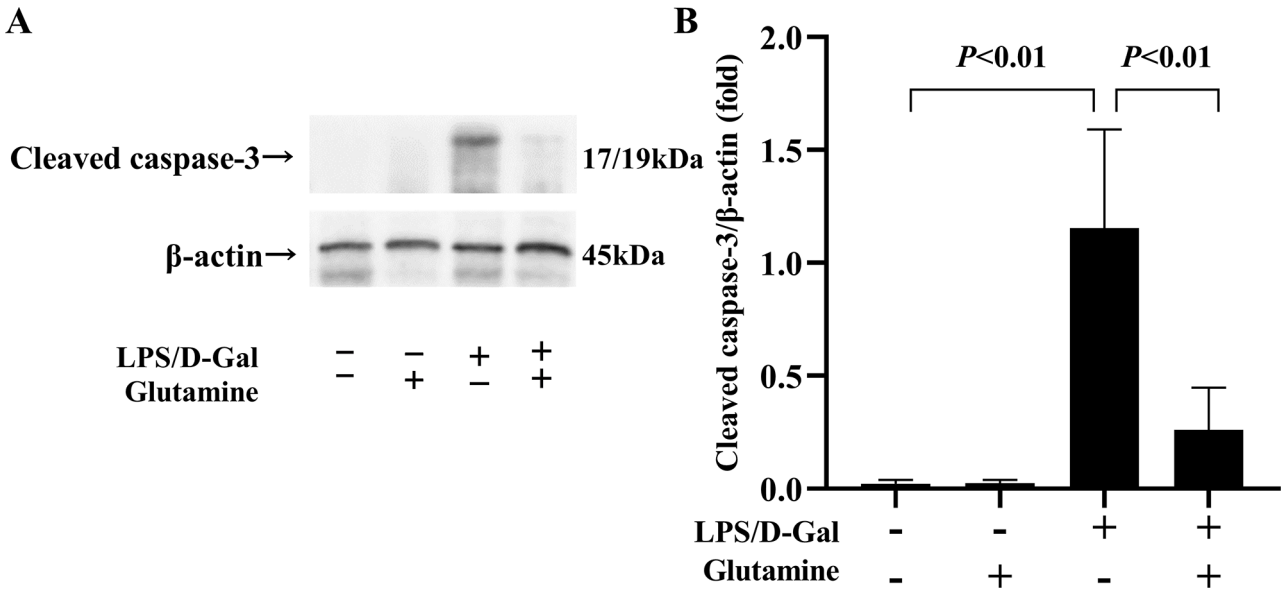

Figure 5. Pretreatment with glutamine suppresses LPS/D-Gal-induced upregulation of cleaved caspase-3. Liver samples were collected 6 h after LPS/D-Gal exposure. (A) The level of cleaved caspase-3 in liver tissue were detected by immunoblot analysis. (B) The blots were semi-quantified. Values are means ± SD, $n = 4$.

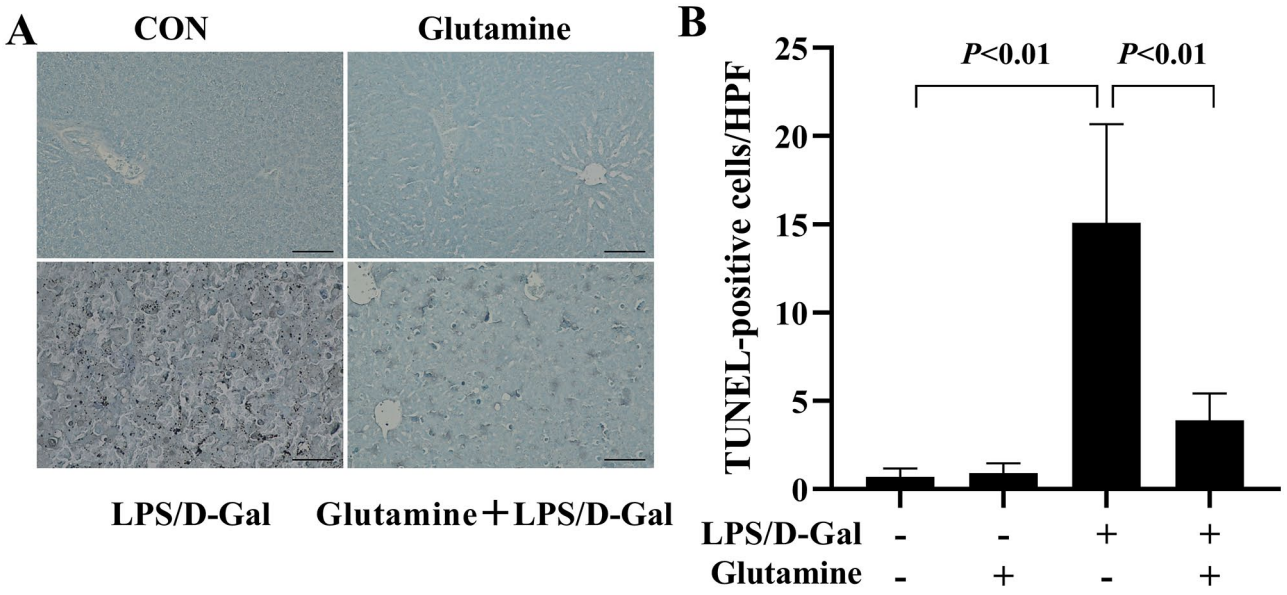

Figure 6. Pretreatment with glutamine decreases hepatocyte apoptosis in LPS/D-Gal-exposed mice. Liver samples were collected 6 h after LPS/D-Gal exposure. Apoptotic cells were detected using the TUNEL method, with the dark-brown nuclei representing the TUNEL-positive cells. (A) The representative liver sections of each group were shown. Scale bar = 100 μm. (B) 10 high power fields were randomly selected to count the number of TUNEL-positive cells. Values are means ± SD, $n = 4$.

Post-treatment with glutamine attenuates LPS/D-Gal-induced liver damage

In order to investigate whether glutamine has therapeutic potential in liver injury induced by LPS/D-Gal, we administered glutamine 1.5 h after LPS/D-Gal injection. Glutamine post-treatment attenuated plasma ALT and AST activities and improved the ultrastructural abnormalities inflicted by LPS/D-Gal (Figure 7). In addition, caspase-3 activity and the TUNEL-positivity of cells in LPS/D-Gal-induced mice were also inhibited by glutamine post-treatment (Figure 8).

Discussion

A compelling body of evidence indicates that glutamine, the most abundant free amino acid in the mammalian body, has appreciable therapeutic potential in several pathological conditions.[12,16,23] The present study demonstrated that treatment with glutamine effectively alleviated LPS/D-Gal-induced fulminant hepatitis, as evidenced by the suppressed plasma ALT and AST activities, and attenuated hepatic histological abnormalities. These findings corroborate and extend previous studies, where glutamine supplementation alleviated hepatic disorders induced by acetaminophen overdose,

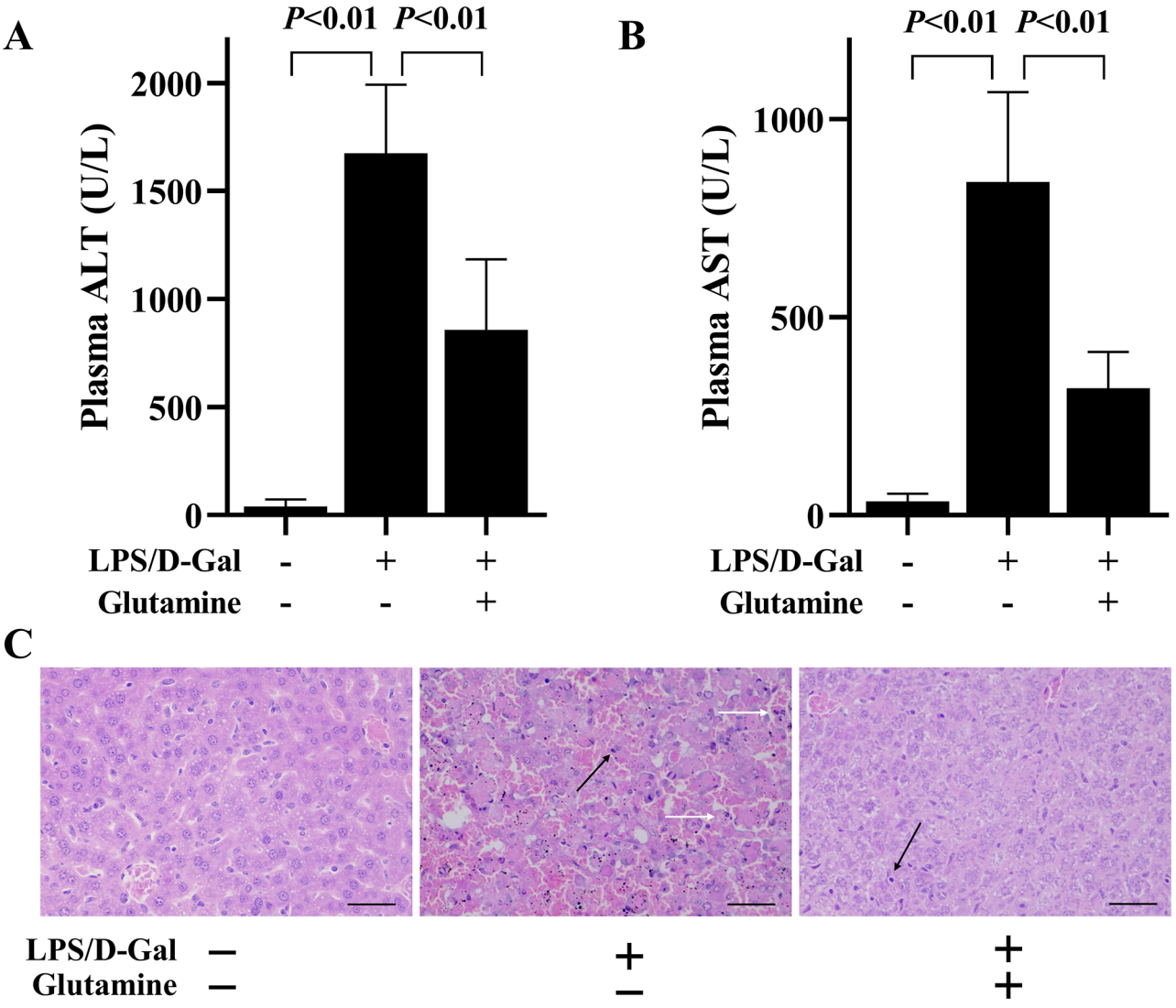

Figure 7. Post-treatment with glutamine protects against LPS/D-Gal-induced liver injury. Glutamine was injected intraperitoneally 1.5 h after LPS/D-Gal exposure. The mice were sacrificed 6 h after LPS/D-Gal injection, and plasma was collected to determine (A) ALT and (B) AST levels. Values are means ± SD, $n = 8$ per group. Representative hematoxylin and eosin-stained liver sections are shown (C). White arrows indicate hepatocyte necrosis; black arrows indicate inflammatory cell infiltration. Scale bar = 50 μm.

carbon tetrachloride (CCl_4) intoxication, thioacetamide (TAA) exposure, and ischemia-reperfusion.[24–27] Therefore, glutamine supplementation might provide hepatoprotective benefits in inflammatory-, oxidative stress- and metabolic disturbance-based liver injury.

In LPS/D-Gal-induced liver injury, inflammatory cells are activated and many pro-inflammatory cytokines are secreted.[28] TNF-α and IL-6 are the main pro-inflammatory cytokines that play crucial roles in the development of liver injury induced by LPS/D-Gal.[19,20] In LPS/D-Gal-induced acute liver injury, TNF-α levels rise rapidly, usually peaking 1–2 h after LPS/D-Gal exposure.[29,30] Subsequently, TNF-α, an early-phase pro-inflammatory factor, can stimulate neutrophils, monocytes, and vascular endothelial cells to release inflammatory factors, such as IL-6, resulting in cascade amplification and hyperactivation of the inflammatory response over the next several hours.[31] Therefore, in this study serum TNF-α and IL-6 concentrations were measured at 1.5 and 6 h after LPS/D-Gal exposure, respectively.[32,33] In this study, glutamine downregulated the levels of TNF-α and IL-6 in LPS/D-Gal-challenged mice. Consistent with the present findings, a previous study showed that glutamine effectively reduced the release of TNF-α and IL-6 in LPS-injected rats.[34] Glutamine also attenuated plasma TNF-α and IL-6 in rats subjected to ischemia- and reperfusion-induced gut injury.[4,35] In addition, glutamine decreased TNF-α production and protected against DSS-induced colitis in mice.[14] These results indicate that the protective effect of glutamine in LPS/D-Gal-induced liver injury may be related to its anti-inflammatory properties.

In addition to the uncontrolled inflammatory response, accumulating evidence indicates that excessive hepatocyte apoptosis is a hallmark of LPS/D-Gal-induced hepatic damage.[4,21,33,36] In the current study, glutamine treatment prevented LPS/D-Gal-induced activation of the caspase cascade, which initiated hepatocyte apoptosis. In addition, glutamine reduced the number of TUNEL-positive cells in the LPS/D-Gal-challenged liver. Collectively, these results demonstrate

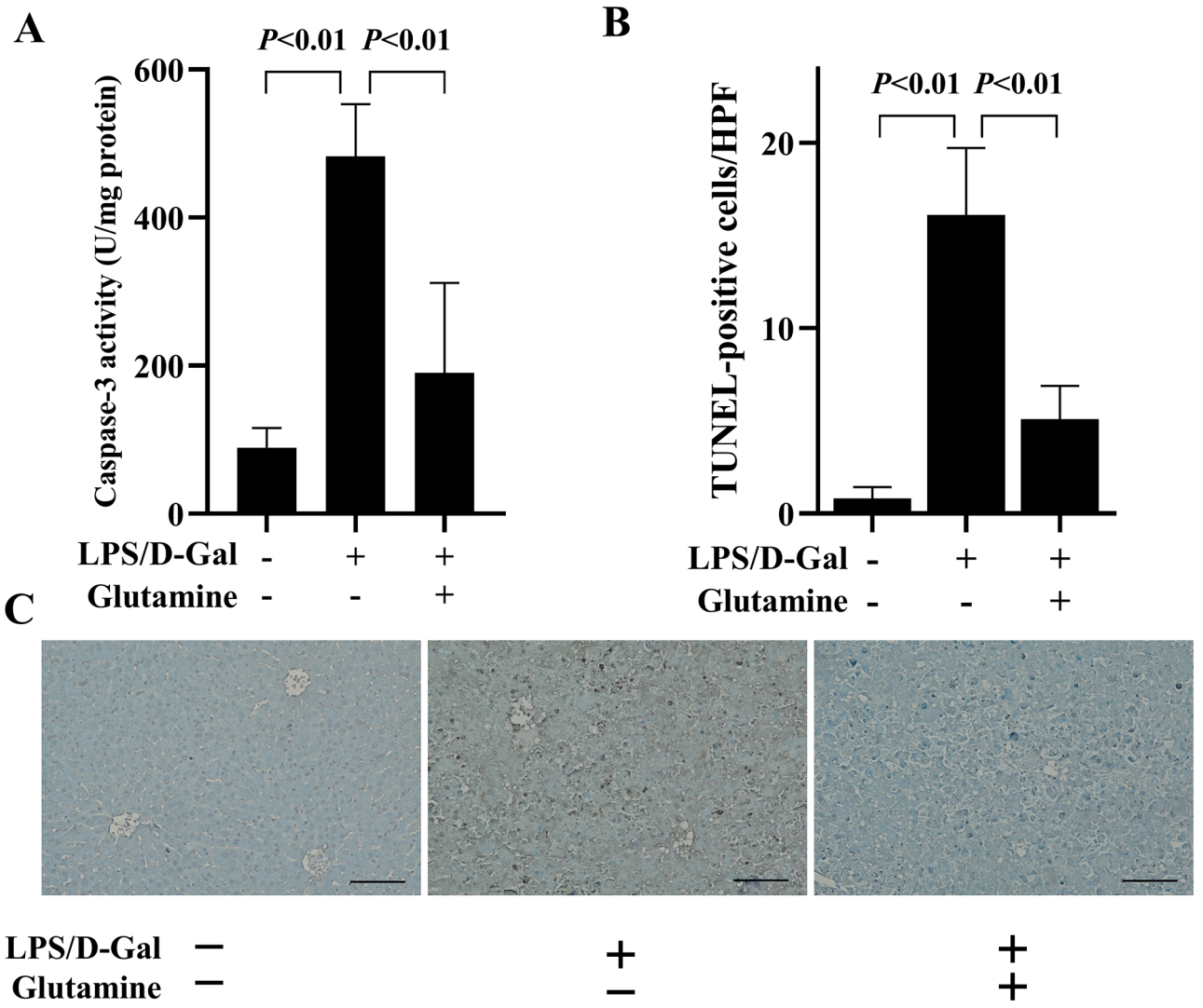

Figure 8. Post-treatment with glutamine decreases hepatocyte apoptosis in LPS/D-Gal exposed mice. Liver was sampled 6 h after LPS/D-Gal exposure to determine caspase-3 activity (A). Apoptotic cells were detected using the TUNEL method, and those with dark-brown nuclei indicated TUNEL-positive cells (B) which were counted in 10 high-power fields. C: Representative sections showing TUNEL-positive cells. Values are means ± SD, $n = 4$. Scale bar = 100 μm.

an anti-apoptotic effect of glutamine in LPS/D-Gal-induced liver injury. Concordant with our results, previous studies have shown that glutamine significantly decreases the number of apoptotic cells induced by ischemia/reperfusion in the liver.[37] Glutamine treatment also attenuated tubular cell apoptosis in kidney tissues of rats with myoglobinuric acute kidney injury.[38] In ischemia/reperfusion injury of the intestine, glutamine reduced the content of pro-apoptotic Bax, increased anti-apoptotic Bcl-2 content, and decreased the number of apoptotic cells in the ileum tissues of rats.[4] In addition, glutamine reduced apoptosis in rats with TNBS-induced colitis.[39] Thus, the protective effect of glutamine in LPS/D-Gal-induced liver injury may also be attributed to its antiapoptotic properties.

Apoptosis is triggered by the activation of death receptors via the extrinsic pathway.[40] Interaction of TNF-α with its death receptor leads to activation of the caspase cascade, culminating in apoptosis.[19,40] In LPS/D-Gal-induced liver injury, the increased expression of TNF-α was suppressed by glutamine, suggesting TNF-α suppression may be a molecular mechanism underlying the anti-apoptotic actions of glutamine. These anti-apoptotic actions were confirmed by *in vitro* experiments.[41] For example, glutamine increased cell survival and decreased apoptosis in TNF-α/IFN-γ-induced young adult mouse colonic epithelial cells.[42] Glutamine also suppressed apoptosis in camptothecin-induced IEC-18 cells following heat shock.[43] In addition, glutamine decreased apoptosis induced by the endoplasmic reticular stressors brefeldin A and tunicamycin in Caco-2 cells.[39] Therefore, glutamine might modulate apoptotic pathways and provide anti-apoptotic benefits in LPS/D-Gal-induced liver injury.

Since glutamine pretreatment would be impractical in most clinical situations, we tested whether glutamine is hepatoprotective when administered after the insult. Interestingly, glutamine post-treatment decreased plasma ALT and AST activities and alleviated the liver histological abnormalities induced by LPS/D-Gal. The findings suggest

that glutamine post-treatment may have therapeutic potential for treatment of acute liver injury.

Collectively, the results of this study demonstrated that treatment with glutamine alleviated LPS/D-Gal-induced liver injury, which may be attributed to its anti-inflammatory and antiapoptotic properties. Although further investigation is required to decipher the molecular mechanisms mediating glutamine hepatoprotection, this study indicates that glutamine might have potential value as a pharmacological intervention in acute liver injury.

AUTHORS' CONTRIBUTIONS

Y-QY and LZ designed the experiments. M-XY and X-YZ conducted the experiments. Data analysis was performed by SZ, R-YS, K-RF, and KH. M-XY, LZ, and Y-QY wrote the manuscript. All authors reviewed the final manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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