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Glucosamine attenuates alcohol-induced acute liver injury via inhibiting oxidative stress and inflammation

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ABSTRACT

Alcohol liver disease (ALD) is a liver disease caused by long-term heavy drinking. Glucosamine (GLC) is an amino monosaccharide that plays a very important role in the synthesis of human and animal cartilage. GLC is commonly used in the treatment of mild to moderate osteoarthritis and has good anti-inflammatory and anti-oxidant properties. In this study, alcoholic injury models were constructed in mice and human normal hepatocyte L02 cells to explore the protective effect and mechanism of GLC on ALD. Mice were given GLC by gavage for 30 days. Liver injury models of both mice and L02 cells were produced by ethanol. Detecting the levels of liver injury biomarkers, lipid metabolism, oxidative stress biomarkers, and inflammatory factors through different reagent kits. Exploring oxidative and inflammatory pathways in mouse liver tissue through Western blot and RT-PCR. The results showed that GLC can significantly inhibit the abnormal increase of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), triglycerides (TG), total cholesterol (TC), very low density lipoprotein (VLDL), low-density lipoprotein cholesterol (LDL-C), and can significantly improve the level of high-density lipoprotein cholesterol (HDL-C). In addition, GLC intervention significantly improved alcohol induced hepatic oxidative stress by reducing the levels of malondialdehyde (MDA) and, increasing the levels of glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) in the liver. Further mechanisms suggest that GLC can inhibit the expression of ethanol metabolism enzyme cytochrome P4502E1 (CYP2E1), activate the antioxidant pathway Keap1/Nrf2/HO-1, down-regulate the phosphorylation of MAPK and NF-κB signaling pathways, and thus reduce the expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6). Therefore, GLC may be a significant candidate functional food for attenuating alcohol induced acute liver injury.

1. Introduction

At present, the International Agency for Research on Cancer (IARC) has classified alcoholic beverages as a carcinogenic substance, which greatly affects the health of people around the world (Seitz et al., 2018). According to data released by the World Health Organization (WHO), alcohol consumption is associated with more than 200 diseases (Axley et al., 2019). At the same time, after alcohol enters the body, most of it is metabolized by the liver. Alcoholic liver disease (ALD) caused by long-term excessive drinking is one of the most common chronic liver diseases in the world, and has become one of the common causes of human death every year in the world (Kong et al., 2019). ALD includes a series of pathological processes such as hepatic steatosis, alcoholic fatty liver disease, alcoholic steatohepatitis and so on. Without treatment and

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intervention, ALD will further deteriorate into cirrhosis and produce other complications, and finally even develop into hepatocellular carcinoma (Bataller et al., 2022).

Glucosamine (GLC) is an endogenous amino monosaccharide synthesized from glucose and is generally found naturally in cartilage and synovial fluid around joints in the form of sulfate or hydrochloride (Mendis et al., 2008). It is an important molecule in the biochemical pathways for the synthesis of glycosylated proteins and lipids, and is also involved in the biosynthesis of cartilage glycoproteins and glycosaminoglycan (Chen et al., 2020). GLC is one of the most common dietary supplements in the United States, and it can also help treat osteoarthritis (Qato et al., 2008; Liu et al., 2017). GLC has been found to have physiological activities such as anti-inflammatory, anti-oxidation, regulation of immune function, inhibition of bacteria, and anti-aging (Zahedipour et al., 2017). In addition, GLC has good safety and no hepatotoxicity and other toxic side effects on human body (Veronese et al., 2020).

In view of the good antioxidant and anti-inflammatory effects of GLC, and the pathogenesis of ALD is closely related to the occurrence of inflammation and oxidative damage, and there is no research or report on the treatment of ALD patients with GLC. Therefore, in this study aimed to explore the protective effect of GLC on acute alcoholic liver injury through in vivo and in vitro acute alcoholic injury models. Objective to explore the protective effect of GLC on ALD by detecting serum inflammation, lipid metabolism, liver oxidative damage and other related indicators. The underlying mechanism was explored by real-time PCR and Western blotting. It lays a theoretical foundation for the development of dietary supplements to prevent ALD.

2. Materials and methods

2.1. Reagents

D-glucosamine hydrochloride (Purity: 98.5%) was purchased from Shanghai McLean Biochemical Technology Co., LTD. Bifendate samples were purchased from Shanghai McLean Biochemical Technology Co., LTD.

2.2. Cell culture

Human normal liver L02 cells were purchased from the Cell Bank of the Chinese Academy of Sciences. Using RPMI 1640 medium (Gibco, USA) containing 10% foetal bovine serum (HyClone, USA) and 1% Penicillin-Streptomycin Solution (10000 Units/mL Penicillin, 10000 μg/mL Streptomycin, HyClone, USA), normal human hepatocytes L02 were cultured in vitro at 37 °C and 5% CO_2. Upon reaching 70–80% confluence, adherent cells were subcultured or frozen (Zhou et al., 2022).

The Cell Counting Kit 8 (CCK-8) method was used to determine the optimal concentration of alcohol required for establishing a liver cell injury model and to screen the dosage of GLC administration.

L02 hepatocytes with normal development were placed in 96 well plates, cultured for 10–12 h. GLC samples were added according to the concentration gradient of 0, 0.25, 0.50, 1.00, 2.00, 4.00 and 6.00 mg/mL, respectively, and cells were divided into 7 experimental groups. After 24 h of culture, CCK-8 method was used to determine the survival rate of cells in each group. With a survival rate higher than 95% as the standard, the final low, medium and high GLC concentrations were 0.25 mg/mL, 0.50 mg/mL, and 1.00 mg/mL, respectively (Table S1).

L02 hepatocytes with normal development were placed in 96 well plates, cultured for 10–12 h. Alcohol was added to each experimental group, and 10 concentrations were set between 0-500 mM, with a difference of 50 mM between each concentration gradient, and cells were divided into 10 experimental groups. CCK-8 method was used to determine the cell survival rate of each group. The selection of the modelling concentration was based on the half-maximal inhibitory concentration (IC50) principle. When the alcohol concentration was 400 mM, the cell survival rate was 50 ± 7%, and the lethality rate was approximately 50%. Therefore, 400 mM was chosen as the alcohol modeling concentration for this experiment (Table S2).

2.3. Determination of L02 cell damage and the oxidation-antioxidant index

The L02 cell experiment was divided into 5 groups: control group (CON), alcohol model control group (MOD), and GLC low (GLC L), GLC medium (GLC M), and GLC high (GLC H), and each group was inoculated into a 6-well plate. In the GLC L, GLC M, and GLC H groups, the doses of GLC were 0.25 mg/mL, 0.50 mg/mL, and 1.00 mg/mL, respectively. Each GLC administration group was treated with 200 μL of medium containing different concentrations of GLC and incubated in incubator for 12 h, and then, 200 μL of 400 mM alcohol was added to the cells; the CON group was treated with the same amount of culture medium as the experimental groups, and 200 μL of 400 mM alcohol was added to the MOD group, and the culture was maintained for another 12 h. The cell culture medium of each group was collected, and the supernatant AST and ALT levels were determined after centrifugation. After extracting cell culture medium, cells were digested with mild trypsinisation and collected to homogenize in 0.1 mol/L, pH7–7.4 phosphate buffer, including MDA, GSH, SOD, and CAT levels, were measured in the cell homogenate. The biological reagent kit used for the above activity indicators was purchased from Nanjing Jiancheng Biotechnology Research Institute. (Jiangsu, China).

2.4. Animal grouping and administration

The experimental animals were male Kunming mice (SPF grade, 7–8 weeks old) provided by Guangdong Medical Laboratory Animal Center (Guangzhou, Guangdong, China) (animal production license number: SCXK (Guangdong) 2018-0002). Mice were housed in the SPF Laboratory of the experimental animal center of Guangdong Pharmaceutical University (license number: SYXK (Guangdong) 2017-0125). This study was approved by the Laboratory Animal Ethics Committee of Guangdong Pharmaceutical University and was performed in strict accordance with the requirements of the "Guidelines for the Ethical Review of Laboratory Animal Welfare" (GB/T35892-2018).

In the Laboratory Animal Research Center of Guangdong Pharmaceutical University, SPF (Specific Pathogen Free) grade Kunming male mice were adaptively reared for 7 days on a conventional diet. All experiments were carried out in the SPF level environment (the animal center feeding environment temperature: 24.0 ± 2.0 °C, relative humidity: 54–65%, air exchange frequency more than 15 times/hour, 12 h of light and dark alternating every day). The normal diet (containing 20% protein, 0% carbohydrate, 4.3% fat, 4.8% cellulose, and 1.96% mineral) was provided by the Laboratory Animal Center of Guangdong Pharmaceutical University. Subsequently, 60 experimental Kunming male mice were divided into 6 groups according to blank control group (CON), ethanol model group (MOD), high, medium, and low dose GLC group (H, M, L), and positive drug control group biphenyl ester group, with 10 mice in each group.

This study referred to the construction method of ALD mouse model and the administration time and dosage of glucosamine in the "Technical Specification for Health Food Inspection and Evaluation" issued by the Chinese Ministry of Health. The recommended human intake for GLC is 720–1440 mg/d/60 kg. In this study, 1440 mg/d/60 kg was used as the recommended dose, and 5, 10, and 20 times the recommended dose were used as low, medium, and high doses, respectively. The specific dosage groups were GLC-L:120 mg/kg BW (Body weight), GLC-M:240 mg/kg BW, GLC-H:480 mg/kg BW, Bifendate positive drug: 150 mg/kg BW(Wu et al., 2019). The drug was dissolved in distilled water to prepare samples with a concentration of 0.1 ml/10 g BW, which were used as drugs of GLC-L, GLC-M and GLC-H groups, respectively. The mice in GLC group and Bifendate group were given corresponding samples through tube feeding method using the same technique for 30

days. For the accuracy of the experiment, a single variable was achieved, the mice in MOD group and CON group were fed with distilled water for 30 days. During this procedure, the weight of mice was recorded once a week. On the 30th day, after the last gavage, the Bifendate group, GLC-H group, GLC-M group, GLC-L group and MOD group were given a single gavage with 14 mL/kg BW of 50% ethanol, and the blank control group was treated with corresponding distilled water according to the weight of the mice. Then all the test animals were fasted for 16 h. After 16 h, all the mice were anesthetized and serum samples were obtained. After that, the mice were euthanized under anesthesia, dissected, and all required organs and tissues were taken out and placed in liquid nitrogen for quick freezing, and then the tissues were stored in an ultra-low temperature freezer at −80 °C for subsequent analysis.

2.5. Serum hepatocyte injury markers, blood lipid indexes, and serum inflammatory factors were detected biochemical analysis

The collected mice blood was added to 2.0 mL sterile Eppendorf (EP) tubes, and the serum was allowed to stand for 1 h at room temperature until significant stratification of the blood can be observed and then centrifuged in a centrifuge at 4 °C (3000 rpm, 15 min) to obtain a supernatant serum sample (Zhou et al., 2023). The results of serum ALT, AST, ALP, LDH, TNF-α, IL-1β, IL-6, TG, TC, HDL-C, LDL-C and VLDL were determined by appropriate kits. TNF-α, IL-1β, IL-6 ELISA kits were purchased from Jiangsu Meimian industrial Co., Ltd. (Jiangsu, China). Other kits were provided by Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

2.6. Histopathological observation of the liver

After the mice were dissected and weighed, 0.5 cm^3 livers were cut out, washed with PBS, placed in an embedding box, then placed in 10% formalin, removed after 24 h, dehydrated with different percentage of ethanol according to the preset procedure, and finally added with xylene for infiltration. After complete drying, they were embedded in high melting point paraffin at 60 °C. The embedded tissues were placed in a cryogenic freezer at −20 °C for 30–60 min, removed and sliced with a microtome, and slices with a thickness of 4 μm were selected. After sectioning, the slides were placed in a tank with warm water at 40 °C for spreading, and then collected by adsorption with slides, baked in a tablet dryer for 2 h, collected and stored in a slice box, and then stained with HE. In addition, the thickness of the sections was set on the freezing microtome to 10 μm, and the slides were placed using adhesive slides after completion. The cut frozen liver sections were collected and stained with oil red O (Feng et al., 2022). The liver injury scoring system comprised: steatosis (0–3), lobular inflammation (0–2), hepatocellular ballooning (0–2), and fibrosis (0–4)(Kleiner et al., 2005). Image Pro Plus 6.0 software was used to calculate the ratio of lipid accumulation area to total liver area (Qiu et al., 2022). The liver injury and lipid droplets scores were performed on each slice by six members of the research group using a blind method. Subsequently, data were collected and statistically analyzed.

2.7. Western blot analysis

L02 liver cells and liver tissue were homogenized with RIPA (Dalian Meilun, China) lysate containing 1% PMSF at 4 °C and 3000 rpm for 15 min at low temperature. After completion, they were placed on ice for 30 min to achieve complete lysis. Then, they were centrifuged at 12000 rpm for 20 min at 4 °C, and the supernatant was separated and aspirated into an EP tube. Quantification of previously extracted proteins using BCA protein concentration detection reagent, dilute the protein to a concentration of 4 μg/μL. Afterwards, add the sample buffer and mix the sample well, and place the sample in a 100 °C metal bath for 10 min. Configure electrophoresis gel in advance as required, and add on the swimlane with protein biomarkers of 3 μL and Protein sample of 10 μL, and after electrophoresis, using electricity to convert the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Merck KGaA, Darmstadt, Germany), which was washed 3 times with 1 × TBST and 10 min each time. Then seal for 1 h with skimmed milk. Based on the molecular weight of the target protein and the localization of protein markers, the primary antibody was added and incubated at 4 °C for 12 h, and then the secondary antibody was added and incubated for 1 h. The antibodies used in the experiments were purchased from Abcam (Cambridge, UK), CYP2E1 (ab28146, rabbit anti-mouse), Keap1 (ab227828, rabbit anti-mouse), HO-1 (ab13243, rabbit anti-mouse), Nrf2 (ab92946, rabbit anti-mouse), NF-kB p65 (ab16502, rabbit anti-mouse), p–NF–kB p65 (ab76302, rabbit anti-mouse), c-Jun N-terminal kinase (JNK) (ab179461, rabbit anti-mouse), p-JNK (ab76572, rabbit anti-mouse), p38 MAPK (ab170099, rabbit anti-mouse), and p-p38 MAPK (ab195049, rabbit anti-mouse). GAPDH (10494-1-AP, rabbit anti-mouse) and secondary antibody HRP-conjugated Affinipure IgG (H + L) (SA00001-2, goat anti-rabbit) were purchased from Proteintech (Wuhan, China). The PVDF membrane was washed 3 times, an HRP-enhanced chemiluminescence (ECL) reagent (Meilun, DaLian, China) was added to develop the blot, which was photographed. Data analysis was performed with an automatic gel imager (ChemiDoc XRS+, BioRad, USA).

2.8. RT-PCR analysis

Total RNA extraction reagent RNAiso plus reagent (Takara, Japan) was added to L02 cells and liver tissues. According to the instructions, the above reagents were centrifuged at 4 °C and 7500 rpm for 5 min, and the supernatant was discarded to extract total RNA. The content and purity of total RNA were determined by nucleic acid ultra micro quantitative analyzer. DNA was removed with a kit (Takara, Japan), and RNA was reverse transcribed into cDNA. Tables S3 and S4 show target gene primer sequences (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China). The PCR program was set as follows: Pre denaturation 95 °C for 30 s, denaturation 95 °C for 5 s, and 60 °C for 30 s in 30 cycles. After the reaction was completed, β-actin was used as the internal reference gene, and the $2^{-\Delta\Delta Ct}$ method was used to analyse the gene expression in the groups and calculate the relative expression of the detected genes (You et al., 2022).

2.9. Statistical analysis

The data and analysis in the experiment were analyzed and processed by GraphPad Prism 8.3.0, and the specific data expression results were analyzed by the means ± SD. The statistical significances of results were analyzed by one-way analysis of variance (ANOVA), and Tukey's multiple comparison test, including a single merged variance, was used to compare the differences between groups. A P value less than 0.05 was considered to indicate a significant difference between the two groups.

3. Results

3.1. Protective effect of GLC on L02 hepatocytes in alcoholic liver injury

L02 hepatocytes were treated with GLC for 12 h, and then treated with alcohol for 12 h. The growth status of cells in different treatment groups is shown in Fig. 1 (a). In comparison with CON group, the morphology of the cells in the MOD group changed greatly, and the number of normal L02 hepatocytes with dark color was less. Compared with the MOD group, the number of cell death in GLC-L group and GLC-M group was slightly reduced, but there were still abnormal cells angle morphology. GLC-H group could significantly improve the growth state and number of L02 hepatocytes.

The survival rate of cells is one of the important indicators for studying drug efficacy. The supernatant of culture medium was collected, and the cell survival rate was measured by CCK-8 method. As

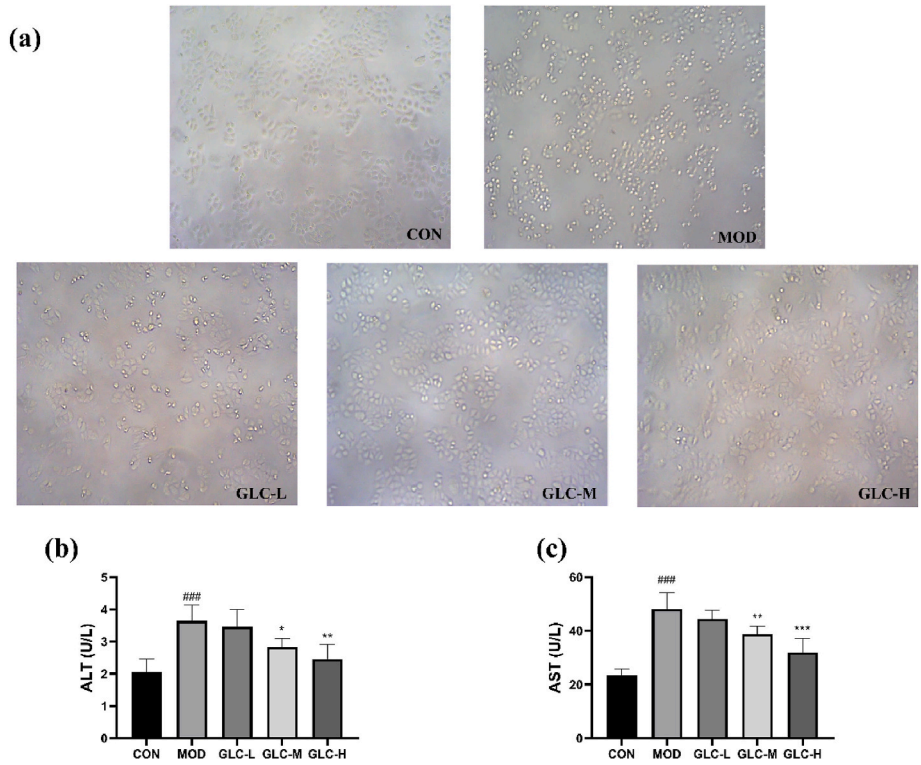

Fig. 1. Effect of GLC on L02 hepatocytes in alcohol intervention. (a) Effect of GLC on the status of L02 hepatocytes in alcohol intervention (200 ×). (b) alanine aminotransferase (ALT) (n = 6, mean ± SD). (c) aspartate aminotransferase (AST) (n = 6, mean ± SD) **Notes:** Compared with the control (CON) group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$; compared with the alcohol model control (MOD) group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

shown in Table S5, the cell survival rate of the MOD group was significantly decreased, while each GLC administration group remarkable improved cell survival rate of L02 cells and reduced the damage of hepatocytes caused by alcohol.

The results of ALT and AST in the alcoholic injury model of L02 hepatocytes were shown in Fig. 1(b) and (c) , GLC administration reduced the production of markers of hepatocyte injury compared with the MOD group. These results suggest that prophylactic administration of GLC protects L02 hepatocytes to some extent from alcohol injury.

3.2. Effect of GLC on biomarkers of oxidative stress in L02 hepatocytes

The effects of GLC on oxidative stress indicators in L02 hepatocytes are shown in Fig. 2. In comparison with CON group, MDA level in MOD group was significantly higher than that in CON group, suggesting that alcohol caused lipid peroxidation in L02 hepatocytes. Compared with the MOD group, the GLC administration group can reduce MDA content, and the high dose of GLC can significantly reduce MDA content. For the three antioxidants GSH, SOD and CAT, the expression levels of MOD group were significantly reduced due to oxidative stress injury caused by

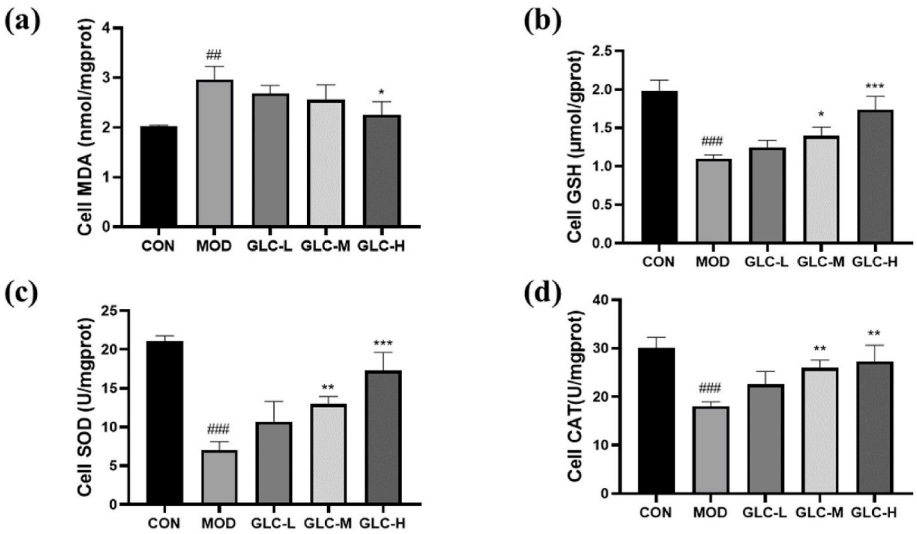

Fig. 2. Effect of GLC on biomarkers of oxidative stress (a) malondialdehyde (MDA), (b) glutathione (GSH), (c) catalase (CAT) and (d) superoxide dismutase (SOD) in L02 hepatocytes (n = 3, mean ± SD). **Notes:** Compared with the MOD group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; compared with the CON group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$.

alcohol, and GLC intervention could significantly increase their expression levels, thereby resisting oxidative stress injury. These results indicate that GLC can reduce alcohol-induced oxidative stress injury in L02 hepatocytes to a certain extent.

3.3. Effect of GLC on serum lipid metabolism in mice

Alcohol metabolism into the liver can cause excessive lipid accumulation and lipid metabolism disorders in hepatocytes, thereby inhibiting fatty acid oxidation and promoting lipid peroxidation. The results are shown in Fig. 3. The serum TC, TG, VLDL and LDL-C in the MOD group were significantly increased, while HDL-C was significantly decreased. Compared with MOD group, GLC administration group had significant improvement on the above lipid metabolism functions. All groups treated with GLC had obvious lipid-lowering effect, and its lipid-lowering effect was better than that of Bifendate. GLC administration groups can significantly reduce the level of TG, and the effect of GlC-M and GlC-H groups is significantly better than that of Bifendate. GLC administration groups can significantly reduce the level of VLDL, and the therapeutic effect of the middle and high dose groups of GLC is equivalent to that of the positive drug Bifendate group. GLC-M and GLC-H groups can significantly increase the level of HDL-C, and GLC-M and GLC-H groups can significantly increase the expression level of LDL-C. These results indicated that GLC could improve the disorder of serum lipid metabolism and maintain the homeostasis of lipid metabolism in ALD mice.

3.4. Effect of GLC on serum hepatocyte injury markers in mice

ALT and AST are the main serum biomarkers to test the degree of liver injury. ALP and LDH are serum measures of the integrity of organelles and membranes in hepatocytes. It can be seen from Fig. 4 that the expressions of ALT, AST, ALP and LDH were significantly increased under the stimulation of ethanol compared with the CON group. Bifendate could significantly reduce the expression of the above liver injury markers. The levels of ALT in GLC-M group and ALT, AST, ALP and LDH in GLC-H group were significantly lower than those in GLC-M group. The results showed that GLC administration could reduce the damage of liver cells induced by alcohol, and had a certain improvement effect on alcoholic liver injury.

3.5. Effect of GLC on serum inflammatory factors in mice

Inflammatory response is a crucial factor in the development of ALD. Reducing and improving inflammatory response is an effective way to treat ALD. TNF-α, IL-6 and IL-1β are important factors to judge the process and severity of liver inflammation. As can be seen from Fig. 4 (e)–(g), the expression levels of TNF-α, IL-6 and IL-1β in the MOD group were significantly higher than those in the CON group, indicating that alcohol had already caused inflammatory stress damage to the liver. The levels of inflammatory cytokines were reduced in all doses of GLC, and the levels of these inflammatory cytokines were significantly reduced in the GLC-H group. In conclusion, GLC can reduce the release of inflammatory factors in the liver, thereby reducing the inflammatory reaction in the liver and alleviating the inflammatory stress injury caused by alcohol.

3.6. Effect of GLC on biomarkers of oxidative stress in mouse liver

MDA is a lipid peroxidation biomarker used to evaluate the severity of oxidative stress, and it is also the most commonly used indicator to measure oxidative stress. It can be observed from Fig. 5(a) that compared with CON group, the lipid peroxidation level of MOD group was significantly increased, and the expression of MDA was significantly reduced in each dose group of GLC, and the therapeutic effect was equivalent to that of Bifendate group.

GSH is one of the most important cellular REDOX buffers for scavenging ROS and the main defense protein against oxidative stress. Excessive ROS produced by alcohol metabolism can easily deplete GSH in the liver, so the detection of GSH is also an important standard to judge the degree of oxidative stress damage. It can be observed from Fig. 5(b) that the GSH expression level of the MOD group was significantly lower than that of the CON group, and the GLC-H administration group was able to significantly increase the GSH expression level and

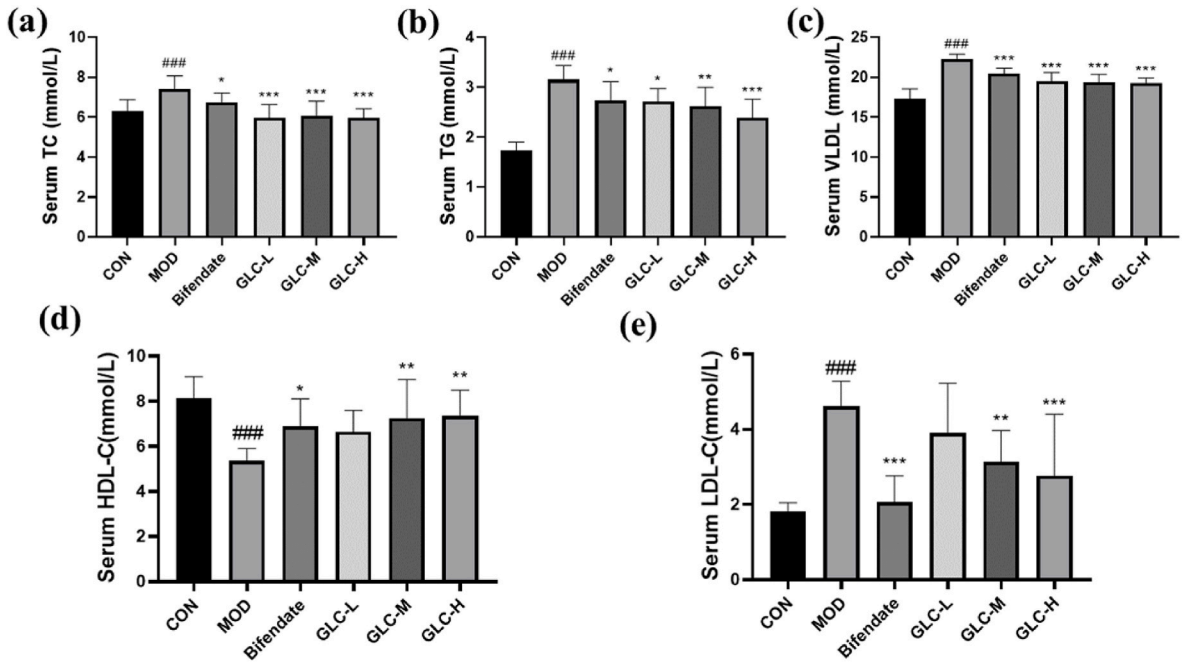

Fig. 3. The influence of GLC on lipid metabolism (a) total cholesterol (TC), (b) triglycerides (TG), (c) very low density lipoprotein (VLDL), (d) high-density lipoprotein cholesterol (HDL-C), (e) low-density lipoprotein cholesterol (LDL-C) (n = 10, mean ± SD). **Notes:** Compared with the MOD group, *p < 0.05, **p < 0.01, and ***p < 0.001; compared with the CON group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$.

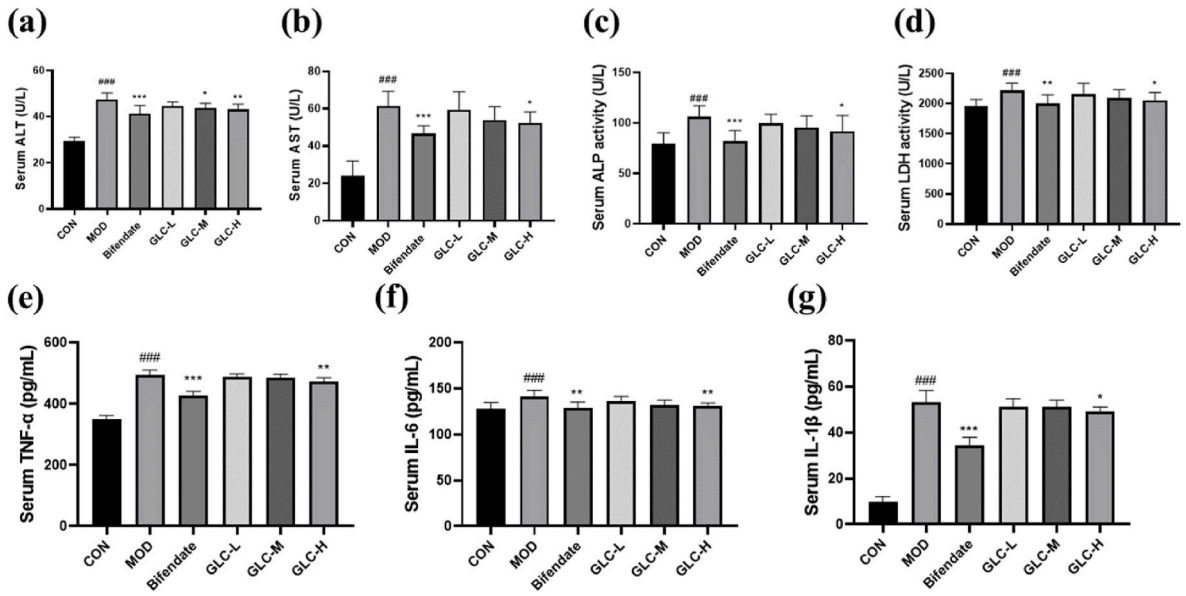

Fig. 4. Influence of GLC on serum hepatocyte injury markers (a) alanine aminotransferase (ALT), (b) aspartate aminotransferase (AST), (c) alkaline phosphatase (ALP), (d) lactate dehydrogenase (LDH), (e) tumor necrosis factor-α (TNF-α), (f) interleukin-6 (IL-6), (g) interleukin-1β (IL-1β) (n = 10, mean ± SD). **Notes:** Compared with the MOD group, *p < 0.05, **p < 0.01, and ***p < 0.001; compared with the CON group, $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, and $^{\#\#\#}$p < 0.001.

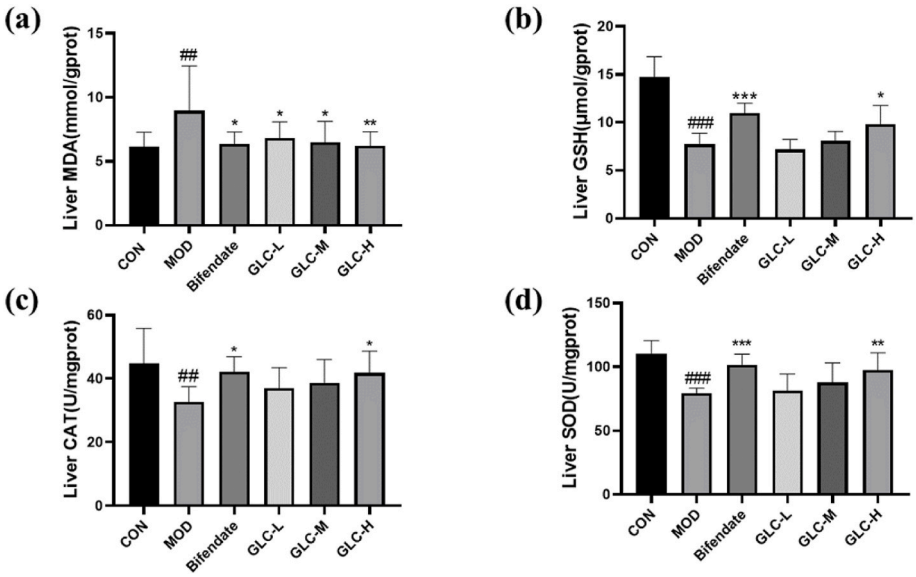

Fig. 5. Effect of GLC on hepatic oxidative stress biomarkers (a) malondialdehyde (MDA), (b) glutathione (GSH), (c) catalase (CAT) and (d) superoxide dismutase (SOD) (n = 10, mean ± SD). **Notes:** Compared with the MOD group, *p < 0.05, **p < 0.01, and ***p < 0.001; compared with the CON group, $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, and $^{\#\#\#}$p < 0.001.

increase the antioxidant capacity of the liver.

CAT and SOD are linked to each other in scavenging superoxide anion free radicals and produce a synergistic effect, which is crucial in maintaining the body's REDOX homeostasis. As can be seen from Fig. 5 (c)–(d), SOD and CAT contents were significantly reduced in alcohol-exposed MOD group, and SOD and CAT contents were increased in each dose group of GLC, among which GLC-H group significantly increased the expression levels of SOD and CAT.

In conclusion, GLC can inhibit the expression of MDA, reduce the oxidative stress caused by ROS, and increase the expression of GSH, SOD and CAT, thereby improving the antioxidant function of liver cells, thereby alleviating the alcohol-induced oxidative damage of liver cells.

3.7. Effect of GLC on liver pathological changes in mice

From Fig. 6, it was observed that the hepatocytes in the CON group were arranged neatly, regularly and tightly, with clear cell boundaries, and no steatosis and inflammatory infiltration were seen. In the MOD group, fat vacuoles and steatosis were observed in the liver, inflammatory infiltration was observed, and the boundaries between liver cells were disordered and arranged in disorder. Likewise, the MOD group exhibited a higher liver injury score compared to the CON group. The GLC-M and GLC-H groups supplemented with GLC exhibited a decline in the liver injury score compared to the MOD group. The inflammatory infiltration and steatosis were improved in each dose group of GLC, and the improvement effect was more obvious in GLC-M and GLC-H groups. The boundary of liver cells tended to be clear, fat vacuoles and

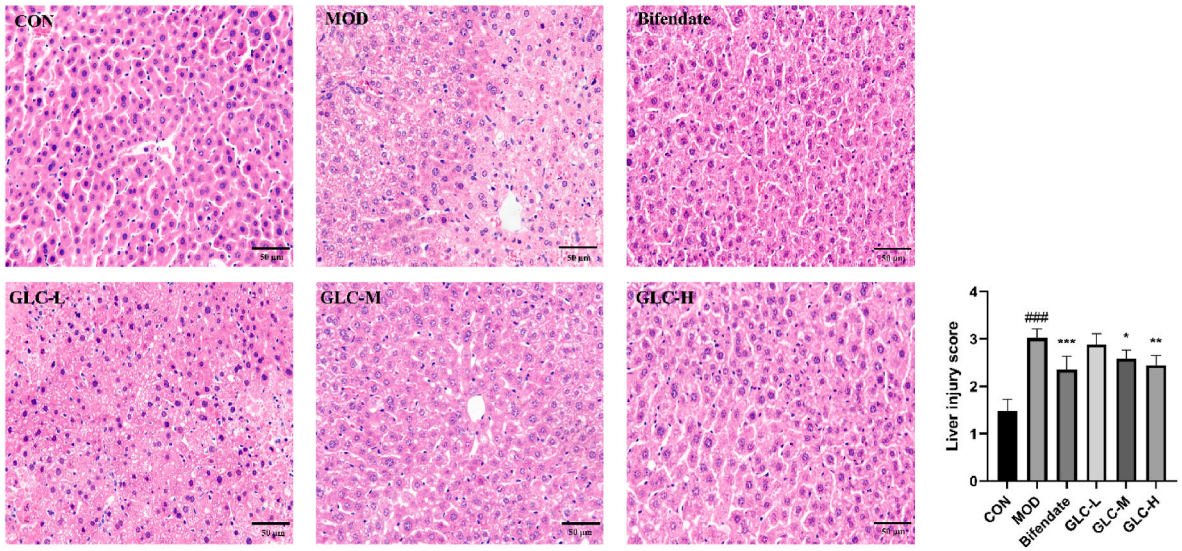

Fig. 6. Histopathological analysis. Pathological image of liver tissue stained with haematoxylin and eosin (H&E) (200X, scale bar = 50 μm). Liver injury score (n = 10, mean ± SD). **Notes:** Compared with the MOD group, *p < 0.05, **p < 0.01, and ***p < 0.001; compared with the CON group, #p < 0.05, ##p < 0.01, and ###p < 0.001.

inflammatory infiltration were reduced, and the pathological condition of liver injury was improved.

Oil red O staining can further observe the lipid degeneration and droplet aggregation in liver pathological sections. Oil red O staining was used to stain frozen sections of liver embedded tissue. Fig. 7 shows that compared with the CON group, there were a large number of obvious bright red lipid droplets in the MOD group. The number and volume of lipid droplets in the positive drug Bifendate group were significantly reduced, and the number and volume of lipid droplets in the GLC-M and GLC-H dose groups were reduced. Compared with the CON group, the MOD group showed higher lipid droplet scores. After GLC interference, it can reduce the lipid droplet score of mouse liver.

The above results of HE staining and oil red O staining of liver pathological sections indicated that GLC administration could improve the liver pathological condition of mice, reduce lipid accumulation and inflammatory infiltration, and alleviate alcohol-induced liver injury.

3.8. Effect of GLC on the expression of alcohol metabolizing enzyme CYP2E1

CYP2E1 is mainly expressed in hepatocytes, and long-term alcohol consumption can induce increased expression of CYP2E1 (Zeng et al., 2018). Therefore, the detection of CYP2E1 expression level can objectively evaluate the protective effect of GLC on alcoholic liver injury.

The expression of CYP2E1 protein was determined by immunoblotting, and the results are shown in Fig. 8(a)-(b) and (d)-(e). The expression of CYP2E1 protein was significantly increased in the MOD-group compared with the CON group, which was the result of alcohol intake. Compared with MOD group, GLC could significantly inhibit the expression of CYP2E1.

In addition, the expression of CYP2E1 mRNA was determined by fluorescence quantitative PCR, as shown in Fig. 8(c) and (f). Compared with the CON group, the expression of CYP2E1 mRNA in the MOD group was significantly increased, and each GLC administration group could

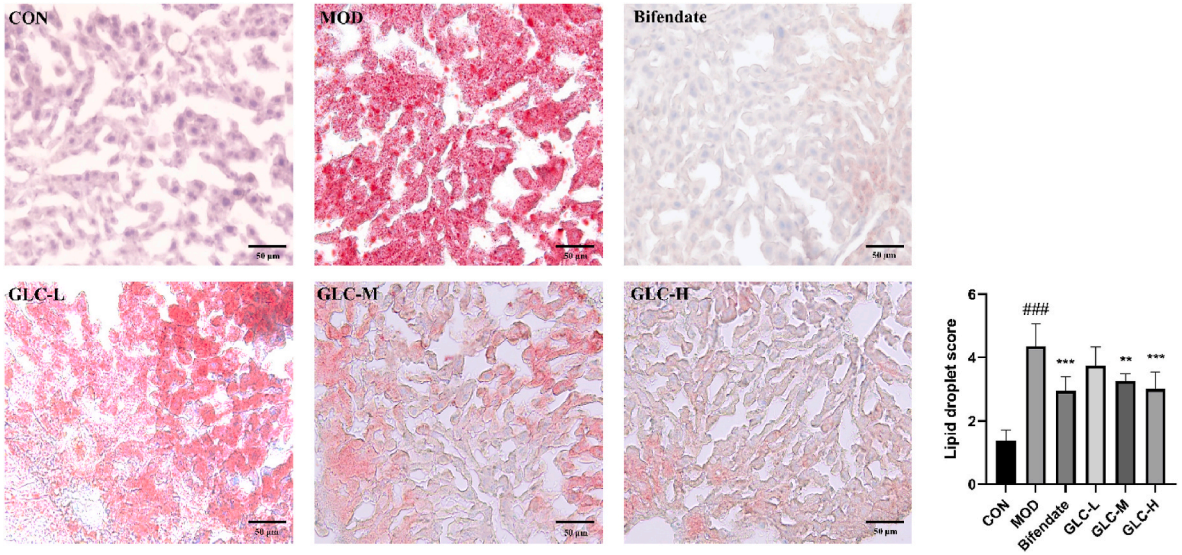

Fig. 7. Pathological image of liver tissue stained with Oil red O (200X, scale bar = 50 μm). Lipid droplet score (n = 10, mean ± SD). **Notes:** Compared with the MOD group, *p < 0.05, **p < 0.01, and ***p < 0.001; compared with the CON group, #p < 0.05, ##p < 0.01, and ###p < 0.001.

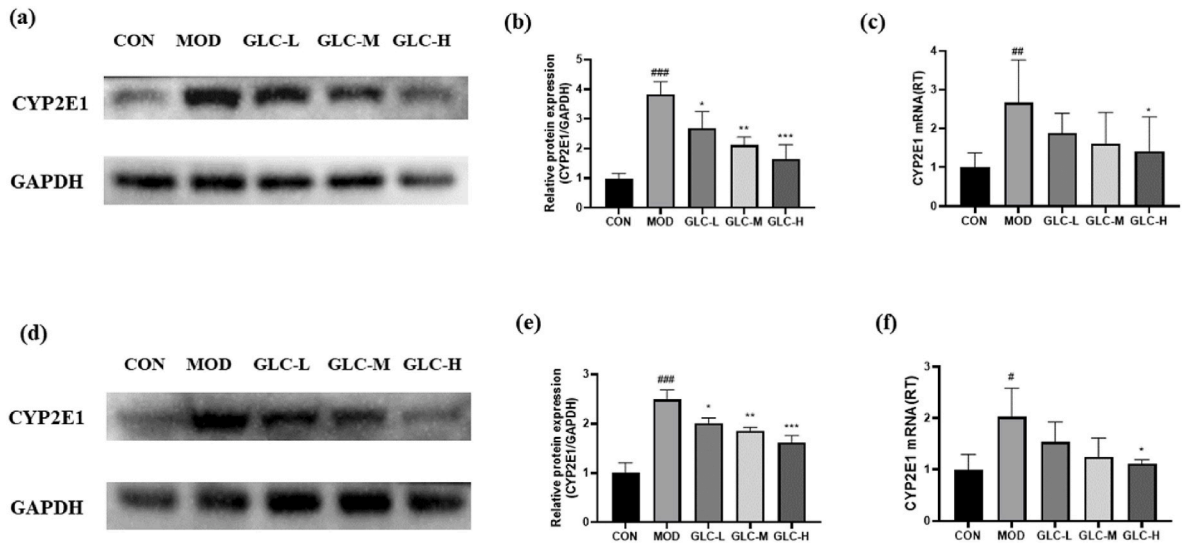

Fig. 8. Effect of GLC on (a), (b) cytochrome P450 proteins 2E1(CYP2E1) protein expression in mouse livers (n = 3, means ± SD). (c) cytochrome P450 proteins 2E1 (CYP2E1) mRNA expression in mouse livers (n = 6, means ± SD). (d), (e) cytochrome P450 proteins 2E1 (CYP2E1) protein expression in L02 liver cells (n = 3, means ± SD). (f) cytochrome P450 proteins 2E1 (CYP2E1) mRNA expression in L02 liver cells (n = 6, means ± SD). **Notes:** Compared with the MOD group, *p < 0.05, **p < 0.01, and ***p < 0.001; compared with the CON group, $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, and $^{\#\#\#}$p < 0.001.

reduce the content of CYP2E1 mRNA. Among them, GLC-H group can significantly reduce CYP2E1 mRNA content.

The above results indicate that GLC can reduce the expression of CYP2E1 to a certain extent, thus playing an antioxidant effect and reducing the liver damage caused by ethanol.

3.9. Effect of GLC on Keap1/Nrf2/HO-1 antioxidant pathway in mouse liver

Nrf2 forms a complex with Keap1 in the cytoplasm. Nrf2 separates from Keap1 when the liver is stimulated by oxidative stress, thus entering the nucleus, and then the subsequent reaction in the nucleus will lead to the expression of downstream target genes such as HO-1, which is crucial for reducing oxidative stress damage caused by alcohol exposure. It is beneficial to the restoration of liver REDOX homeostasis (Sun et al., 2018; Xu et al., 2018; Galicia-Moreno et al., 2020).

As can be seen from the results in Fig. 9, the expression of Keap1 protein in the MOD group was significantly higher than that in the CON group. Compared with the MOD group, the protein expression of Keap1 was inhibited in all GLC administration groups, and the middle and high dose groups showed a significant downward trend. In addition, MOD significantly inhibited the expression levels of Nrf2 and HO-1 compared with CON group, while GLC reversed this inhibition and increased the expression levels of antioxidant proteins Nrf2 and HO-1 in the liver. The results showed that GLC could increase the degree of dissociation between Nrf2 and Keap1, make a large amount of Nrf2 enter the nucleus to play an antioxidant role, induce the expression of HO-1 antioxidant protein, and alleviate oxidative stress in the liver.

3.10. Effect of GLC on MAPK pathway expression

Studies over the past three decades have shown that the MAPK

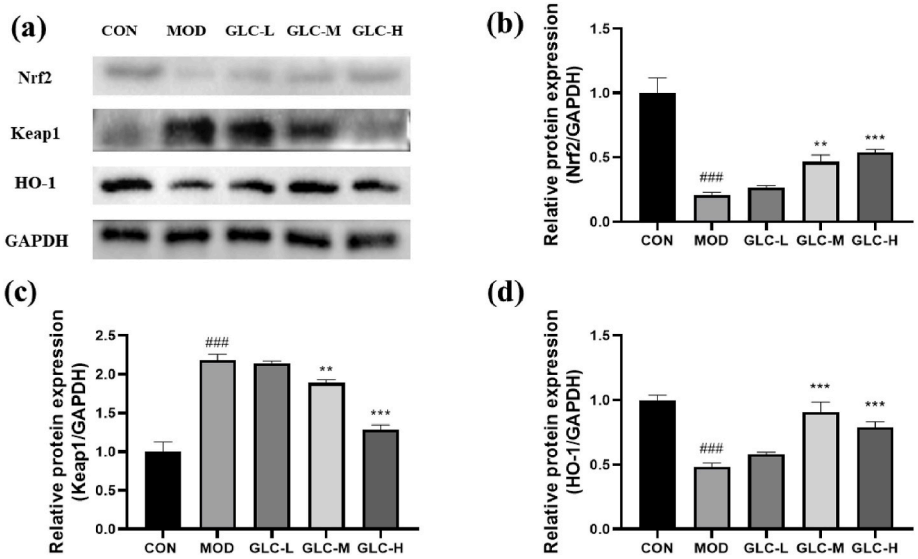

Fig. 9. Effect of GLC on (a)–(d) the expression of proteins in the kelch-like ECH-associated protein 1 (Keap1)/nuclear factor erythroid-2-related factor 2 (Nrf2)/haem oxygenase-1 (HO-1) pathway in mouse livers (n = 3, means ± SD). **Notes:** Compared with the MOD group, *p < 0.05, **p < 0.01, and ***p < 0.001; compared with the CON group, $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, and $^{\#\#\#}$p < 0.001.

pathway can translate extracellular stimuli into intracellular responses, playing a key role in cell growth, migration, proliferation, apoptosis and differentiation, inflammatory response and oxidative stress. Among them, p38 MAPK and JNK are the most important in regulating cell apoptosis, ameliorating oxidative stress and inflammatory response, while ERK cascade mainly deals with cell growth factor-stimulated signal transduction (Yue and López, 2020). Phosphorylated protein expression is increased upon stimulation of p38 MAPK and JNK following alcohol intake. Excess ROS and oxidative stress also alter mitochondrial membrane permeability and transition potential (Shalbueva et al., 2013). These results indicated that inhibition of p38 MAPK and JNK phosphorylation expression could alleviate oxidative stress and hepatocyte apoptosis caused by ALD (Zhang et al., 2019).

We measured the levels of p38 MAPK and JNK phosphorylation in mouse liver and L02 cells (Fig. 10) by Western blot. It was found that the phosphorylated protein expression of p38 MAPK and JNK was significantly higher in MOD group than in CON group. After administration of GLC, the phosphorylation of these two proteins was reduced. GlC-M and GLC-H could significantly inhibit the increase of phosphorylated proteins of p38 MAPK and JNK in the liver in a dose-dependent manner. The results showed that GLC could improve oxidative stress and inflammatory response caused by alcohol by reducing the protein expression of p38 MAPK and JNK.

3.11. Effect of GLC on NF-κB pathway expression

It has been reported that there is a positive correlation between the phosphorylation expression of NF-κB and the severity of ALD, and nuclear translocation of NF-κB is related to the phosphorylation level of NF-κB and can activate the NF-κB pathway. At the same time, a positive correlation was observed between the expression level of NF-κB and serum liver function indexes in patients with alcoholic fatty liver disease (Kong et al., 2021). Excessive alcohol intake can lead to the disorder of intestinal flora, the destruction of intestinal barrier function, and the change of intestinal permeability, resulting in excessive accumulation of LPS into the liver, and then binding to TLR4 receptor and activating NF-κB inflammatory signaling pathway (Chen et al., 2015; Liu et al., 2021). At the same time, the phosphorylation of NF-κB can cause the production of many pro-inflammatory factors TNF-α, IL-1β, and IL-6. Causes inflammation and damage to the liver (Liu et al., 2020).

The phosphorylation level of NF-κB p65 was detected in L02 cells and mouse liver (Fig. 11), and the results showed that the phosphorylation level of NF-κB p65 in MOD group was significantly higher than that in CON group, indicating that alcohol caused inflammatory damage in liver by activating the inflammatory signaling pathway of NF-κB. The expression of phosphorylated NF-κB p65 protein decreased after GLC intervention, while GlC-M and GlC-H could significantly inhibit the phosphorylation of NF-κB p65. These results suggest that GLC may alleviate ethanol-induced liver injury by inhibiting the NF-κB pathway and reducing inflammatory factors in liver tissue.

3.12. Effect of GLC on the expression of inflammatory cytokines

The enhanced inflammatory response leads to the excessive production of pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α(Wang et al., 2022). In the previous study, it was found that GLC could inhibit the hyperphosphorylation of NF-κB protein expression and improve the liver inflammatory injury caused by alcohol. Therefore, in this section, the mRNA of TNF-α, IL-1β, and IL-6 were analyzed and detected by PCR technology to further explore the mechanism of GLC in reducing the inflammatory response.

In L02 cells (Fig. 12(a)-(c)), the mRNA levels of TNF-α, IL-1β and IL-6 in the MOD group were significantly higher than those in the CON group, which indicated that alcohol caused L02 hepatocytes to produce a large number of pro-inflammatory factors and induce inflammatory damage. After the intervention of GLC administration, the levels of TNF-α, IL-1β and IL-6 were significantly decreased, and the inflammatory response was alleviated and improved. In mouse liver (Fig. 12(d)–(f)), the mRNA expression levels of TNF-α, IL-1β and IL-6 in MOD group were significantly higher than those in CON group due to alcohol intake. GLC administration can significantly reduce the mRNA content of these three types of inflammatory factors and reduce the damage of inflammatory factors to the liver. In conclusion, GLC can reduce the excessive release of inflammatory factors caused by ethanol, which explains the anti-inflammatory effect of GLC on alcoholic liver injury from the level of cytokines.

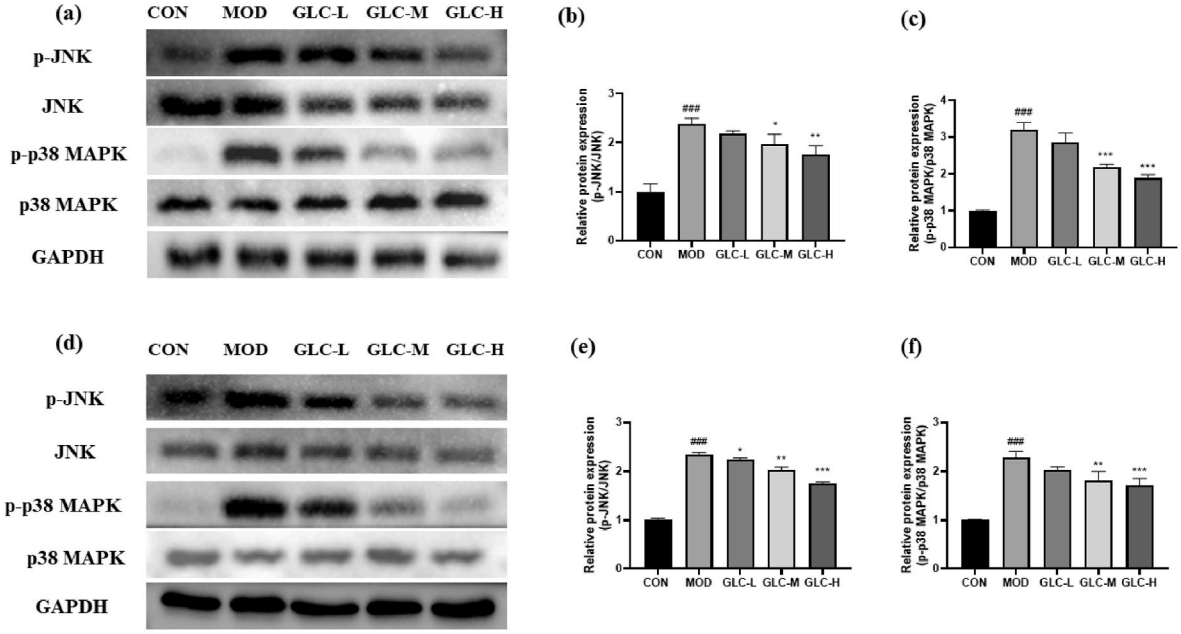

Fig. 10. (a)–(c) Effects of GLC on the mitogen-activated protein kinase (MAPK) pathways in mouse liver (n = 3, means ± SD). (d)–(f) Effects of GLC on the mitogen-activated protein kinase (MAPK) pathways in L02 liver cells (n = 3, means ± SD). **Notes:** Compared with the MOD group, *p < 0.05, **p < 0.01, and ***p < 0.001; compared with the CON group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$.

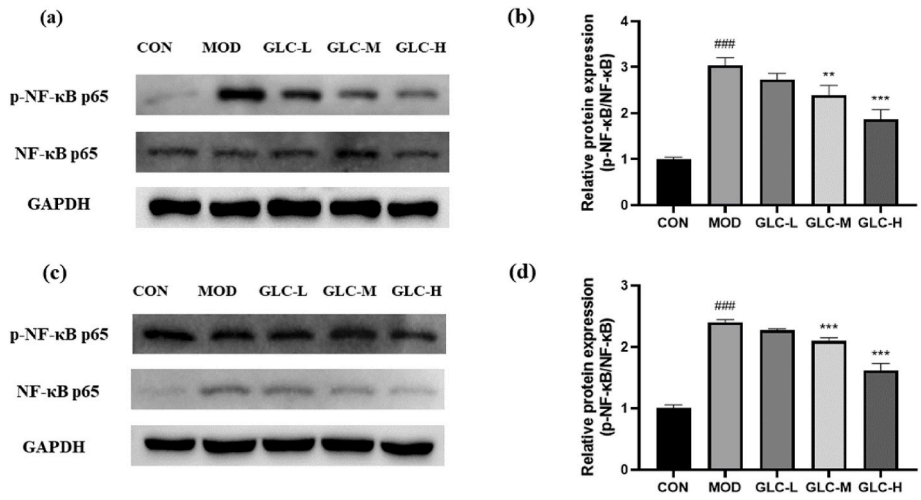

Fig. 11. (a), (b) Effects of GLC on the nuclear factor kappa-B (NF-κB) pathways in mouse liver (n = 3, means ± SD). (c), (d) Effects of GLC on the nuclear factor kappa-B (NF-κB) pathways in L02 liver cells (n = 3, means ± SD). **Notes:** Compared with the MOD group, *p < 0.05, **p < 0.01, and ***p < 0.001; compared with the CON group, $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, and $^{\#\#\#}$p < 0.001.

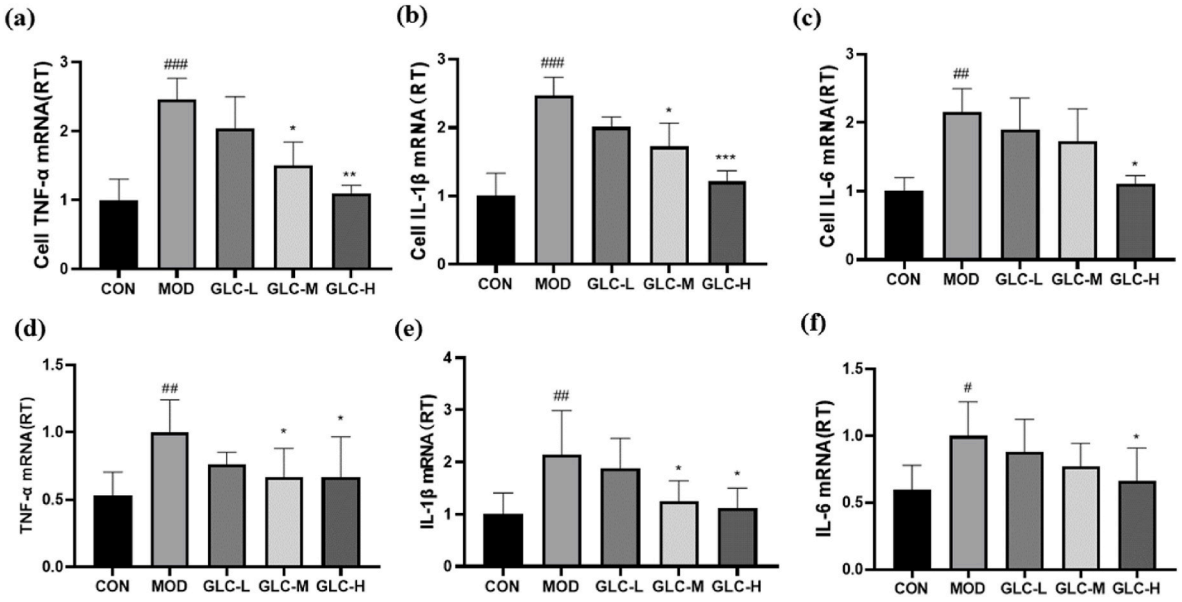

Fig. 12. (a)–(c) Effects of GLC on the expression of pro-inflammatory cytokines in L02 liver cells (n = 6, mean ± SD). (d)–(f) Effects of GLC on the expression of pro-inflammatory cytokines in mouse liver (n = 6, mean ± SD). **Notes:** Compared with the MOD group, *p < 0.05, **p < 0.01, and ***p < 0.001; compared with the CON group, $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, and $^{\#\#\#}$p < 0.001.

4. Discussion

In this study, the mouse alcoholic liver injury model and L02 hepatocyte alcoholic injury model were constructed respectively, and the protective effect and specific mechanism of GLC on alcoholic liver disease were explored from the perspective of in vivo and in vitro. Our results indicate that GLC can alleviate acute liver injury caused by alcohol by improving liver cell damage, lipid metabolism, enhancing liver antioxidant capacity, inhibiting liver inflammation and cell apoptosis. This study provides a theoretical basis and preliminary basis for the research on the protection of GLC against alcoholic liver disease, and GLC has the potential and prospect to become a new dietary supplement drug for the treatment of ALD.

Glucosamine is a widely used supplement typically taken for osteoarthritis and joint pain (Ma et al., 2020). Because of its good anti-inflammatory effect, it has attracted our extensive attention. In our previous study, we found that GLC improved lipid metabolism and antioxidant capacity in NAFLD mice, and through LPS/TLR4/NF-κB signaling pathway ameliorates liver inflammation in NAFLD mice (Li et al., 2023). However, the protective effect of GLC on alcohol induced acute liver injury is still unknown.

The liver is a key hub for many physiological processes. It is an important organ for energy metabolism, fatty acid metabolism, immune regulation, bile acid biosynthesis, alcohol metabolism, and inflammatory response (Trefts et al., 2017). However, excessive alcohol consumption may lead to liver related dysfunction, including lipid metabolism disorders and high levels of oxidative stress, accompanied by inflammation (Guo et al., 2022).

Measuring AST and ALT levels is widely used to assess the risk of alcoholic liver disease (Sutoh et al., 2020). In addition, the increase of ALP and LDH levels also means the aggravation of liver injury (Zhou et al., 2022). GLC administration intervention can increase cell survival

rate, reduce cell death, restore cell growth morphology and improve cell growth status. At the same time, it can significantly reduce the contents of liver transaminases ALT and AST, and reduce the damage caused by alcohol to L02 hepatocytes. Moreover, animal experiments showed that GLC had a significant inhibitory effect on serum ALT, AST, ALP and LDH in mice. This indicated that GLC ameliorated alcohol induced liver injury to a certain extent.

Excessive drinking can seriously affect the liver. Disorder of lipid metabolism is one of the manifestations of alcoholic liver disease. Excessive alcohol intake promotes the synthesis of TC and TG in the liver. TC and TG can be transferred from the liver to peripheral sites through HDL-C, increasing the liver lipid content, thus promoting the deterioration of ALD (Yang et al., 2021). Compared with the MOD group, GLC can significantly reduce the levels of serum TC, TG, VLDL, and LDL-C, increase the content of HDL-C, improve the disorder of serum lipid metabolism in mice, and restore the homeostasis of lipid metabolism.

Oxidative stress plays an important role in alcoholic liver disease (Cederbaum et al., 2009). MDA, GSH, SOD, and CAT, as oxidative stress biomarkers, are often important indicators reflecting the degree of liver oxidative damage (Wang et al., 2023). The detection results of liver oxidative stress biomarkers MDA, GSH, SOD and CAT showed that GLC could inhibit the expression of MDA, promote the expression of GSH, SOD and CAT, enhance the antioxidant function of liver cells, and alleviate the damage caused by oxidative stress caused by alcohol. CYP2E1 can produce ROS such as "O^{2-}, H_2O_2, and ·OH" (Leung and Nieto, 2013). Therefore, overexpression of CYP2E1 causes an imbalance in the REDOX system. In L02 hepatocytes and mouse liver, compared with MOD group, GLC administration group can significantly reduce the expression of CYP2E1 protein and mRNA, and alleviate the oxidative damage to hepatocytes. Nrf2 is a critical regulatory transcription factor in the antioxidant system and is located upstream of the system, playing a major role in regulating REDOX balance (Sun et al., 2018). The role of Keap1/Nrf2 signaling in resisting oxidative stress has been validated in many animal models of ALD (Zheng et al., 2019; Xu et al., 2022). GLC could activate the antioxidant pathway Keap1/Nrf2/HO-1, reduce the protein expression of Keap1, and increase the levels of the key antioxidant protein Nrf2 and its downstream antioxidant enzyme HO-1.

Inflammation is generally considered to be an important biomarker of the degree of alcoholic liver disease (Wen et al., 2021). Our results showed that GLC can significantly reduce the expression of TNF-α, IL-6, IL-1β and other pro-inflammatory factors, reduce the damage of alcohol on liver cells, and improve alcoholic liver disease to a certain extent. The MAPK and NF-κB signaling pathway plays a crucial role in regulating inflammatory responses during liver injury (Cui et al., 2019; Nowak and Relja, 2020). The phosphorylation of key proteins in MAPK and NF-κB pathways showed that GLC could inhibit the phosphorylation of JNK and p38 MAPK, reduce the expression of NF-κB p65 phosphorylation protein, and inhibit the activation of MAPK and NF-κB pathways, thereby inhibiting liver inflammation and apoptosis. The mRNA expression of TNF-α, IL-1β, and IL-6 in L02 hepatocytes and mouse liver tissues indicated that GLC could significantly reduce the mRNA expression levels of TNF-α, IL-1β, and IL-6, which indicated that GLC reduced the excessive release of downstream inflammatory factors caused by alcohol intake.

In summary, GLC has been shown to improve hepatocyte injury, lipid metabolism, inflammatory response, oxidative stress and other excellent activities in both in vivo and in vitro models, and can improve alcoholic liver disease. Further mechanisms suggest that GLC plays a protective role mainly in oxidative stress and inflammatory response: GLC can inhibit the expression of ethanol metabolism enzyme CYP2E1, activate the antioxidant pathway Keap1/Nrf2/HO-1, up-regulate the expression of antioxidant enzymes, down-regulate the phosphorylation of MAPK and NF-κB signaling pathways, and thus reduce the expression of TNF-α, IL-1β, IL-6 and other inflammatory factors. This study preliminarily suggests that dietary supplementation of GLC may alleviate acute liver injury caused by alcohol by inhibiting oxidative stress and inflammation, and GLC has the potential and prospect to become a new dietary supplement drug for the treatment of ALD.

In future studies, the protective mechanisms of GLC against alcoholic liver disease needs to provide more credible references by further research. First, in terms of lipid metabolism, this study only detected the levels of TC, TG, VLDL and LDL-C, and the specific lipid metabolism mechanism still needs to be explored. Secondly, alcohol may cause damage to the intestinal barrier and disturbance of intestinal flora after entering the intestine (Lamas-Paz et al., 2020; Schneider et al., 2022). The research data on intestinal histopathology and intestinal flora need further exploration. Finally, even though it has been explored in previous studies, the research on NF-κB pathway in this study is not deep enough. We can further explore the regulatory mechanism of gut liver axis on LPS-TLR4-NF-κB, so as to provide a more reliable reference for the development of a promising functional food to improve or prevent alcoholic liver disease.

Alcoholic liver disease is accompanied by oxidative damage and inflammation of the liver, and dietary supplements that are safe and able to provide feedback on both aspects may have potential for treating ALD and further research. This may suggest that for the mechanism of disease occurrence, we can choose products with corresponding characteristics, which are already mature and have good safety for further research, and broaden their indications.

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CRediT authorship contribution statement

Weiwen Lai: Conceptualization, Methodology, Software, Investigation, Formal analysis, Writing – original draft. **Shipeng Zhou:** Data curation, Writing – original draft. **Yan Bai:** Visualization, Investigation. **Qishi Che:** Resources, Supervision. **Hua Cao:** Software, Validation. **Jiao Guo:** Visualization, Writing – review & editing, Funding acquisition, Resources. **Zhengquan Su:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Weiwen Lai reports financial support was provided by Guangdong Pharmaceutical University. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

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Abbreviations Used

ALD	alcoholic liver disease
GLC	glucosamine
CYP2E1	cytochrome P450 proteins 2E1
Keap1	kelch-like ECH-associated protein 1
Nrf2	nuclear factor erythroid-2-related factor 2
HO-1	haem oxygenase-1
ALT	alanine aminotransferase
AST	aspartate aminotransferase
MDA	malondialdehyde
GSH	glutathione
SOD	superoxide dismutase
CAT	catalase
ALP	alkaline phosphatase
LDH	lactate dehydrogenase
TNF-α	tumor necrosis factor-α
IL-1β	interleukin-1β
IL-6	interleukin-6
TC	serum total cholesterol
TG	triglyceride
VLDL	very low density lipoprotein
ROS	reactive oxygen species
MAPK	mitogen-activated protein kinase
JNK	C-Jun N-terminal kinase
NF-κB	nuclear factor kappa-B
p38 MAPK	p38 mitogen-activated protein kinase

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100699.

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