MicroRNA-141-3p Attenuates Oxidative Stress-induced Hepatic Ischemia Reperfusion Injury via Keap1/Nrf2 Pathway

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Research Article

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Abstract

Hepatic ischemia reperfusion injury (IRI) is a major factor affecting the prognosis of liver transplantation through a series of severe cell death and inflammatory responses. MicroRNA-141-3p (miR-141-3p) has been reported to be associated with hepatic steatosis and other liver diseases. However, the potential role of miR-141-3p in hepatic IRI is currently unknown. In the present study, we found that miR-141-3p levels were negatively correlated with alanine aminotransferase (ALT)/aspartate aminotransferase (AST) in liver transplantation patients. The results demonstrated that miR-141-3p was decreased in mouse liver tissue after hepatic IRI in mice and in hepatocytes after hypoxia/reoxygenation (H/R). Overexpression of miR-141-3p directly decreased Kelch-like ECH-associated protein 1 (Keap1) levels and attenuated cell apoptosis *in vivo* and *in vitro*, while inhibition of miR-141-3p facilitated apoptosis. Further experiments revealed that overexpression of miR-141-3p also attenuated oxidative stress-induced damage in hepatocytes under H/R conditions. Taken together, our results indicate that miR-141-3p plays a major role in hepatic IRI through the Keap1 signaling pathway, and the present study suggests that miR-141-3p might have a protective effect on hepatic IRI to some extent.

Introduction

Liver transplantation is an effective treatment for end-stage liver disease [1]. The liver is susceptible to hypoxia due to being highly dependent on oxygen. Hepatic ischemia reperfusion injury (IRI) is a common consequence of liver transplantation and liver resection. Hepatic IRI includes two stages: ischemic injury and inflammatory-regulated reperfusion. A series of events occur during hepatic IRI, including reactive oxygen species (ROS) generation, peroxidation of DNA and proteins, which can cause a series of cascading reactions, such as inflammation, cell death, and hepatic failure [2,3]. As previously reported, some treatment measures for hepatic IRI have been studied, such as ischemic preconditioning, surgical interventions, targeted therapy, and gene therapy [4,5]. However, these strategies are controversial due to their poor validity.

Kelch-like ECH-associated protein 1 (Keap1) can interact with nuclear factor erythroid 2-related factor 2 (Nrf2), the master transcriptional regulator of the cellular antioxidant program [6]. Under physiological conditions, Keap1 targets Nrf2 for degradation. However, under conditions of oxidative stress, Keap1-Nrf2 binding is suppressed, which stabilizes Nrf2 [7]. Nrf2 can induce transcriptional activation of antioxidant genes [8], including heme oxygenase-1 (HO-1). HO-1 exerts anti-inflammatory and antioxidative effects by catalyzing the degradation of heme into biliverdin and carbon monoxide [9].

MicroRNAs (miRNAs) are a family of highly conserved small endogenous noncoding RNA molecules (18-22 nucleotides) [10]. MiRNAs play significant roles in many disease processes, including proliferation, tumorigenesis, and oxidative stress [11,12]. Recent findings have suggested that many miRNAs participate in modulating hepatic IRI, such as miR-146a and miR-450b-5p [13,14].

MiR-141 belongs to the miR-200 family. It has been reported that miR-141 strongly influences nonalcoholic fatty liver disease (NAFLD) development by negatively regulating expression of the SIRT1 gene and protein while at the same time ameliorating liver function [15]. MiR-141 has also been proposed as a molecular biomarker of various hepatic disorders [16]. As previously reported, miR-141 suppresses Keap1 levels, which activate the Nrf2-dependent pathway and confer 5-FU resistance in hepatocellular carcinoma (HCC) [17]. However, the underlying function of miR-141 in hepatic IRI is not clear.

In this study, we demonstrated that miR-141-3p expression is decreased in liver transplantation patients after surgery and hepatocytes under H/R conditions. MiR-141-3p overexpression protects against liver IRI. Moreover, Keap1 might be a target of miR-141-3p during mice hepatic IRI and hepatocyte hypoxia/reoxygenation (H/R) stress. This study provides new ideas for the development of novel treatment strategies for hepatic IRI.

Materials And Methods

Materials and reagents

Antibodies used in this research include rabbit anti-keap1 (cat. #ab227828, Abcam, Cambridge, UK); rabbit anti-Nrf2 (cat. #ab62352, Abcam, Cambridge, UK); rabbit anti-heme oxygenase 1 (cat. #ab1889491, Abcam, Cambridge, UK); rabbit anti-NQO1 (cat. #11451-1-AP, Proteintech Group, Inc, Rosemont, USA); anti-Bax (cat. #50599-2-lg, Proteintech Group, Inc, Rosemont, USA), anti-Bcl-2 (cat. #3498, CST, Danvers, MA, USA) and anti-cleaved caspase-3 (cat. #9661, CST, Danvers, MA, USA); rabbit anti-GAPDH (cat. #AB0037, Abways Technology, Shanghai, China); rabbit anti-Lamin B (cat. #WL01775, Wanleibio, Shanghai, China). Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Beyotime Biotechnology (cat. #S0033S, Shanghai, China).

Serum collection

Whole blood from 27 liver transplantation patients was collected preoperatively, 4 h after reperfusion, and on days 1, 2, and 3 after surgery. Blood was collected allowed to stand and clot at room temperature (RT). Then the tube was centrifuged at 3000 g for 15 min at 4°C, and stored at -80°C. All procedures were approved and were performed with the patient's informed consent, and this research was approved by the human ethics committee of the First Affiliated Hospital of Chongqing Medical University.

Construction of the hepatic I/R injury model

C57BL/6J mice (male, 18-20 g, 6-8 months old) were purchased from Chongqing Medical University Experimental Animal Center (Chongqing, China). Animals were maintained in a specific pathogen-free (SPF) setting with 12 h light/12 h dark conditions. All animal experiments were approved by the institutional animal care and use committee.

A warm partial (70%) liver I/R injury model was established as previously described [18]. After anesthesia with the sodium pentobarbital (intraperitoneal injection, 50 mg/kg), we opened the abdomen and clamped the left liver and middle liver artery. The clamp was removed after 60 min of ischemia and then reperfusion was performed (reperfusion time: 0 h, 1 h, 3 h, 6 h, 9 h) (n=5 in each time point). The control group was the sham operation group (n=5). Mice were euthanized with an overdose of sodium pentobarbital (100 mg/kg intravenous). Subsequently, the liver tissues and 1 ml venous blood were harvested for subsequent experiments.

Overexpression of miR-141-3p in vivo

Mouse pre-miRNA-141-3p lentivirus gene transfer vectors were constructed by Hanbio (Shanghai, China). The pre-miRNA-141-3p lentivirus was prepared at 1×10^8 transfection units/ml according to the instructions. Mice were divided into 4 groups: sham, I1R6, I1R6+vector control and I1R6+miR-141-3p. The pre-miRNA-141-3p lentivirus and NC lentivirus were transfected with approximately 2×10^7 transfection units *in vivo* by tail vein injection into mice.

Cell culture

The human liver LO2 cell line was purchased from Zhong Qiao Xin

Zhou Biotechnology Co., Ltd (Shanghai, China). Cells were incubated in RPMI 1640 basic medium (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (Biological Industries, Israel). For the H/R model, cellular hypoxia was induced by incubation in serum-free medium and culturing cells in a tri-gas incubator (Thermo, MA, USA) with 94% N_2, 1% O_2, and 5% CO_2. Cells were exposed to hypoxia for 2 h, 4 h, 8 h, 12 h and 24 h followed by reoxygenation for 6 h.

Cell transfection

Sequences of miRNA-141-3p mimic and inhibitor were as follows: miRNA-141-3p mimic sense 5'-UAACACUGUCUGGUAAAGAUGG-3', miRNA-141-3p inhibitor sense 5'-CAGUACUUUUGUGUAGUACAA -3'. MiRNA-141-3p mimic and miRNA-141-3p inhibitor were diluted in serum-free medium, as well as the Lipofectamine 2000 (Invitrogen, Carlsbad, USA) transfection reagent, and then cells were transferred to cell culture medium after mixing. Six hours later, the medium was replaced. Total RNA was extracted 24-48 h after transfection.

Hematoxylin and eosin (HE) staining

After the left lobe of the liver was obtained, part of the liver tissue was fixed in 10% buffered formalin. After deparaffinization and rehydration, hematoxylin/eosin staining and mounting were performed and sections were observed at 200× magnification.

Reverse transcription-quantitative PCR (RT-qPCR) analysis

Total RNA extraction from serum was performed using a miRcute serum/plasma miRNA isolation kit (Tiangen, Beijing, China). Total RNA was extracted from liver tissues and cells with TRIzol reagent (Takara Bio Inc, Japan). The primers U6, Keap1 and GAPDH primers were purchased from Takara. The primer sequences of miR-141-3p (RiboBio Co., Guangzhou, China) are proprietary information from the company. U6 primer: forward 5'-AGAGAAGATTAGCATGGCCCCTG-3' and reverse 5'-ATCCAGTGCAGGGTCCGAGG-3'. Keap1 primer: forward 5'- CCCAATGCTGACACGAAGG-3' and reverse 5'- GCTGAATTAAGGCGGTTTGTC-3'. GAPDH primer: forward 5'-CACTCCTCCACCTTTGACGC-3' and reverse 5'-CTGTTGCTGTAGCCAAATTCGT-3'. Relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were collected from patients as described above. Interleukin-6 (IL-6) (4A Biotech, China) and interleukin-1β (IL-1β) (NeoBioscience, China) concentrations in the serum were determined by ELISA kits according to the instructions.

Western blot analysis

Total protein and nuclear protein were extracted primarily using RIPA and nuclear protein extraction kit (Beyotime, Shanghai, China). Protein concentration was measured by BCA (Beyotime, Shanghai, China). After separation in gels, blocking was performed for 15 min using quick western blocking solution. After overnight incubation with primary antibodies (1:1,000 with diluent), proteins were combined with the primary antibody. Then, we incubated the proteins with secondary antibody (1:10,000 diluent) for 1.5 h. We visualized the proteins using Fusion FX7.

Luciferase reporter assay

The 3'-UTR of Keap1 was amplified and cloned into the pGL3 vector (Promega, WI, USA), creating wild-type (WT) pGL3-Keap1 3'-UTR. Point mutations in the potential miR-141-3p binding site were performed to create mutated pGL3-Keap1 3'-UTR. For the luciferase assay, LO2 cells were transfected with pGL3-Keap1 3'-UTR-wt/mutant and miR-141-3p mimic/NC. Luciferase activities were measured using the Dual Luciferase kit (Promega, WI, USA).

Measurement of intracellular ROS accumulation

DCFH-DA fluorescence dye was used to examine intracellular ROS accumulation. Briefly, LO2 cells were seeded in plates and treated with the miRNA-141-3p mimic and inhibitor. After incubation, DCFH-DA was added to serum-free culture medium, which was added to the cells and then incubated for 15 min in the dark at 37°C. For flow cytometry analysis, dichlorofluorescein (DCF) fluorescence intensity was measured using a BD FACSAria II flow cytometer (USA). For confocal laser scanning microscopy (CLSM), after DCFH-DA staining, cells were incubated with Hoechst for 15 min at 37°C, and ROS levels were observed under CLSM (ZESS, Germany) at 488 nm by comparing the fluorescence intensity (green signal).

Serum levels of aminotransferase

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are indexes of liver injury. ALT and AST assay kits (Nanjing Jiancheng, China) and microplate readers (Biotek, USA) were used to measure mouse serum ALT and AST levels.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Differences between groups were analyzed using one-way analysis of variance (ANOVA). Correlation analysis was performed using Spearman's rank correlation method. SPSS 18.0 software (SPSS, Chicago, IL, USA) was used to perform statistical analyses, and $p < 0.05$ was considered statistically significant.

Results

MiR-141-3p is correlated with the recovery of liver function in the serum of patients with liver transplantation

To examine the significance of miR-141-3p in liver transplantation, we first collected serum from 27 liver transplantation patients at the following time points: preoperation, 4 h after reperfusion, and on postoperative days 1, 2 and 3. Basic patient characteristics are summarized in Table 1, and the laboratory data of patients are summarized in Table 2. Next, we assessed miR-141-3p expression. We found that expression of miR-141-3p 4 h after perfusion was lower than that at preoperation, and over time, miR-141-3p increased (Fig. 1a). In contrast, AST and ALT levels, which reflect liver function, were increased 4 h after perfusion compared to preoperation and decreased gradually from days 1 to 3 (Fig. 1b). As shown in Fig. 1c, IL-1β and IL-6 were increased after liver transplantation and gradually decreased after postoperative day 2. Moreover, we analyzed the correlation between expression of miR-141-3p and ALT/AST levels. We found a significant negative correlation between miR-141-3p expression and ALT/AST levels (Fig. 1d-e). Expression of miR-141-3p was strongly correlated with ALT/AST levels 4 h after perfusion ($r>0.80$, $p<0.001$). These data all suggest that miR-141-3p is negatively correlated with the recovery of liver function.

Table 1
Patient characteristics and baseline laboratory data

Parameter	Value
Total number (n)	27
Gender	
Male, n (%)	23 (85.2%)
Female, n (%)	4 (14.8%)
Ages (years)	47 (30-64)
Type of transplant, n (%)	
Deceased donor liver transplant	27 (100%)
Indication for liver transplant, n (%)	
Hepatocellular carcinoma	11 (40.7%)
Cirrhosis with liver decompensation	13 (48.2%)
Acute hepatitis	3 (11.1%)
Continuous variables are expressed as median (range).	

Table 2
Baseline laboratory parameters

Parameter		Value
ALT (U/L)	Pre-operation	46 (15-644)
	4h after reperfusion	471 (144-2089)
	Day1	401 (118-1594)
	Day2	314 (76-1176)
	Day3	229 (64-841)
AST (U/L)	Pre-operation	46 (11-320)
	4h after reperfusion	872 (278-2838)
	Day1	789 (78-2305)
	Day2	348 (37-1411)
	Day3	178 (25-684)
Bilirubin (μmol/L)	Pre-operation	37.4 (5.1-516.5)
	4h after reperfusion	71.5 (11.2-251.9)
	Day1	48.7 (7.5-287.3)
	Day2	42.2 (6.1-248.7)
	Day3	44.1 (6.8-253.5)
MELD score		24 (7-40)
HBV DNA (IU/mL)		
HBV DNA at time of transplant		
HBV DNA< 1*103		25 (92.6%)
HBV DNA>1*103		2 (7.4%)
AST, aspartate aminotransferase; ALT, alanine aminotransferase; MELD, model for end-stage liver disease		
Continuous variables are expressed as median (range)		

MiR-141-3p is downregulated in liver tissue in the hepatic IRI model

To determine expression of miR-141-3p in the hepatic IRI model, we established a mouse hepatic IRI model. We found that levels of mouse serum ALT or AST in the IRI groups were significantly increased compared to the sham group (Fig. 2a). Levels of both serum ALT and AST gradually increased with

reperfusion time. HE analysis revealed large areas of hepatocyte necrosis in hepatic IRI group mice compared to sham group mice (Fig. 2b). MiR-141-3p expression was decreased in the IRI groups compared to the sham group (Fig. 2c). We chose 6 h as our reperfusion time with consideration of the expression between miR-141-3p and ALT/AST levels. These data demonstrate that the miR-141-3p levels are decreased in the hepatic IRI model.

MiR-141-3p inhibits IRI-triggered hepatic dysfunction and cell apoptosis in vivo

To further establish the influence of miR-141-3p on hepatic IRI, mice were transfected with lentivirus. We found that miR-141-3p expression in liver tissues was significantly decreased in both the model and vector control groups, while it was increased in the miR-141-3p treatment group (Fig. 3a). HE analysis revealed large areas of hepatocyte necrosis in the model group mice compared to the sham group, and the degree of necrosis in the miR-141-3p treatment group was less than that in the model and vector control groups (3b). Furthermore, Bcl-2 expression in the miR-141-3p group was higher than in the vector control group, and Bax and cleaved-caspase3 expression in the miR-141-3p group was lower than in the vector control group (Fig. 3c). Finally, western blot analyses showed that Keap1 expression in the miR-141-3p group was lower than in the vector control group (Fig. 3d). Therefore, miR-141-3p ameliorates IR-triggered hepatic dysfunction and apoptosis.

Mir-141-3p Inhibits Apoptosis In H/r-induced Injury Of Lo2 Cells

A search of the TargetScan 7.2 database (http://www. targetscan.org/) indicated that there were binding sites between miR-141-3p and the 3’-UTR of Keap1 mRNA (Fig. 4a). The luciferase reporter assay showed that miR-141-3p attenuated luciferase activity of the reporter containing WT Keap1 3’-UTR but not mutant Keap1 3’-UTR (Fig. 4b). Then we found that miR-141-3p levels were decreased after H/R-induced injury in LO2 cells (Fig. 4c), and Keap1 mRNA and protein levels were increased after H/R (Fig. 4d). Moreover, miR-141-3p mimic/inhibitor and corresponding NC were utilized to modulate endogenous miR-141-3p levels in LO2 cells, and miR-141-3p levels were increased in the H/R +miR-141-3p mimic group and decreased in the H/R +miR-141-3p inhibitor group (Fig. 4e). Bcl-2 expression in the H/R +miR-141-3p mimic group was increased compared to the NC group (Fig. 4f). In contrast, Bax and cleaved-caspase3 expression in the H/R +miR-141-3p mimic group was decreased (Fig. 4f). Keap1 protein levels were decreased in the H/R +miR-141-3p mimic group. However, nuclear Nrf2 levels were increased in the H/R +miR-141-3p mimic group (Fig. 4g). Furthermore, protein expression of two critical Nrf2-dependent genes, HO-1 and NAD(P)H quinone dehydrogenase 1 (NQO1), was increased in the H/R +miR-141-3p mimic group (Fig. 4g). These data indicate that miR-141-3p inhibits apoptosis in LO2 cells, which may be related to Keap1/Nrf2 signaling.

MiR-141-3p inhibits the generation of intracellular ROS in H/R-induced injury

We tested ROS generation mediated by miR-141-3p in LO2 cells. Intracellular ROS levels were measured using the DCFH-DA fluorescence method after treatment. Flow cytometry data showed that ROS induction in the H/R +miR-141-3p mimic group was decreased compared to the NC group and was

increased in the H/R +miR-141-3p inhibitor group (Fig. 5a). Meanwhile, we measured intracellular ROS with CLSM after DCFH-DA and Hoechst staining. Our data revealed that ROS levels were decreased in the H/R +miR-141-3p mimic group (Fig. 5b). This indicates that miR-141-3p also inhibits ROS generation in LO2 cells.

Discussion

IRI is a condition in which ischemic organs experience restored blood flow, which leads to more serious damage, further inducing organ dysfunction [19]. Hepatic IRI often occurs in hypovolemic shock, hepatic resections and liver transplantation [20]. In the current study, we measured the function of miR-141-3p in hepatic IRI. Our results showed that Keap1 signaling might be the signaling pathway through which miR-141-3p attenuates oxidative damage and apoptosis *in vivo* and *in vitro*.

Some miRNAs have been reported to participate in modulating apoptosis and oncogenesis [21]. A previous study indicated that ischemia preconditioning attenuates hepatic post-ischemia TNF release from Kupffer cells, reducing liver injury following hepatic IRI and that the effect of preconditioning is mediated by NO [22]. MiR-141 belongs to the miR-200 family, and the miR-200 family is upregulated early after ischemic preconditioning and is neuroprotective primarily by downregulating proly1 hydroxylase 2 levels [23]. Therefore, we speculated that miR-141-3p overexpression exerts protective effects similar to ischemic preconditioning in hepatic IRI. In the present study, our vision shifts onto the expression profile and local action of miR-141-3p throughout the whole stage of hepatic IRI instead during ischemic preconditioning. It is worth mentioning that in the early stage of liver transplantation, differential expression of miR-141-3p in the serum is meaningful in the present study, as it was negatively correlated with ALT/AST levels. Expression of miR-141-3p induced a certain decreasing trend from postoperative day 1 to 3, and we speculate that the reason for this decline may be a degree of depletion of miR-141-3p with the recovery of liver function. As clinical prognosis data require long-term follow-up, prognostic analysis was not performed in this study, but relevant analyses will appear in our future clinical studies. Consistent with the serum results, we also found that miR-141-3p levels were decreased after hepatic IRI or hepatocyte H/R both *in vivo* and *in vitro*.

Reports have shown that miR-141 also participates in modulating oxidative stress-induced apoptosis. MiR-141 attenuates UV-induced damage through Nrf2 stabilization and activation in retinal pigment epithelium cells [24]. MiR-141-3p interacts with CHD8, which plays critical roles in cardiomyocyte apoptosis induced by H/R [25]. The Keap1-Nrf2 complex may ameliorate hepatic IRI in orthotopic liver transplantations, as Keap1 negatively regulates Nrf2, and Keap1-Nrf2 regulates Akt activation, which is beneficial to cell survival [26]. Nrf2 is a transcription factor that protects organs against oxidative stress [27]. There is evidence that depletion of Nrf2 increases susceptibility to liver injury [28], indicating that Nrf2 plays a major role in the hepatic protective pathway. HO-1 is a stress-induced isoform that attenuates oxidative damage, and previous studies have reported that HO-1 protects against liver, neurological, renal, and intestinal IRI [29, 30]. These studies have shown that miR-141-3p regulates oxidative stress by targeting Keap1. In our study, it was confirmed that miR-141-3p alleviates oxidative

stress damage in the liver through Keap1. In addition, a previous report showed that miR-141 attenuates myocardial IRI via antithetical regulation of ICAM-1 and inflammatory cells [31]. This report indicates that miR-141 also ameliorates hepatic IRI through other pathways, but this hypothesis requires further investigation.

In conclusion, in this study, we combined clinical and basic experimental research methods and found that miR-141-3p ameliorates hepatic IRI both *in vivo* and *in vitro*. The potential mechanism of this protection is related to the inhibition of Keap1 and resulting reduced degradation of Nrf2, which attenuated oxidative stress-induced damage and apoptosis. The study suggests that miR-141-3p might be a potential test index molecule and therapeutic target in hepatic IRI.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Authors' Contributions

ZW and TL participated in designing the experiments and editing the article. TL, XW and QC participated in the collection of samples. TL, QC, JD, ZH, YL, JP and HY participated in performing the studies. TL, ZH and TM wrote the manuscript.

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Compliance with Ethical Standards

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Figures

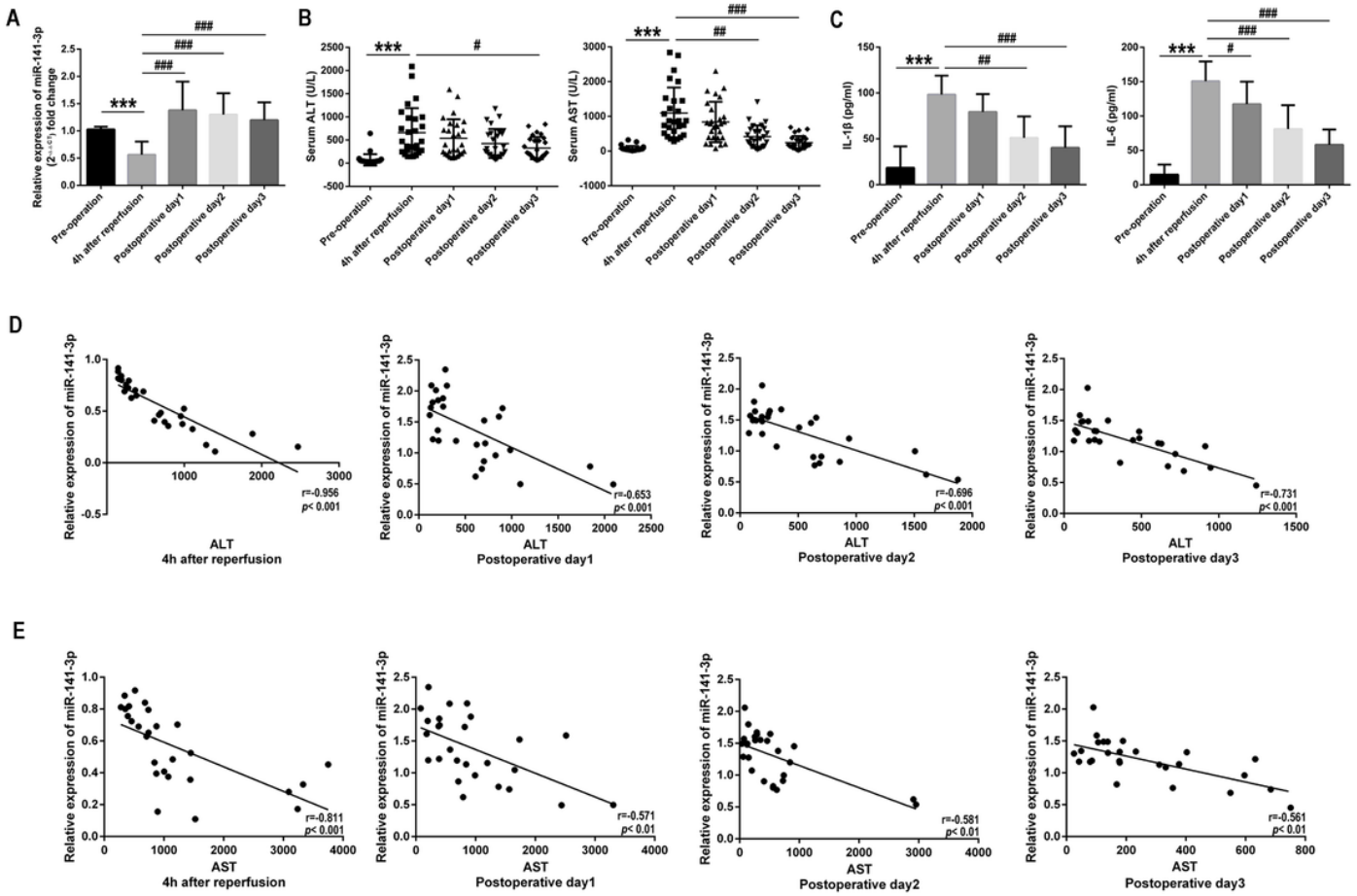

Figure 1

MiR-141-3p is correlated with the recovery of liver function in liver transplantation patients. a RT-qPCR analysis of serum miR-141-3p levels (U6 as the internal control) at the following time points of liver transplantation: preoperation, 4 h after reperfusion, and postoperative days 1, 2 and 3. b Serum levels of ALT and AST at the above time points. c Serum expression of IL-1β and IL-6 was quantified. d Correlation analysis between miR-141-3p and ALT levels. e Correlation analysis between miR-141-3p and AST levels. ***P<0.001 compared to the preoperation group. #P<0.05, ##P<0.01, and ###P<0.001 compared to the reperfusion group 4 h after reperfusion.

Figure 2

MiR-141-3p is downregulated in liver tissue of the hepatic IRI model. a Mouse serum ALT and AST levels after IRI. b HE staining analysis of liver injury after mouse IRI. Original magnification ×200. Scale bar=200μm. c MiR-141-3p levels of liver tissues were analyzed by RT-qPCR. n=5 for each group. *P<0.05, ***P<0.001 compared to the sham group.

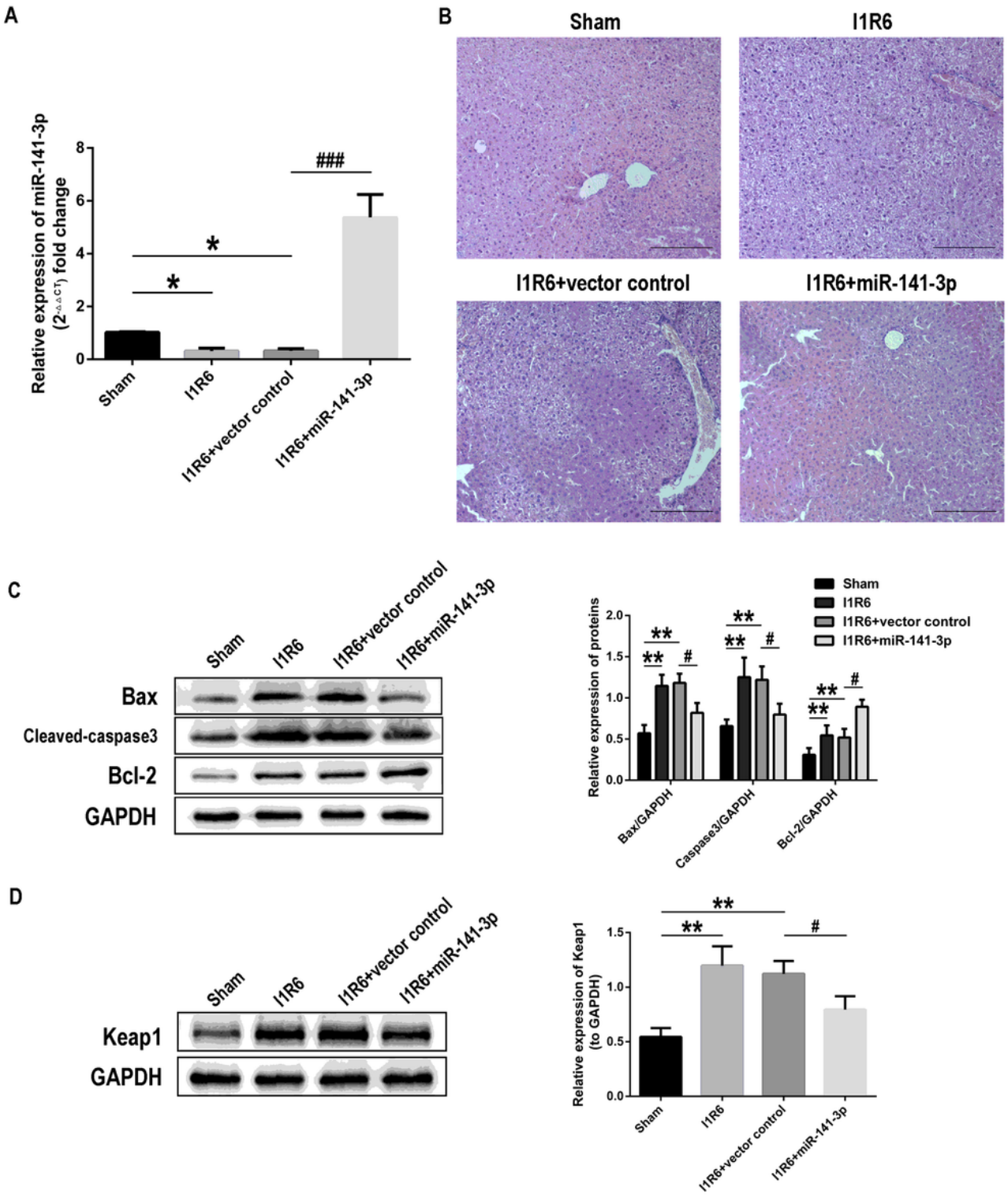

Figure 3

MiR-141-3p inhibits IRI-triggered hepatic dysfunction and apoptosis in vivo. a MiR-141-3p levels in liver tissues were analyzed by RT-qPCR in the sham, I1R6, I1R6+vector control and I1R6+miR-141-3p groups. b HE staining analysis of liver injury. Original magnification ×200. Scale bar=200μm. c Western blot analysis showing Bax, cleaved caspase-3. d Western blot analysis of Keap1 expression. *P<0.05,

**P<0.01 compared to the sham group. n=6 for each group. #P<0.05, ###P<0.001 compared to the I1R6+vector control group.

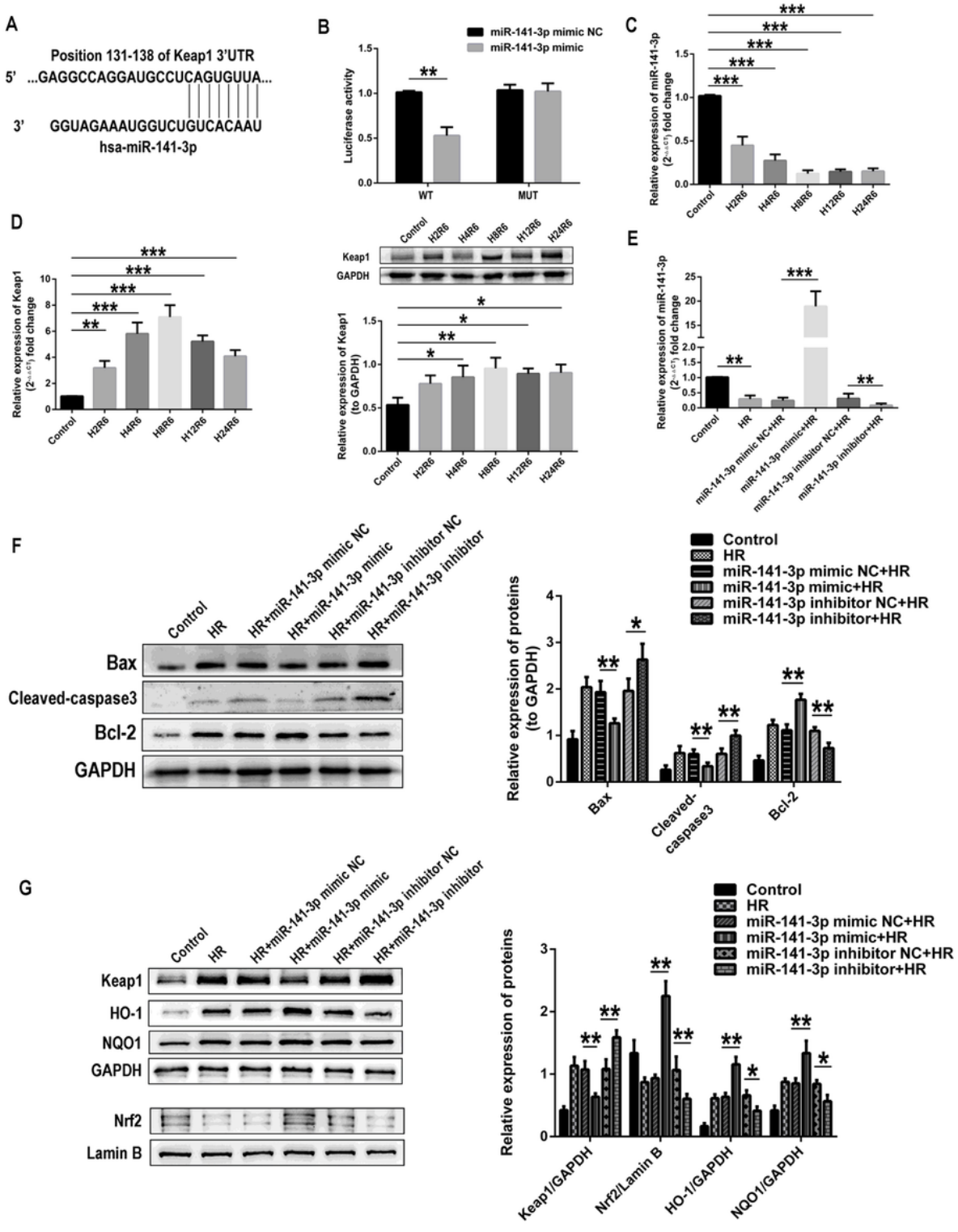

Figure 4

MiR-141-3p inhibits apoptosis in H/R-induced injury of LO2 cells. a MiR-141-3p selectively targets 131-138 of the Keap1 3'UTR. b Luciferase activity of each group was detected. c Level of miR-141-3p were analyzed by RT-qPCR after H/R in LO2 cells. d mRNA and protein levels of Keap1 were analyzed by

RT-qPCR and western blot after H/R. *P<0.05, **P<0.01, ***P<0.001 compared to control group. e MiR-141-3p levels were analyzed after treatment with miR-141-3p mimic and inhibitor. f Western blot analysis of Bax, cleaved-caspase3 and Bcl-2 expression. g Western blot analysis of Keap1, Nrf2, HO-1 and NQO1. n=6 for each group *P<0.05, **P<0.01, ***P<0.001 compared to the NC group.

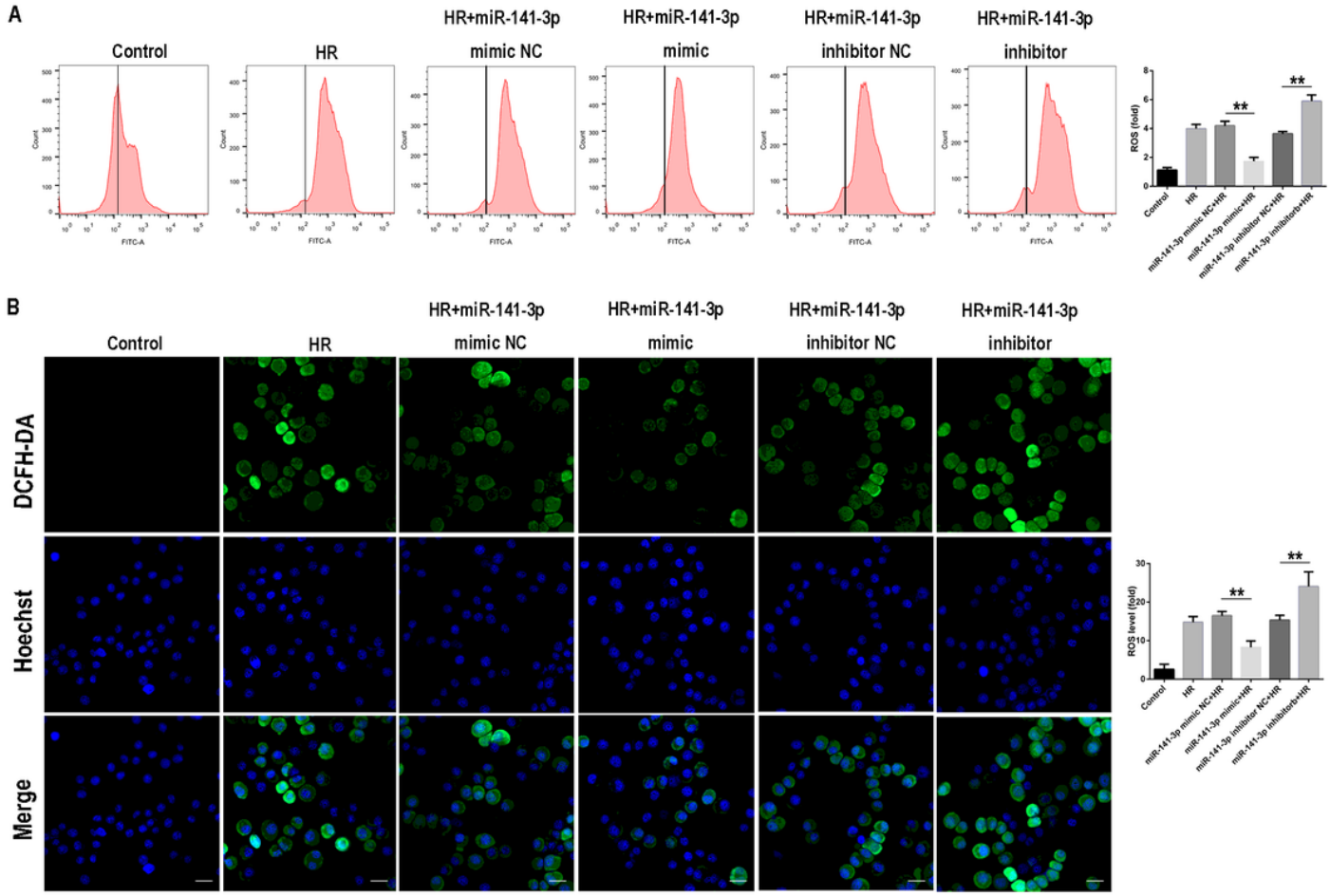

Figure 5

MiR-141-3p inhibits the generation of intracellular ROS in H/R-induced injury. a Flow cytometry analysis of ROS induction in response to treatment with miR-141-3p mimic and inhibitor. b Confocal laser scanning microscopy analysis of intracellular ROS (200x magnification). Scale bar=30μm. **P<0.01 compared to the NC group.