Drug Design, Development and Therapy

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ORIGINAL RESEARCH

Gracillin Protects Liver Ischemia-Reperfusion Injury from Oxidative Stress-Induced Apoptosis

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Purpose: To explore the therapeutic effects of gracillin on liver ischemia-reperfusion (IR) injury.

Methods: The effects of gracillin on mouse liver function were evaluated by pathological analysis and measurement of serum biochemical indicators including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH). In addition, apoptosis-related gene expression levels were assessed using quantitative real-time PCR and western blotting. The following oxidative stress related indices were detected by reactive oxygen species (ROS) content, malondialdehyde (MDA) content, and glutathione peroxidase (GSH-Px) activity, and superoxide dismutase (SOD) content. An H_2O_2-mediated oxidative stress model was developed to test the therapeutic effects of gracillin. The Akt inhibitors LY294002 was used to explore the role of the Akt/GSK3β signaling pathway in gracillin-induced protective effects.

Results: Gracillin protected against IR-induced liver dysfunction. Gracillin pretreatment significantly inhibited pathological liver damages and decreased serum ALT, AST, ALP, and LDH levels. Gracillin pretreatment increased the mRNA and protein levels of anti-apoptotic factor Bcl-2, while reducing those of pro-apoptotic factor Bax mRNA and protein levels. Additionally, H_2O_2-induced the oxidative stress and H_2O_2-enhanced hepatocyte apoptosis were markedly inhibited by gracillin pretreatment. Mechanistically, gracillin pretreatment activated the Akt/GSK3β signaling pathway. Inhibition of the Akt/GSK3β signaling pathway reversed the protective effects induced by gracillin.

Conclusion: These results provide evidences that gracillin exerts beneficial effects against liver dysfunction during liver IR. The mechanisms underlying the beneficial effects may be suppression of oxidative stress and apoptosis via the Akt/GSK3β signaling pathway activation. These results suggest that a potential therapeutic role for gracillin in protecting against liver IR injury.

Keywords: liver ischemia-reperfusion injury, oxidative stress, apoptosis, Akt/GSK3β signaling pathway

Introduction

Liver resection and liver transplantation are critical treatments for end-stage liver diseases. Liver ischemia-reperfusion (IR) occurs inevitably during liver graft preservation and liver surgery. However, liver IR injury causes liver dysfunction, which leads to early allograft dysfunction, liver graft rejection, and liver failure.[1–4] Numerous studies have revealed that mitochondrial dysfunction, inflammatory responses, oxidative stress, autophagy, and apoptosis are involved in liver IR pathological processes.[5–10] Among these factors, oxidative stress and apoptosis are important factors that influence liver IR-mediated hepatocytes injury.[7,11–13] Several studies have suggested that these mechanisms play critical regulatory roles in hepatocyte damage.[14,15] Overproduction of reactive oxygen species (ROS) can cause oxidative stress, resulting in damage to biomolecules and organelles damage, further inducing cell apoptosis and tissue injury.[7] A previous study revealed that nano-selenium inhibited hepatocyte apoptosis through ROS-PARP1 signaling.[16] Therefore, suppressing of oxidative stress and apoptosis may provide an effectively therapeutic strategy for the ameliorating of liver IR injury.

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Received: 29 July 2024
Accepted: 15 August 2025
Published: 12 November 2025

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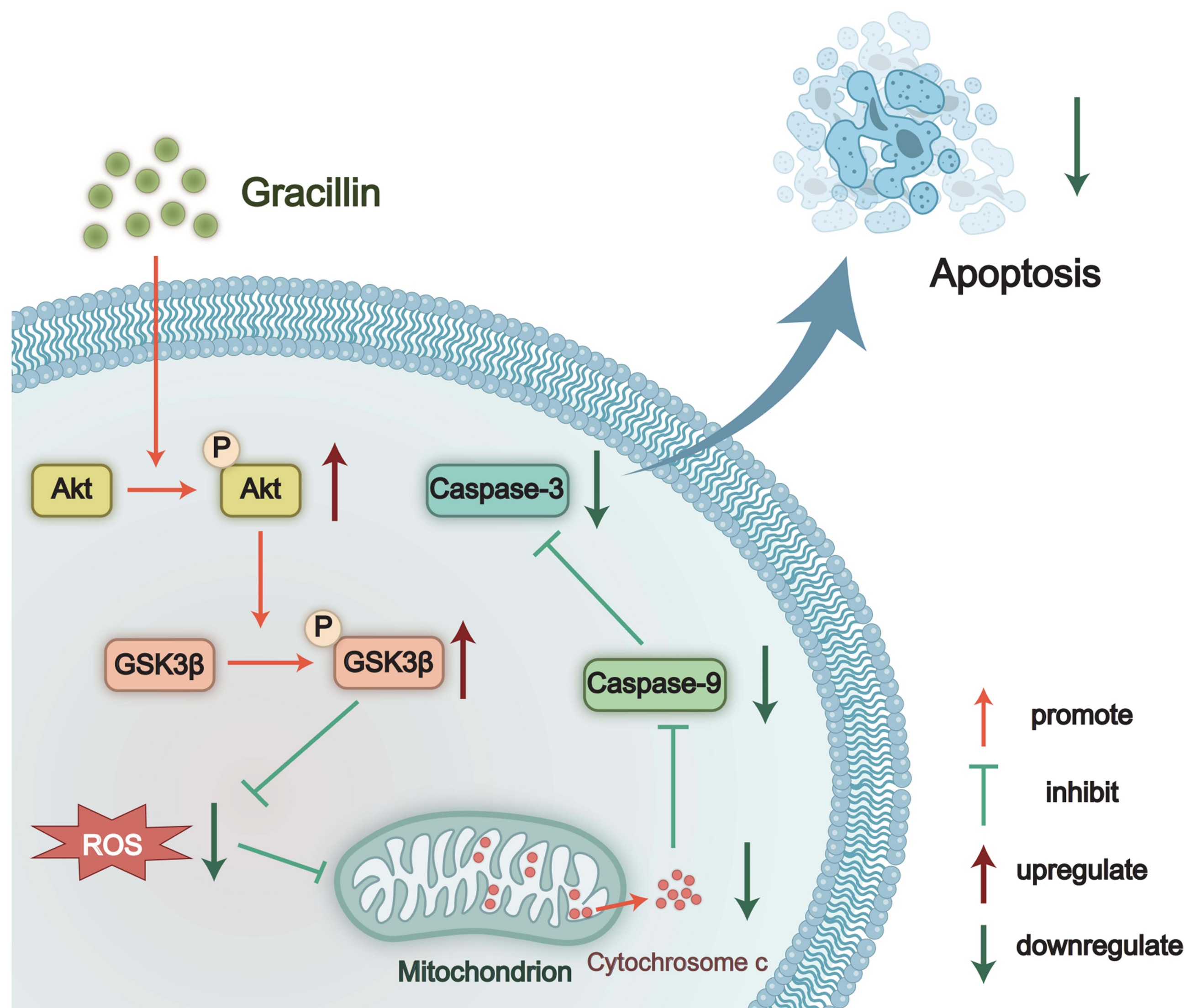
Graphical Abstract



Akt/GSK3β signaling pathway has been reported to regulate various function by inhibiting of GSK3β activity.[17] Akt causes inactivation of GSK3β by phosphorylating GSK3β at Ser9 to decrease oxidative stress-mediated apoptosis.[18] GSK3β can regulate mitochondrial function.[19] Several studies indicated that GSK3β has protective effects against liver IR-induced injury,[18,19] suggesting the Akt/GSK3β signaling pathway as a potential therapeutic target.

Gracillin, a natural steroid saponin compound found in *Dioscorea villosa, Solanum xanthocarpum* and *Acontum carmichaeli*, possesses anti-inflammatory and anti-cancer effects.[20–22] A recent study indicated that gracillin protects against LPS-induced myocardial injury by inhibiting apoptosis and inflammation.[20] Pharmacological studies have indicated that gracillin exerts protective effects on atopic dermatitis by suppressing IL-4 production and mast cells infiltration.[23] In addition, gracillin has been shown to exert anticancer properties by inducing apoptosis.[21,22]

Gracillin has been reported to show hepatoprotective effects of in both animal and cell models. Gracillin inhibits the metastasis of liver cancer in animal models.[24] Previous study has suggested that gracillin suppresses the proliferation of human liver cancer cells.[25] However, whether gracillin exerts protective effects against liver IR injury remains unclear. We hypothesized that gracillin could alleviate liver IR injury by suppressing oxidative stress-induced apoptosis and

regulating oxidative stress-related signaling pathways. To confirm this hypothesis, we sought evaluated the protective role of gracillin in liver IR injury and to explore whether this protection is attributable to activation of Akt/GSK3β signaling pathway. Our findings validate the therapeutic use of gracillin to alleviate liver IR injury, identifying the Akt/GSK3β signaling pathway as a therapeutic target of gracillin for ameliorating of liver IR injury.

Materials and Methods

Animals

The calculation of sample sizes in the animal experiments were calculated using the resource equation approach used in previous studies.[26,27] The calculation method is presented in Supplementary Material.

Healthy C57BL/6 mice (8-week-old males) were obtained from the Vital River Laboratory Animal Technology Company (Beijing, China). Animals were lived in comfortable environments with 12 h/12 h light/dark cycle, temperature of 25 °C, and humidity of 40–70%. All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. All the animal experimental procedures were approved by the Animal Ethics Committee of the Affiliated Hospital of Qingdao university.

Animal Experimental Design and the Liver Ischemia-Reperfusion Injury Model

To investigate the protective effects of gracillin in mice. The mice were randomly divided into the following groups (n=5 for each group): (1) sham group; (2) sham + gracillin 10 mg/kg (sham + gracillin-L) group; (3) sham + gracillin 20 mg/kg (sham + gracillin-H) group; (4) liver IR group; (5) liver IR + gracillin 10 mg/kg (liver IR + gracillin-L) group; and (6) liver IR + gracillin 20 mg/kg (liver IR + gracillin-H) group. Gracillin (TargetMol, Boston, MA, USA) was dissolved in dimethyl sulfoxide (DMSO) and diluted with corn oil. Mice in the liver IR + gracillin group were intragastrically administered 10 or 20 mg/kg gracillin 1 h before induction of liver ischemia. The sham and liver + IR group were administered with an equivalent volume of corn oil supplemented with DMSO.

To explore the mechanism underlying the hepatoprotective effects of gracillin, the mice were randomly divided into the following groups: (1) sham group; (2) liver IR group; (3) liver IR + gracillin group; and (4) liver IR + gracillin + LY294002 group. LY294002 (0.5 mg/kg, TargetMol) was administered to the mice of the IR + gracillin + LY294002 group 90 min before gracillin pretreatment.

Mice were anesthetized using an animal anesthesia machine (Beijing Zhongshi Dichuang Technology Development Co., Ltd, Beijing, China) and subjected to midline laparotomy, and interruption of arterial and portal venous blood flow was interrupted for 1 h. The atraumatic clip was removed to restore the blood flow. Mice in the sham group were subjected to the same operation and without blood flow interruption of blood flow. The mice were sacrificed after 12 h of reperfusion, and the liver and blood samples were collected.

Histological Changes

The degrees of damage to the liver tissues was measured using hematoxylin and eosin (H&E) staining. The liver tissues were collected at the 12 h of reperfusion. The liver tissues were fixed in formalin (BioChannel Biological Technology Co., Ltd, Beijing, China), embedded in paraffin, and cut into liver samples slices. The sections were stained with hematoxylin and eosin (Beijing Leagene Biotechnology Co., Ltd, Beijing, China). Pathological changes in liver tissues were observed and photographed under a light microscope.

Measurement of Liver Function

Mouse blood samples were collected and centrifuged to separate the serum after 12 h of reperfusion. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) levels were determined using an autobiochemical analyzer system (Siemens, Tarrytown, NewYork, USA).

Detection of Oxidative Stress

Levels of oxidative damage markers, including malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) were determined using corresponding assay kits according to the manufacturer's protocols (all from Beijing Solarbio Science & Technology Co., Ltd, Beijing, China). Reactive oxygen species (ROS) levels in liver tissues were estimated by the dihydroethidium (DHE) staining (Solarbio). In belief, liver sections were incubated with fluorescent dye DHE at 37 °C for 30 min. The intensity of DHE fluorescence in the liver tissues and hepatocytes was assessed using Image J (NIH, Bethesda, MD, USA).

Quantitative Reverse Transcription-PCR (qRT-PCR)

The extraction of total RNA was extracted using by TRIzol reagent (BioriginBeijing Inc, China). The RNA was used to synthesize cDNA using the StarLighter Script RT all-in-one mix for qPCR (FS-P1001, Beijing Foreverstar Biotech, Beijing, China). qRT-PCR was performed using SYBR Green qPCR Master Mix (APExBIO, Houston, USA). The mRNA relative expression levels were analyzed using delta-delta Ct method. The specific primers were listed as follows: β-actin forward: GTGACGTTGACATCCGTAAAGA and reserve: GCCGGACTCATCGTACTCC; Bcl-2 forward: ATGCCTTTGTGGAACTATATGGC and reserve: GGTATGCACCCAGAGTGATGC; Bax forward: AGGATGCGTCCACCAAGAAGCT and reserve: TCCGTGTCCACGTCAGCAATCA.

Western Blotting

Mouse hepatocytes were lysed in RIPA lysis buffer (Sangon Biotech Co., Ltd, Shanghai, China) containing PMSF (Wuhan Fine Biotech Co., Ltd, Hubei, China) and cocktail (Share-bio, Shanghai, China). Protein concentration was analyzed using a BCA protein assay kit (Shandong Sparkjade Biotechnology CO., Ltd, Shandong, China). Mitochondrial proteins and cytoplasmic proteins were extracted using corresponding commercial kits (Beyotime Institute of Biotechnology, Shanghai, China). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a prestained protein marker (Sparkjade), and transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). The PVDF membranes were blocked with no-fat milk and incubated with primary antibodies. These primary antibodies used in our study were as follows: Bax (Biosynthesis Biotechnology Inc, Beijing, China), Bcl-2 (AtaGenix Laboratories Co., Ltd, Wuhan, China), Cytochrome C (Hangzhou HUABIO Biotechnology Co., Ltd, China), COX IV (Zen bioscience, Chengdu, China), Akt (Biogot technology Co., Ltd, Jiangsu, China), p-Akt (Hangzhou HUABIO), GSK3β (ELK biotechnology, Wubei, China), p-GSK3β (Hangzhou HUABIO), and β-actin (Biopm Biotechnology Co., Ltd, Wuhan, China). The PVDF membranes were then incubated with the corresponding secondary antibodies (Sparkjade). The blots were analyzed using chemiluminescence (Life-iLab Biotech Co., Ltd, Shanghai, China).

Caspase-3 Activity and Caspase-9 Activity Assays

The liver tissue and AML12 cells were collected, lysed, and centrifuged. The caspase-3 activity and caspase-9 activity in samples were assessed using the corresponding caspase-3 activity and caspase-9 activity kits (Elabscinece Biotechnology Co., Ltd, Wuhan, China).

Cell Culture and Treatments

The AML12 mouse hepatocytes were obtained from the Conservation Genetics Chinese Academy of Sciences (Shanghai, China). Cells were seeded in culture dishes (Guangzhou Jet Bio-Filtration Co., Ltd, Guangzhou, China) and incubated in Dulbeccos modified Eagles Medium/F12 (DMEM/F12) medium (Lonsera, Suzhou Shuangru Biotechnology Co., Ltd, Suzhou, China) supplemented with 10% fetal bovine serum (CellMax Cell Technology Co., Ltd, Beijing, China), ITS supplement (Shanghai QiDa Biotechnology Co., Ltd, Shanghai, China), and dexamethasone (Keycell Biotechnology Co., Ltd, Wuhan, China). The AML12 cells were incubated at 37 °C with 5% CO_2 humified atmosphere. The cells were stored in cell saving medium (Cellregen Life Science and Technology, Beijing, China) and perserved cells in cryopreservation tube (NEST Biotechnology Co.,Ltd, Wuxi, China).

The AML12 cells were pretreated with or without gracillin for 6 h prior to H_2O_2 stimulation. To evaluate the effects of gracillin on oxidative stress-induced apoptosis, AML12 cells were divided into three groups: (1) sham group; (2) H_2O_2 group; (3) H_2O_2 + gracillin 5 μM (H_2O_2 + gracillin-L) group; and (4) H_2O_2 + gracillin 10 μM (H_2O_2 + gracillin-H) group. To explore the underlying mechanisms of gracillin-mediated protective effects, the AML12 cells were divided into four groups: (1) sham group; (2) H_2O_2 group; (3) H_2O_2 + gracillin 10 μM (H_2O_2 + gracillin) group; and (4) H_2O_2 + gracillin 10 μM + LY294002 30 μM (H_2O_2 + gracillin + LY294002) group. AML12 cells were treated with the Akt inhibitors LY294002 (Solarbio) for 1 h before gracillin pretreatment. The LY294002 concentrations was based on a previous study.[28]

Cell Viability

The proliferation of hepatocytes was assessed using the Cell Counting Kit-8 (CCK-8) assay. Hepatocytes were seeded in 96-well plates (NEST). Hepatocytes were treated with different concentrations of gracillin for 24 h. The AML12 cells were pretreated with gracillin for 6 h and exposed to H_2O_2 for 6 h. After these treatments, The AML12 cells were incubated with CCK-8 solution (Absin, Biotechnology Co., Ltd, Shanghai, China) and evaluated by measuring the *OD450*.

Assessment of LDH Release

Hepatocytes damage was assessed by measuring LDH release in the cell supernatant. The preparation and examination of hepatocyte medium supernatant was prepared and examined according to the manufacturer's instructions (Elabscience).

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Staining (TUNEL) Staining

The percentage of apoptotic in hepatocytes and paraffin-embedded liver sections was measured using TUNEL assay kit (Wuhan Sunncell Biotechnology Co.,Ltd) followed manufacturer's instructions. The nuclei were stained with Hoechst 33342 (Coolaber Science & Technology, Beijing, China). The apoptotic cells were imaged using a fluorescence microscope (Carl Zeiss, Jena, Germany).

Flow Cytometry

The percentage of apoptotic hepatocytes was determined using an Annexin V and propidium iodide (PI) apoptosis detection kit (Chamot Biotechnology Co., Ltd, Shanghai, China). Hepatocytes were collected and incubated with Annexin-V and PI. The percentage of apoptotic hepatocytes was evaluated using the CytoFLEX flow cytometer (Beckman, Coulter, Brea, CA, USA). The results of cell apoptosis was analyzed using the FlowJo software (Tree Star, San Carlos, USA).

Statistical Analysis

All data are presented as the mean standard deviation (SD). GraphPad Prism 9.0 (San Diego, CA, USA) was used for statistical analysis. Because each group contained a small sample size, the Shapiro–Wilk test and Q-Q plots were used to measured normality and Bartletts's test was used to assess variance homogeneity. For data with normally distributed and homogeneity of variance. Multiple groups were compared using one-way ANOVA with Tukey's multiple comparison HSD test. For data disallowed from normally distribution and unequal variance, the Kruska-Wallis test was applied, followed by Dunn's test. A p value <0.05 was considered statistically significant.

Results

Gracillin Relieved Liver Ischemia-Reperfusion Injury

The protective effects of gracillin were examined using a mouse liver IR model, in which mice were subjected to ischemia for 1 h and reperfusion for 12 h. The results suggested that low and high doses of gracillin have no toxic effects on the livers of mice (Figure 1A–F). We assessed the degree of liver damage in the mouse that underwent liver IR. The H&E staining results indicated that gracillin pretreatment decreased the necrotic area of the liver (Figure 1A and B). Mice pretreated with gracillin showed decreased the serum ALT, AST, ALP, and LDH levels compared with those in the liver IR group (Figure 1C–F). These data demonstrated that gracillin pretreatment restored liver IR-induced hepatocellular damage. Moreover, the hepatoprotective

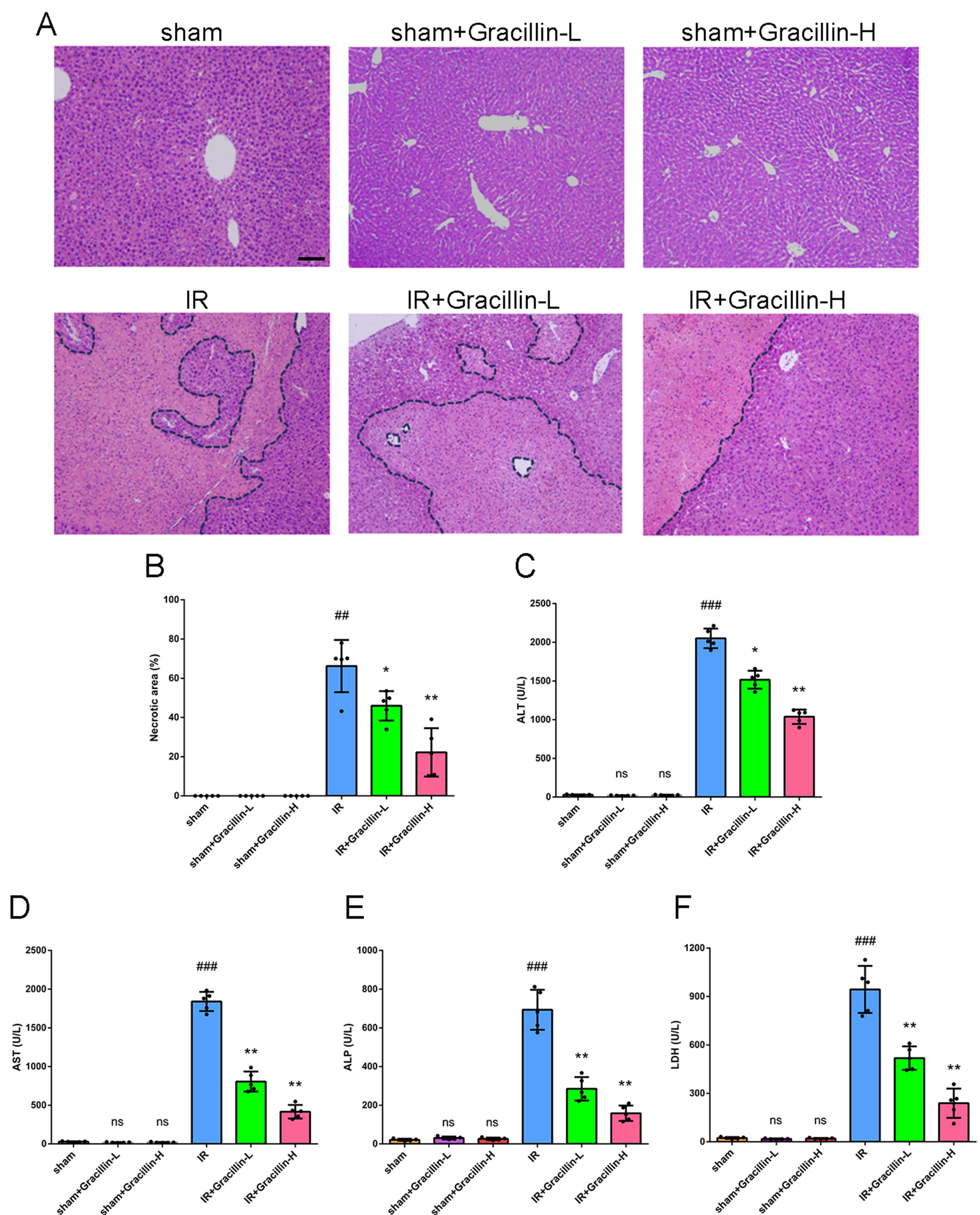


Figure 1 Gracillin pretreatment reduced liver IR injury. (**A**) Representative pathological changes of liver tissues (n=5/each group), scale bar represents 100mm. (**B**) Necrotic areas of liver tissues (n=5/each group). The serum levels of (**C**) ALT, (**D**) AST, (**E**) ALP, and (**F**) LDH (n=5/each group). Compared with the sham group, ##*p*<0.01, ###*p*<0.001, ns: no significant; Compared with the liver IR group, **p*<0.05, ***p*<0.01.

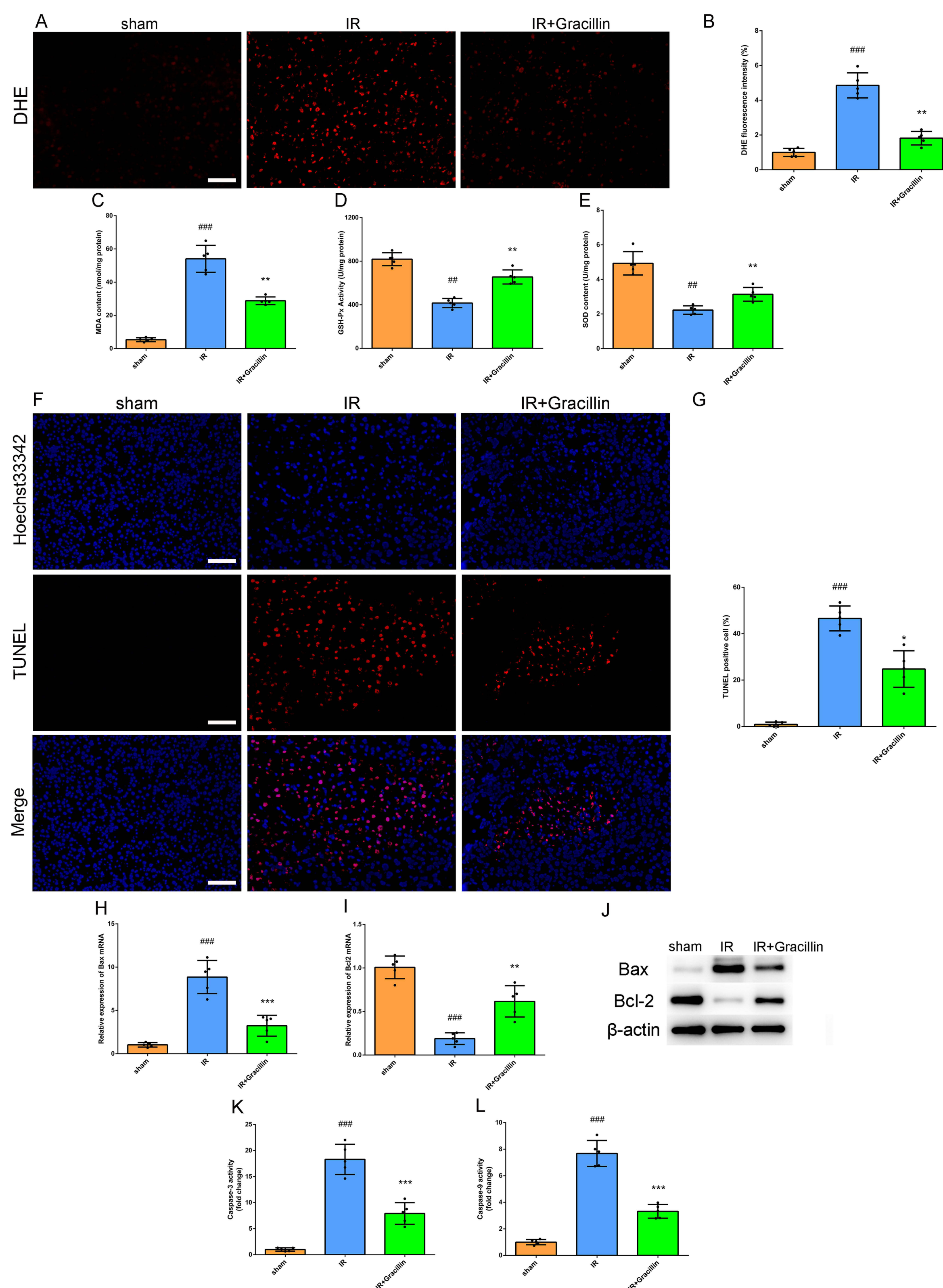


Figure 2 Gracillin pretreatment inhibited oxidative stress and apoptosis. (**A**) The liver tissues ROS levels were measured by DHE fluorescence staining (n=5/each group), scale bar represents 150mm. (**B**) The fluorescence density analysis of ROS (n=5/each group). (**C**) The MDA contents, (**D**) GSH-Px activity, and (**E**) SOD contents (n=5/each group). (**F**) The TUNEL staining (n=5/each group), scale bar represents 150mm. (**G**) The analysis of TUNEL staining (n=5/each group). (**H**) Bax and (**I**) Bcl-2 mRNA levels were examined by qRT-PCR (n=5/each group). (**J**) The protein levels of Bax and Bcl-2 were examined by western-blotting (n=5/each group). (**K**) Caspase-3 activity and (**L**) Caspase-9 activity (n=5/each group). Compared with the sham group, ##*p*<0.01, ###*p*<0.001; Compared with the liver IR group, **p*<0.05, ***p*<0.01, ****p*<0.001.

https://doi.org/10.2147/DDDT.S479009

effects of high doses of gracillin were more superior to those of low doses of gracillin. Therefore, high doses of gracillin were used to explore its protective effects against liver IR injury.

Gracillin Pretreatment Attenuated Oxidative Stress

Oxidative stress is an important process during liver IR, and liver IR injury can be relieved by inhibiting oxidative stress. We assessed the effects of gracillin on IR-induced oxidative stress in the liver. We measured the ROS contents in the liver tissues by DHE staining. The results of DHE staining suggested that gracillin pretreatment markedly decreased ROS levels compared to those in mice subjected to liver IR (Figure 2A and B). As shown in Figure 2C, the MDA contents was lower in the gracillin pretreatment group than that in the liver IR group. In addition, the GSH-Px activity and SOD content were higher in the gracillin pretreatment group than in the liver IR group (Figure 2D and E). These results indicated that gracillin pretreatment could attenuated IR-induced oxidative stress in the liver.

Gracillin Pretreatment Alleviated Apoptosis

To investigate whether gracillin could alleviate IR-induced liver apoptosis, we measured apoptosis in liver tissues using TUNEL staining, qRT-PCR and Western blotting. Our results revealed that gracillin pretreatment decreased TUNEL-positive cells compared to that in the IR group (Figure 2F and G). Compared to the liver IR group, gracillin pretreatment dramatically decreased the mRNA expression of the pro-apoptotic factor Bax and increased the mRNA expression of the anti-apoptotic factor Bcl-2 (Figure 2H and I). To confirm the above data, Bax and Bcl-2 protein levels were measured by western blotting, which showed that the upregulation of Bax protein levels and downregulation Bcl-2 protein levels of were markedly inhibited in the gracillin pretreatment group (Figure 2J) compared to those in the liver IR group. Furthermore, we investigated the effect of gracillin on the caspase-3 activity and caspase-9 activity. The results shown in Figure 2K and L suggested that the liver IR markedly increased caspase-3 activity and caspase-9 activity compared to those in the sham group. However, gracillin pretreatment significantly decreased caspase-3 activity and caspase-9 activity compared to the liver IR group (Figure 2K and L). These results indicated that apoptosis induced by liver IR was inhibited by gracillin pretreatment.

Gracillin Ameliorated H_2O_2-Induced Oxidative Stress and Apoptosis in AML12 Cells

To select suitable concentrations of gracillin, its cytotoxicity against gracillin on AML12 cells was examined using the CCK-8 assay. These results suggested that gracillin did not cause cell cytotoxicity in AML12 cells at concentrations ranging from 0.625 μM to 10 μM (Figure 3A). Therefore, we selected gracillin concentrations of 5 μM and 10 μM. The CCK-8 assay was used to investigate the effects of different concentrations of H_2O_2 on the viability of AML12 cells. As shown in Figure 3B, the viability of AML12 cells was reduced at H_2O_2 concentrations from 200 μM to 800 μM. An H_2O_2 concentrations of 400 μM was chosen for further experiments.

As shown in Figure 3C, H_2O_2-induced cytotoxicity was alleviated at concentrations of 5 μM and 10 μM, with which 10 μM showing the optimum effect. H_2O_2 treatment induced LDH release in the cell supernatant compared to the sham group (Figure 3D). In addition, gracillin pretreatment inhibited LDH release compared to the H_2O_2 treatment (Figure 3D). Liver IR promotes the oxidative stress,[29,30] so we measured the oxidative stress-related indicators in H_2O_2-induced AML12 cells using DHE staining. DHE staining indicated that H_2O_2-induced oxidative stress was significantly decreased by gracillin pretreatment (Figure 3E and F). In addition, gracillin pretreatment significantly decreased the MDA contents (Figure 3G). The SOD contents and GSH-Px activity were increased after gracillin pretreatment compared to those in the H_2O_2 group (Figure 3H and I). These results indicated that gracillin exerts antioxidant effects. Considering 10 μM gracillin has favorable antioxidant effects, this concentrations of gracillin of 10 μM was selected for the next in vitro experiments.

Liver IR induces ROS production, which then causes opening of the mitochondrial permeability transition, thereby further promoting cytochrome c and contributing to hepatocyte apoptosis.[31] To further explore the protective effects of gracillin against oxidative stress-induced apoptosis, the changes in hepatocyte apoptosis were examined. Using TUNEL staining, we found that AML12 exposed to H_2O_2 showed increased hepatocyte apoptosis. In contrast, gracillin pretreatment partially reversed this apoptosis induced by H_2O_2 (Figure 4A and B). H_2O_2 increased and decreased the mRNA

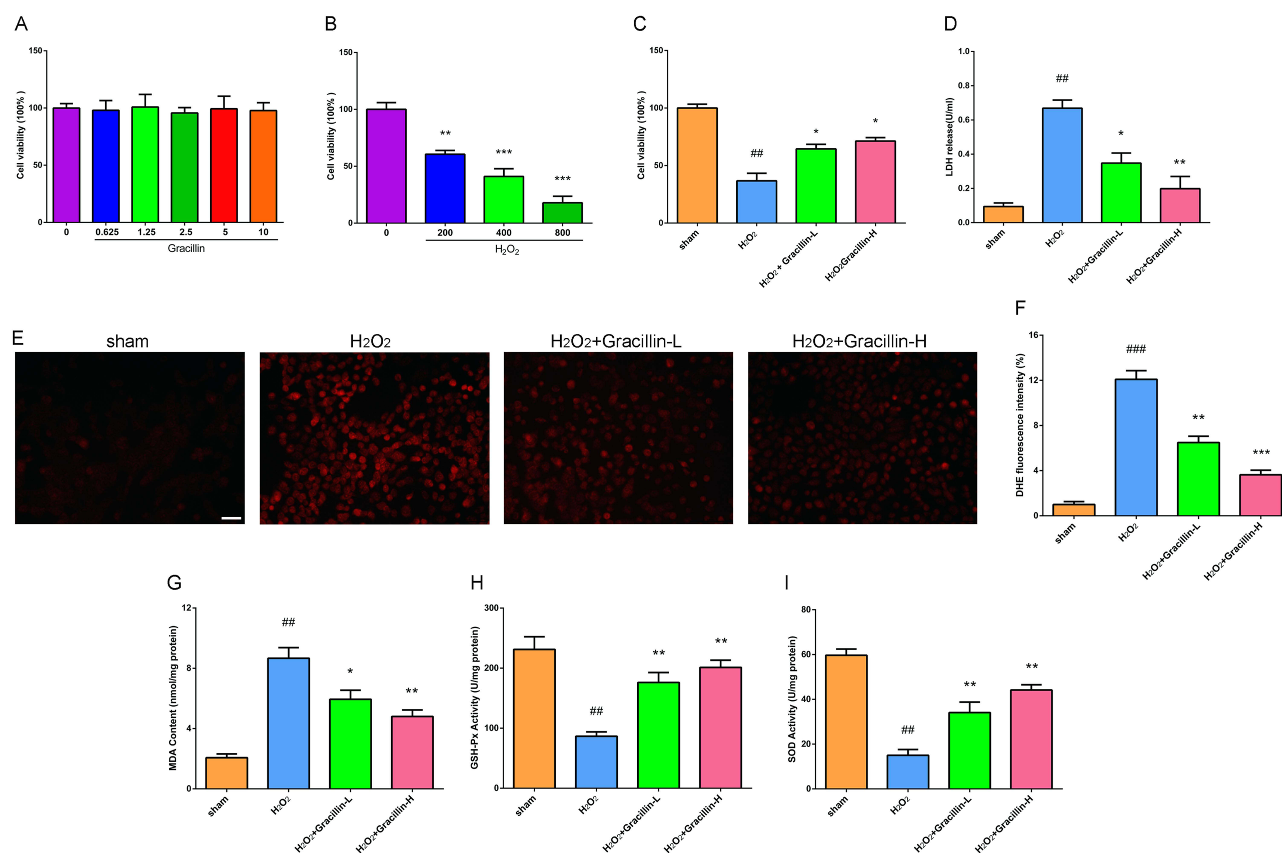


Figure 3 Gracillin pretreatment suppressed oxidative stress. (**A**) Effect of gracillin on AML12 cells (n=5/each group). (**B**) Effect of H_2O_2 on AML12 cells (n=5/each group). (**C**) The viability of AML12 cells after treated with gracillin and H_2O_2 (n=5/each group). (**D**) The LDH levels in cell supernatant (n=5/each group). (**E**) The ROS levels were measured by DHE fluorescence staining (n=5/each group), scale bar represents 20mm. (**F**) The fluorescence density analysis of ROS (n=5/each group). (**G**) The MDA contents, (**H**) GSH-Px activity, and (**I**) SOD contents (n=5/each group). Compared with the sham group, ##p<0.01, ###p<0.001; Compared with the H_2O_2 group, *p<0.05, **p<0.01, ***p<0.001.

expression levels of Bax and Bcl2 respectively, compared to those in the sham group. Moreover, gracillin pretreatment significantly reversed these changes in Bax and Bcl-2 expression (Figure 4C and D). As shown in Figure 4E, gracillin pretreatment inhibited the decrease in Bcl-2 protein levels and increase in Bax protein levels. Western blotting indicated that cytoplasmic cytochrome c protein levels were higher in the H_2O_2 group than in the sham group. Gracillin pretreatment decreased the cytoplasmic cytochrome c protein levels compared to those in the H_2O_2 group (Figure 4F). H_2O_2 stimulation decreased mitochondrial cytochrome c protein levels compared to those in the sham group, whereas gracillin pretreatment decreased mitochondria cytochrome c release. These results indicated that gracillin inhibits cytochrome c release from the mitochondrial into the cytoplasm. Considering that cytochrome c release is related to caspase-3 and caspase-9 activation, we assessed the caspase-3 activity and caspase-9 activity. As expected, the caspase-3 activity and caspase-9 activity in the gracillin-pretreated group was lower than those in the H_2O_2 group (Figure 4G and H). Flow cytometry results suggested that gracillin could inhibited H_2O_2-induced apoptosis (Figure 4I and J). These results indicated that gracillin inhibited the oxidative stress-induced apoptosis.

Activation of the Akt/GSK3β Signaling Pathway is Associated with the Protective Effects of Gracillin

The Akt/GSK3β signaling pathway has been acknowledged as a pivotal regulator in liver IR injury.[32,33] To explore the role of the Akt/GSK3β signaling pathway in the gracillin-induced protective effects. We test whether gracillin pretreatment affected liver IR-induced phosphorylation of Akt and downstream molecule GSK3β . As shown in Figure 5A, the phosphorylation of Akt and phosphorylation of GSK3β were increased in liver tissues at 12h after liver IR. However, the total of Akt and GSK3β were not changed. Moreover, gracillin pretreatment further upregulated liver IR-mediated

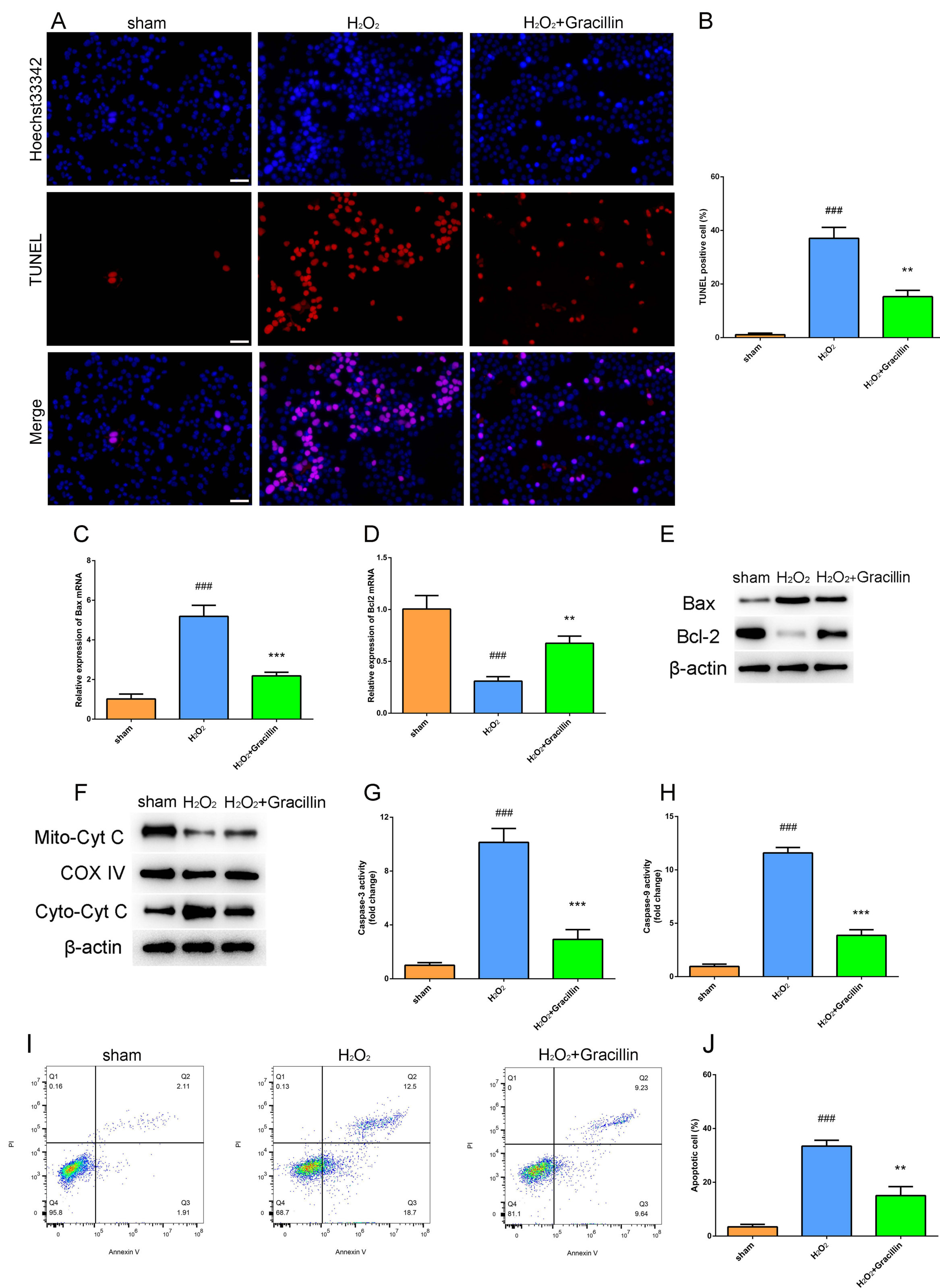


Figure 4 Gracillin alleviated oxidative stress-mediated apoptosis. (**A**) The apoptotic cells were examined by TUNEL staining (n=5/each group), scale bar represents 20mm. (**B**) The percentage of apoptotic cells (n=5/each group). (**C**) Bax and (**D**) Bcl-2 mRNA expression levels (n=5/each group). (**E**) Bax and Bcl-2 protein expression levels (n=5/each group). (**F**) Cytochrome c protein expression levels in mitochondrial and cytoplasm (n=5/each group). (**G**) Caspase-3 activity and (**H**) Caspase-9 activity (n=5/each group). (**I**) Flow cytometric assays of apoptotic hepatocytes. (**J**) The percentage of apoptotic hepatocytes (n=5/each group). Compared with the sham group, ###p<0.001; Compared with the H_2O_2 group, **p<0.01, ***p<0.001.

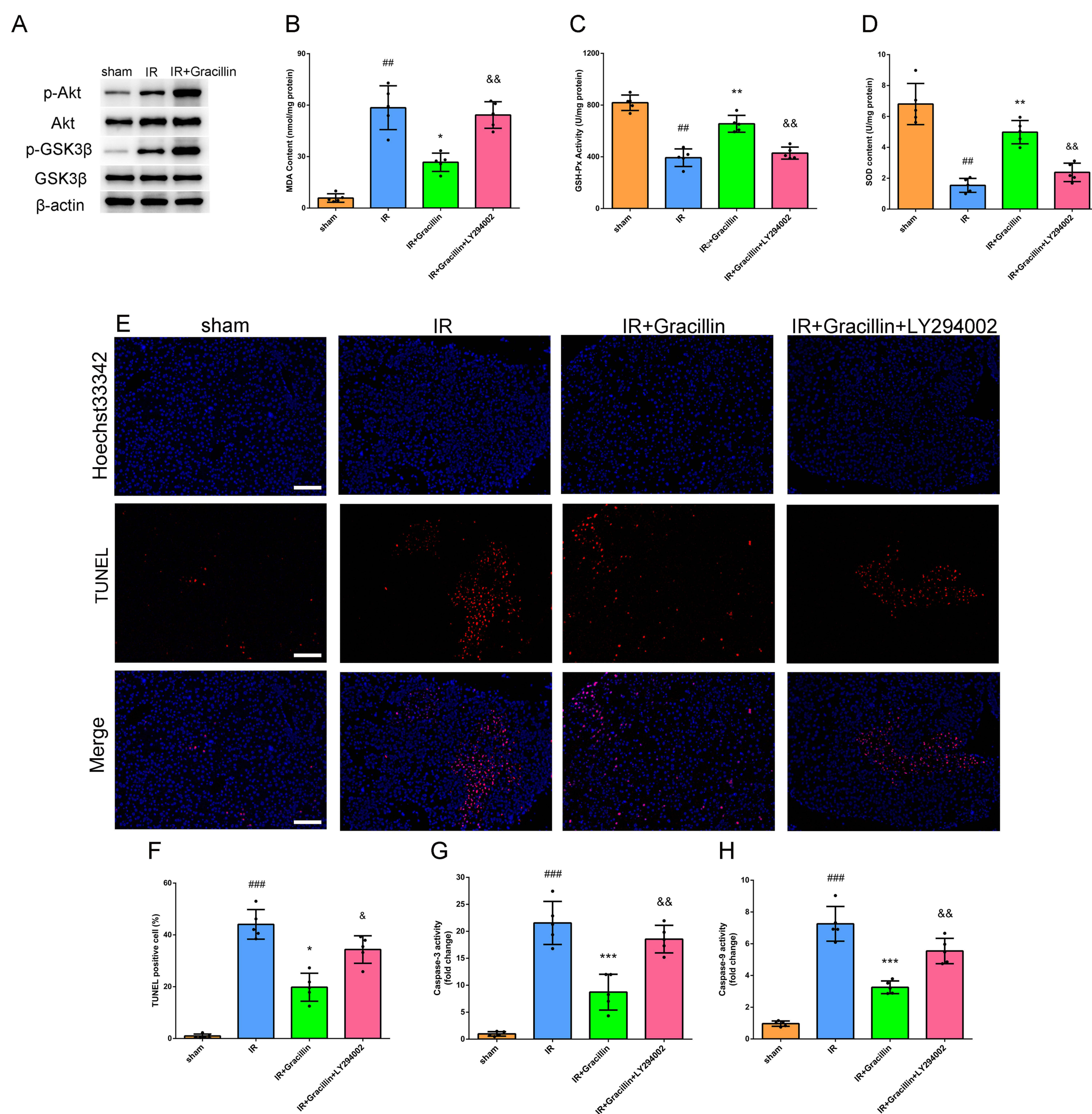


Figure 5 Gracillin ameliorates liver IR injury by activating Akt/GSK3β signaling pathway. (**A**) Akt, p-Akt, GSK3β, and p-GSK3β protein levels in liver tissues after liver IR (n=5/each group). The detection of (**B**) MDA content, (**C**) GSH-Px activity, and (**D**) SOD activity in liver tissues (n=5/each group). (**E**) The TUNEL staining in different mice liver tissues (n=5/each group), scale bar represents 150mm. (**F**) The analysis of TUNEL positive cells (n=5/each group). (**G**) Caspase-3 activity and (**H**) Caspase-9 activity in liver tissues (n=5/each group). Compared with the sham group, ##p<0.01, ###p<0.001; Compared with the IR group, *p<0.05, **p<0.01, ***p<0.001; Compared with the IR + Gracillin group, $^{\&}p$<0.05, $^{\&\&}p$<0.01.

phosphorylation of Akt and phosphorylation of GSK3β (Figure 5A). To further confirm gracillin pretreatment can exert hepatoprotective effects by activating Akt/GSK3β signaling pathway, LY294002 was used to treat mice. As shown in Figure 5B–D, LY294002 reversed the gracillin-induced inhibitory effects on oxidative stress. In addition, LY294002 reversed gracillin-induced the inhibition of apoptosis (Figure 5E–H). These results indicated that gracillin alleviates liver IR injury by activating Akt/GSK3β signaling pathway.

To validate whether gracillin attenuates oxidative stress-related injury was carried out by activating the Akt/GSK3β signaling pathway, we tested the protein expression levels of Akt, phosphorylated-Akt, GSK3β, and phosphorylated-GSK3β.

Western blotting indicated that phosphorylated-Akt and phosphorylated-GSK3β were increased in the H_2O_2 group. Moreover, gracillin further increases phosphorylation of Akt and GSK3β (Figure 6A). AML12 cells were pretreated with an Akt inhibitor (LY294002) before exposure to H_2O_2. LY294002 markedly aggravated H_2O_2-induced hepatocellular injury, as evidenced by reduced cell viability and increased LDH release (Figure 6B and C). LY294002 administration reduced GSH-Px activity and SOD activity (Figure 6D–F) and occluded gracillin-induced anti-apoptotic effects in AML12 cells (Figure 6G–L). Taken together, these results suggested that the Akt/GSK3β signaling pathway was the downstream target of gracillin.

Discussion

Our results indicated that gracillin pretreatment ameliorated liver IR-induced oxidative stress, apoptosis, liver tissue damage and liver dysfunction. Moreover, we found that gracillin pretreatment could activate the Akt/GSK3β signaling pathway after liver IR injury and H_2O_2 stimulation. However, the anti-apoptotic effect of gracillin was decreased after administration of the Akt inhibitor. We verified that gracillin inhibited oxidative stress-mediated apoptosis through the Akt/GSK3β signaling pathway. Hence, our results suggested that Akt/GSK3β signaling pathway may be a target of gracillin, suggesting it as a promising therapeutic medicine to alleviate of liver IR injury.

Accumulating evidence has demonstrated that oxidative stress is an important pathological process that impairs liver function following liver IR injury.[7] Hypoxia and ischemia lead to excessive ROS production during liver IR.[34,35] Antioxidant enzymes such as SOD and GSH-Px are important regulators of ROS clearance. The oxidative stress state of the liver may be due to an imbalance between the overproduction of ROS and the inactivation or depletion of antioxidants enzymes.[7] Larger amounts of ROS can cause oxidative damage to proteins, DNA, and lipids. Therefore, inhibiting oxidative stress is an effective strategy to alleviate liver IR injury. In this study, we found that oxidative stress was accompanied by liver dysfunction. After pretreatment with gracillin, the ROS and MDA production were reduced, and the GSH-Px activity and SOD contents were induced. We established an H_2O_2-induced hepatocyte model to mimic oxidative stress, and found that gracillin had antioxidant effects. We speculated that gracillin markedly mitigated liver IR injury, which is associated with the inhibition of oxidative stress.

Oxidative stress is an important factor that induces of mitochondrial-mediated apoptosis during liver IR. A recent study has suggested that liver IR induced oxidative stress, resulting in the opening of mitochondrial permeability transition pores, thereby promoting membrane potential depolarization, ATP depletion, and cytochrome-c release.[29] The release of cytochrome c is an important step in the initiation of caspase dependent apoptosis.[36] Suppressing of oxidative stress is considered as a promising protective method for alleviating liver IR. In this study, an H_2O_2-induced oxidative stress AML12 cell model was used to explore the protective mechanism of gracillin. Our results indicated that anti-apoptotic gene expression was increased, whereas pro-apoptotic gene expression, cytochrome c release, and caspase-3 activity and caspase-9 activity were decreased after gracillin pretreatment in hepatocyte. Taken together, gracillin pretreatment exerted hepatoprotective effects by inhibiting oxidative stress-mediated mitochondrial dysfunction and apoptosis.

A Previous study has confirmed that the Akt agonist insulin-like growth factor can relieve liver IR, whereas the Akt inhibitors LY294002 can aggravate liver IR.[37] Akt is upstream of GSK3β. The activation of Akt increased phosphorylation of GSK3β at Serine and resulted in inactivation of GSK3β. The enzymatic activity of Serine phosphorylation of GSK3β is restricted. Inhibiting GSK3β alleviates liver IR by regulating the diversity of cellular functions including oxidative stress, apoptosis, and inflammation.[38–42] GSK3β inactivation alleviates oxidative stress-induced hepatocyte apoptosis.[38–40] GSK3β inactivation protects against liver IR injury by inhibiting inflammation.[41,42] Moreover, inactivation of GSK3β suppresses opening of the mitochondrial permeability transition pore and protects against mitochondrial-related cell apoptosis.[43] These studies revealed that activation of Akt/GSK3β signaling pathway has beneficial effects on liver IR injury, therefore activating Akt/GSK3β signaling pathway contributes to the improvement of liver dysfunction. Consistent with these studies, we also found that Akt was activated in mice liver tissues and hepatocytes and further inactivated GSK3β to protect against liver IR injury. Because oxidative stress is a major driver of liver IR injury, and inactivation of GSK3β inhibited oxidative stress-indued apoptosis.[43] We therefore explored whether gracillin protects mice and hepatocytes against oxidative stress-induced apoptosis by regulating Akt/GSK3β signaling during liver IR injury. Our results revealed that gracillin doses indeed alleviated oxidative stress-induced apoptosis in liver IR injury.

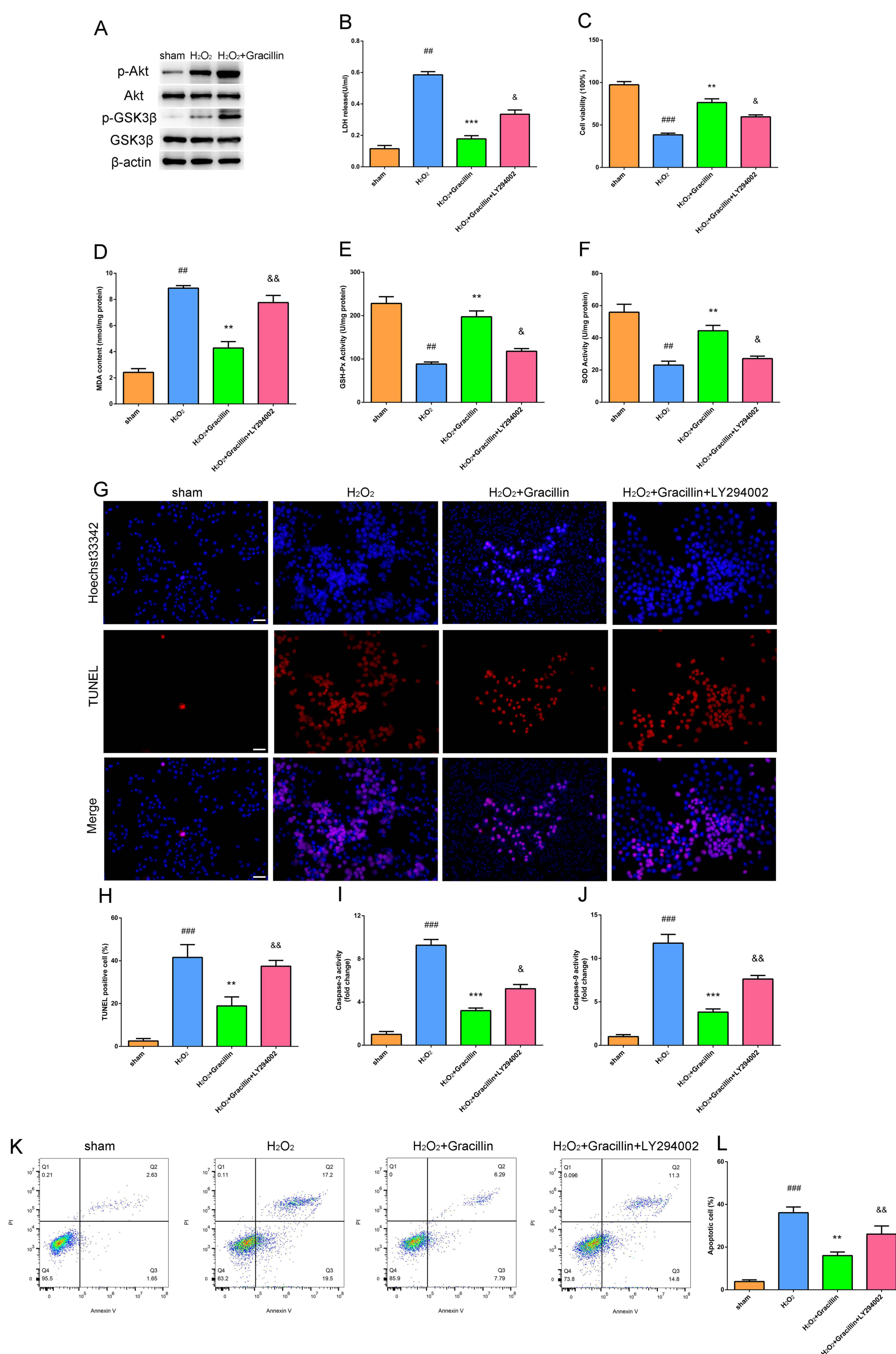


Figure 6 Gracillin inhibited oxidative stress-mediated apoptosis by activating Akt/GSK3β signaling pathway. (**A**) Akt, p-Akt, GSK3β, and p-GSK3β protein levels in AML12 cells pretreated with or without gracillin before exposed to H_2O_2 (n=5/each group). (**B**) The cell viability of AML12 cells (n=5/each group). (**C**) The LDH levels in cell supernatant (n=5/each group). (**D**) H_2O_2-induced intracellular MDA content, (**E**) GSH-Px activity, and (**F**) SOD activity in AML12 cells (n=5/each group). (**G**) The apoptotic cells were examined by TUNEL staining (n=5/each group), scale bar represents 20mm. (**H**) The percentage of apoptotic cells (n=5/each group). (**I**) Caspase-3 activity and (**J**) Caspase-9 activity in AML12 cells (n=5/each group). (**K**) Flow cytometric assays of apoptotic hepatocytes. (**L**) The percentage of apoptotic hepatocytes (n=5/each group). Compared with the sham group, ##p<0.01, ###p<0.001; Compared with the H_2O_2 group, **p<0.01, ***p<0.001; Compared with the H_2O_2 + Gracillin group, $^{\&}p$<0.05, $^{\&\&}p$<0.01.

 https://doi.org/10.2147/DDDT.S479009

A previous study also indicated that oxidative stress-induced apoptosis is reversed by inactivation of GSK3β.[44] In addition, we inhibited Akt using LY294002 and found that the protective effects of gracillin against liver IR injury were reversed by Akt inhibition. Our results also indicated that gracillin inhibited H_2O_2-induced hepatocytes apoptosis, whereas pretreatment with LY294002 partially reversed the protective effects of gracillin. These results indicate that activation of the Akt/GSK3β signaling pathway may be a key mechanism underlying the protective effects of gracillin against oxidative stress-induced apoptosis.

The gracillin have beneficial effects on hepatocytes to reduce liver IR injury. These beneficial effects are verified by improved mouse liver injury, reductions in oxidative stress and apoptosis. These results suggest that gracillin possesses anti-oxidant and anti-apoptotic properties with clinical applications in relieving the severity of liver IR injury. In addition, gracillin offers the advantages of convenient implementation, readily availability, and higher protective efficiency. However, the stability, solubility, half-life, and adverse effects of gracillin restrict its clinical application of gracillin. To solve these problems, further experiments should be conducted to evaluate the optimal doses, and frequency of administration, as well as the adverse effects profile of gracillin.

In summary, we demonstrated that gracillin could improve liver IR injury. In the meanwhile, we also found that gracillin pretreatment attenuated oxidative stress-mediated apoptosis in IR injury through activation of the Akt/GSK3β signaling pathway.

Conclusion

Our findings suggest that gracillin suppresses oxidative stress-induced apoptosis by regulating Akt/GSK3β signaling pathway, thereby exerting hepatoprotective effects for liver ischemia-reperfusion injury. This study highlights the therapeutic value of gracillin in liver IR injury.

Abbreviations

IR, Ischemia-reperfusion; Bcl-2, B-cell lymphoma 2; ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; LDH, Lactate dehydrogenase; CAT, Catalase; GSH-Px, Glutathione peroxidase; SOD, Superoxide dismutase; MDA, Malondialdehyde; DHE, Dihydroethidium; ROS, Reactive oxygen species; GSK3β, Glycogen synthase kinase-3β.

Data Sharing Statement

Reasonable inquiries about raw data can be directed to the corresponding author.

Acknowledgments

We thank Luo Yanan for her kindly assistance with this study.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the vision to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

The research work was supported by grants from the Shandong Provincial Natural Science Foundation (ZR2022MH292) and the National Natural Science Foundation of China (82103662, 82370666).

Disclosure

The authors declare that they have no known competing financial interests or personal relationships that cloud have appeared to influence the work reported in this paper.

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