International Journal of Immunopathology & Pharmacology

Original Research Article

D-Pinitol protects against endoplasmic reticulum stress and apoptosis in hepatic ischemia-reperfusion injury via modulation of AFT4-CHOP/GRP78 and caspase-3 signaling pathways

International Journal of Immunopathology and Pharmacology
Volume 35: 1–15

DOI: 10.1177/20587384211032098
journals.sagepub.com/home/iji
SAGE

Lei Yan[1,2], Heng Luo[3], Xingsheng Li[4] and Yongyong Li[4]

Abstract

Hepatic ischemia-reperfusion injury (IRI) is a major unavoidable clinical problem often accompanying various liver surgery and transplantation. D-Pinitol, a cyclic polyol, exhibits hepatoprotective efficacy. The objective of this study is to determine the possible mechanism of action of pinitol against endoplasmic reticulum (ER) stress regulation-mediated hepatic IRI and compare its effects with thymoquinone (TQ) in experimental rats. Male Sprague Dawley rats were pre-treated orally with either vehicle (DMSO) or D-Pinitol (5, 10, and 20 mg/kg) or TQ (30 mg/kg) for 21 days and subjected to 60 min of partial hepatic ischemia followed by 24 h of reperfusion. Pre-treatment with pinitol (10 and 20 mg/kg) effectively ($P < 0.05$) protected against IRI-induced hepatic damage reflected by attenuation of elevated oxidative stress and pro-inflammatory cytokines. Additionally, western blot and ELISA analyses suggested that pinitol significantly ($P < 0.05$) down-regulated expression of endoplasmic reticulum stress apoptotic markers, namely glucose-regulated protein (GRP)-78, CCAAT/enhancer-binding protein homologous protein (CHOP), activating transcription factor (AFT)-4 and -6α, X-box binding protein-1, and caspase-3, 9, and 12. Additionally, pinitol pre-treatment effectively ($P < 0.05$) improved mitochondrial function and phosphorylation of Extracellular signal-regulated kinase (ERK)-1/2 and p38. Pinitol markedly ($P < 0.05$) protected hepatic apoptosis determined by flow cytometry. Further, pinitol provided effective ($P < 0.05$) protection against hepatic histological and ultrastructural aberrations induced by IRI. TQ showed more pronounced protective effect against attenuation of IRI-induced hepatic injury as compared to D-Pinitol. Pinitol offered protection against endoplasmic reticulum stress-mediated phosphorylation of ERK1/2 and p38, thereby inhibiting AFT4-CHOP/GRP78 signaling response and caspase-3 induced hepatocellular apoptosis during hepatic ischemia-reperfusion insults. Thus, Pinitol can be considered as a viable option for the management of hepatic IRI.

Keywords

AFT-6α, caspase-3, liver injury, oxidative stress, XBP-1

Date received: 11 April 2021; accepted: 23 June 2021

[1]Clinical Experimental Centre, Xi'an International Medical Center Hospital, Xi'an, Shaanxi, China
[2]Xi'an Engineering Technology Research Center for Cardiovascular Active Peptides, Xi'an, Shaanxi, China
[3]Reproductive Medicine Center, Tangdu Hospital, Fourth Military Medical University, Xi'an, Shaanxi, China
[4]Department of Gerontology, The Second Affiliated Hospital of Chongqing Medical University, Yuzhong, Chongqing, China

Corresponding author:
Yongyong Li, Department of Gerontology, The Second Affiliated Hospital of Chongqing Medical University, No. 76 Linjiang Road, Yuzhong District, Chongqing 400010, China.
Emails: yy_licqmu@163.com

Introduction

Hepatic ischemia-reperfusion injury (IRI) is a major pathophysiological phenomenon that commonly occurs in an array of clinical conditions such as trauma, circulatory shock, liver transplantation, and resection surgery leading to liver dysfunction, even failure.[1] Hepatic ischemia-reperfusion is an inevitable process during liver surgery or transplantation; thus, numerous advanced techniques such as hepatic pedicle clamping (Pringle's maneuver) and hemihepatic vascular occlusion (HHO) have been introduced to control the hepatic arterial and portal venous flow.[2] Despite implementing such techniques, hepatic ischemia-reperfusion is considered one of the most challenging elements during hepatic surgery and is associated with a significant number of postoperative morbidity and mortality.[2] A mild hepatic IRI often causes multiple organ dysfunction, including lungs, heart, and kidneys, secondary to liver damage.[3]

Despite significant pharmaceutical industry development, safe and effective treatment options for clinical management of hepatic IRI remain challenging. Moreover, various surgical techniques have been evaluated during hepatic reperfusion however, none could prevent mortality associated with IRI.[2] Thus, there is a need for alternative options to prevent mortality and improve patient survival during hepatic IR insult. Several researchers have used a preconditioning strategy by implementing various moieties such as lipoic acid and isoflurane to protect against hepatic IRI.[4,5] These agents have been shown to inhibit elevated postsurgical levels of aspartate transferase and alanine transferase, thus improving the condition of patients with hepatic IR. Furthermore, intravenous administration of *N*-acetylcysteine in patients undergoing liver transplantation showed improved liver function.[6,7] However, the mechanism of this protective effect is not yet fully elucidated, and clinical outcomes about the effect of these treatment options against hepatic warm IRI are still ambivalent.[8] Thus, an effective treatment strategy for clinical management of hepatic IRI is urgently needed. Researchers have used various experimental models to evaluate the potential number of therapeutic moieties against hepatic IRI.[9,10] However, partial warm hepatic ischemia induced by occlusion of the hepatic artery and the portal vein is well established and widely used in animal models which mimic most of the clinical features of human hepatic IRI.[9,11] In the present investigation, we have also implemented animal model to evaluate the potential of D-Pinitol against hepatic IRI.

Pinitol (3-*O*-methyl-*chiro*-inositol) is a cyclic polyol that has shown its clinical potential in patients with non-alcoholic fatty liver disorder (NAFLD) and Type 2 diabetes mellitus.[12,13] In 12 weeks randomized, double-blind, placebo-controlled study, administration of pinitol (500 mg/d) in subjects with NAFLD showed inhibition of elevated AST (aspartate transaminase), ALT (alanine aminotransferase), and oxidative stress levels as a testimony to its hepatoprotective potential.[12] The antidiabetic effect of pinitol was mediated by a significant diminution in the generation of reactive oxygen species (ROS) in Type 2 diabetes mellitus patients during a 12-week, double-blind, randomized trial.[13] Its wide range of pharmacological activities include antihyperlipidemic, hepatoprotective, cardioprotective, antidiabetic, anti-inflammatory, antioxidant, and anticancer.[14,15] Inhibitory potentials of pinitol against various pro-inflammatory cytokines including TNF-α (tumor necrosis factor-alpha), and ILs (interleukins) has been significantly implemented for its antiarthritic potential.[16] Furthermore, pinitol exerts its antiapoptotic potential via modulation of expression of Bcl-2 (B-cell lymphoma-2) and Bcl-xL (B-cell lymphoma-extra-large) in-vitro.[14] The hepatoprotective effect of pinitol against D-galactosamine induced,[17] high-fat diet-induced,[18] and streptozotocin-induced[19] hepatotoxicities have been well established. However, the potential of pinitol against IRI remains largely unknown. Thus, the present investigation was undertaken to determine the possible mechanism of action of pinitol and to compare its effects with thymoquinone against the regulation of endoplasmic reticulum (ER) stress during hepatic IRI in experimental rats.

Materials and methods

Drugs and chemicals

D-Pinitol (purity 95%) was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Rats-specific TNF-α, IL-1β, and IL-6 enzyme-linked immunosorbent assay (ELISA) Kit were obtained from Bethyl Laboratories Inc. (Montgomery, TX, USA). The primary antibodies of caspase-3, caspase-9,

caspase-12, AFT4 (Activating transcription factor-4), AFT6α (Activating transcription factor-6α), XBP-1 (X-box binding protein 1), ERK-1/2 (Extracellular signal-regulated kinase-1/2), and p38 were purchased from Abcam (Cambridge, MA, USA).

Animals

Adult male Sprague Dawley rats (180–200 g) were purchased from Second Affiliated Hospital of Chongqing Medical University Animal House, China, and kept in quarantine for 1-week in-house at the institute animal house under standard laboratory conditions, that is, a temperature of 24°C ± 1°C, relative humidity of 45–55% and 12 h light/dark cycle. Animals had free access to standard chow pelleted food and water. The experimental protocol was approved by the Institutional Animal Ethics Committee of Second Affiliated Hospital of Chongqing Medical University (CQMU-efy-2020021), China, and performed in accordance with the guidelines of the National Institute of Health Guide for Care and Use of Laboratory Animals.

Selection of sample size

The sample size calculation was based on the resource equation approach.[20] The acceptable range of degrees of freedom (DF) for the error term in an analysis of variance (ANOVA) is between a minimum and a maximum number of animals per group. To determine the sample sizes per group, the present study considered the glucose-regulated protein (GRP)-78 as the primary outcome to compared between various treatment groups.

Induction of hepatic IRI

The rats were made to fast for 12 h prior to the experiment but were provided with tap water ad libitum. Rats were anesthetized with an intraperitoneal (i.p.) injection of pentobarbital (5%), a midline abdominal incision was performed vertically at a length of 3.5–4 cm. The liver was then separated from its surrounding ligaments to expose the hilar, and then they subjected to surgery as described previously.[10,11] Briefly, a partial warm hepatic ischemia model (70% of liver mass) was induced by occlusion of the hepatic artery with a microvascular clamp for 60 min. This selective interruption of the blood supply avoided accompanying gastrointestinal congestion and hemodynamic instability. After removal of the clamps, reperfusion was initiated for the next 24 h. During the reperfusion, warm saline was injected into the abdomen, and the abdomen was closed temporarily with a continuous 4-0 silk suture. All rats were placed in a designed warm container (HTP-1500 Heat Therapy Pump, Adroit Medical Systems, USA) to maintain their temperature at 29°C.

Experimental groups

Animals were divided randomly into various groups ($n = 12–16$) as follows:

Group 1: Sham group: Rats were subjected to the surgical procedure but without the occlusion of the hepatic pedicle. They received pre-treatment of a vehicle (10 g/kg of 1% aqueous DMSO (Dimethyl sulfoxide) solution, p.o.) for 21 days.

Group 2: IRI control group: Rats were subjected to 60 min of partial ischemia (70%) followed by 24 h reperfusion. They received pre-treatment of a vehicle (10 g/kg of 1% aqueous DMSO solution, p.o.) for 21 days.

Group 3: Thymoquinone (30 mg/kg) treated group: Rats were subjected to 60 min of partial ischemia (70%) followed by 24 h reperfusion. They received pre-treatment of thymoquinone (30 mg/kg, p.o.) for 21 days.

Group 4: D-Pinitol (5 mg/kg) treated group: Rats were subjected to 60 min of partial ischemia (70%) followed by 24 h reperfusion. They received pre-treatment of D-Pinitol (5 mg/kg, p.o.) for 21 days.

Group 5: D-Pinitol (10 mg/kg) treated group: Rats were subjected to 60 min of partial ischemia (70%) followed by 24 h reperfusion. They received pre-treatment of D-Pinitol (10 mg/kg, p.o.) for 21 days.

Group 6: D-Pinitol (20 mg/kg) treated group: Rats were subjected to 60 min of partial ischemia (70%) followed by 24 h reperfusion. They received pre-treatment of D-Pinitol (20 mg/kg, p.o.) for 21 days.

Previous reports were used to determine the treatment doses of pinitol (5, 10, 20 mg/kg).[15,16]

Serum biochemistry

On day 21, at the end of the reperfusion period (24 h), blood was withdrawn by a retro-orbital plexus, and serum was obtained by centrifugation at 8350*g* for 10 min, at 4°C. The serum AST and ALT levels were measured using reagent assay kits (Accurex Biomedical Pvt. Ltd., Mumbai, India) with an ultraviolet-visible spectrophotometer (JASCO-V-530, JASCO Corp., Tokyo, Japan).

Biochemical estimation

Tissue homogenate preparation, estimation of oxidative stress, and pro-inflammatory markers. All animals were sacrificed at the end of the study, that is, on the 21st day, the liver was immediately isolated. Tissue homogenates were prepared with 0.1 M Tris-HCl buffer (pH 7.4), and supernatant of homogenates were employed to estimate superoxide dismutase (SOD), reduced glutathione (GSH), lipid peroxidation (MDA, i.e. Malondialdehyde content), and nitric oxide (NO) content as described previously.[21] Another portion of aliquot was used for estimation of hepatic pro-inflammatory markers using rats-specific TNF-α, IL-1β, IL-6, glucose-regulated protein 78 (GRP78), CCAAT/enhancer-binding protein homologous protein (CHOP) ELISA Kit (Bethyl Laboratories Inc., Montgomery, TX, USA).

Mitochondrial enzymes estimation

Liver mitochondrial isolation was performed according to a previously described method.[22] Mitochondrial complex-I activity was measured spectrophotometrically according to a previously described method.[23] Mitochondrial Complex-II activity Succinate dehydrogenase (SDH) was measured spectrophotometrically according to an already described method.[23] Mitochondrial redox activity (Complex-III), that is, the MTT reduction rate, was used to assess the activity of mitochondrial respiratory chain in isolated mitochondria. It was determined according to an already established method.[24] Mitochondrial complex-IV (Cytochrome oxidase assay) activity was assayed in liver mitochondria according to a previously described method.[25]

Reverse transcriptase PCR

The mRNA expressions of GRP78 (Forward: 5′-CTGAGGCGTATTTGGGAAAG-3′, Reverse: 5′-TCATGACATTCAGTCCAGCAA-3′), CHOP (Forward: 5′-CTTGAGCCTAACACGTCGATT-3′, Reverse: 5′-TGCACTTCCTTCTGGAACACT-3′), and β-actin (Forward: 5′-GTCACCCACACTGT GCCCATCT-3′, Reverse: 5′-ACAGAGTACTTG CGCTCAGGAG-3′) were analyzed in liver tissue using quantitative reverse transcription-polymerase chain reaction (qRT–PCR) according to a method described elsewhere.[26] PCR was performed using 1 X forward and reverse primers and 2.5 U Taq polymerase (MP Biomedicals India Private Limited, India). Amplification of β-actin served as a control for sample loading and integrity.

Western blot procedure

The protein expressions of caspase-3, caspase-9, caspase-12, AFT4, AFT6α, XBP-1, ERK-1/2, p38, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were estimated in liver tissue according to a method described elsewhere.[27] Briefly, liver tissue was dissected and sonicated in Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Inc., India). The lysates were centrifuged at 10,000*g* for 10 min at 4°C. Protein concentrations were determined using a Bicinchoninic Acid (BCA) assay kit (Beyotime Shanghai, China) on ice for 30 min. An equal amount of extracted protein samples (50 μg) were separated by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dry milk at 37°C for 1 h and incubated overnight at 4°C with the respective primary antibodies that recognized caspase-3, caspase-9, caspase-12, AFT4, AFT6α, XBP-1, ERK-1/2, p38, and GAPDH. Anti-rabbit horseradish-linked IgG was used as the secondary antibody, incubated for 37°C for 2 h. Protein bands were visualized using the Chemiluminescent kit (Bio-Rad Laboratories, Inc.). GAPDH served as the loading control.

Preparation of single-cell (SC) suspensions and flow cytometry analysis

Preparation of single-cell (SC) suspensions and determination of apoptotic cell populations were

carried out as previously described.[26] At the end of treatment, the liver of rats was collected and mixed with 0.4% collagenase and 0.25% Trypsin at 37°C for 30 min and dissociated, ground and obtained homogenate was passed through a 70 µm nylon mesh. Single-cell (SC) suspension was washed three times with phosphate-buffered saline (PBS). In order to determine SC apoptosis, the isolated SCs were incubated with rabbit anti-cow S-100 antibody, followed by staining with APC-goat anti-rabbit IgG (both from BD) with FITC-Annexin V and PI (Sigma-Aldrich, St. Louis, MO, USA). The percentages of expression of Fas and Annexin-V on gated S-100 positive SC were analyzed by a FACS Calibur cytometer using CELL Quest software (Becton & Dickinson, San Diego, USA).

Histological and electron microscopic analysis

Histopathological analysis of liver tissue was carried out using hematoxylin and eosin (H&E) stain under a light microscope (Nikon E200, Japan) whereas, the liver ultrastructural studies were performed under a transmission electron microscope (H-7000 Hitachi) according to a method described previously.[21]

Statistical analysis

GraphPad Prism 5.0 software (GraphPad, San Diego, CA) was used to perform data analysis. Data are expressed as mean ± standard error mean (SEM) and analyzed using One-Way ANOVA followed by Tukey's multiple range post hoc analysis (for parametric tests) as well as Kruskal-Wallis test for post hoc analysis (non-parametric tests). A value of $P < 0.05$ was considered to be statistically significant.

Results

Effect of IRI-induced alterations in body weight, liver index, and hepatic function tests in rats

Induction of IRI was associated with significant ($P < 0.05$) reduction in body weight and increased liver index in the IRI control group compared to the sham group. When compared with the IRI control group, administration of TQ and pinitol (10 and 20 mg/kg) showed a significant ($P < 0.05$) attenuation of IRI-induced alterations in body weight and liver index. However, pinitol (20 mg/kg) showed more significant ($P < 0.05$) attenuation in the prevention of IRI-induced increased liver index as compared to the TQ group (Table 1).

There was marked elevation ($P < 0.05$) in the hepatic function test (AST and ALT) in the IRI control group as compared to the sham group. These elevations of AST and ALT levels were significantly prevented by TQ and pinitol (10 and 20 mg/kg) treatment compared with the IRI control group. However, prevention in elevation of AST and ALT levels were more noticeably ($P < 0.05$) inhibited by the TQ group compared to the pinitol group (Table 1).

Effect of IRI-induced alterations in hepatic oxidative stress in rats

The hepatic SOD and GSH levels decreased significantly ($P < 0.05$), whereas hepatic MDA and NO levels elevated prominently ($P < 0.05$) in the IRI control group when compared to the sham group. Pre-treatment with TQ significantly ($P < 0.05$) inhibited IRI-induced elevated hepatic oxidative stress compared to the IRI control group. Pinitol (10 and 20 mg/kg) treatment also markedly improved ($P < 0.05$) hepatic SOD and GSH levels, whereas hepatic MDA and NO levels decreased significantly ($P < 0.05$) as compared to the IRI control group. The inhibition of IRI-induced hepatic oxidative stress was more significant ($P < 0.05$) in the TQ group as compared to the pinitol treated group (Table 1).

Effect of IRI-induced alterations in a hepatic mitochondrial complex in rats

IRI-induces a significant ($P < 0.05$) reduction in hepatic mitochondrial complex (I–IV) levels in the IRI control group as compared to the sham group. IRI-induced reduction in hepatic mitochondrial complex (I–IV) levels was prominently ($P < 0.05$) inhibited by TQ pre-treatment as compared to the IRI control group. Pinitol (10 and 20 mg/kg) administration also showed significant ($P < 0.05$) improvement in hepatic mitochondrial complex (I–IV) levels compared to the IRI control group. However, hepatic mitochondrial complex (I–IV) levels were more effectively ($P < 0.05$) increased by the TQ group compared to pinitol treatment (Table 2).

Table 1. Effect of pinitol treatment on IRI-induced alterations in body weight, liver index, AST, ALT, and hepatic oxidative stress in rats.

Parameters	Treatment					
	Sham	IRI control	TQ (30)	P (5)	P (10)	P (20)
Body weight (g)	234.00 ± 3.73	220.70 ± 2.36#	233.70 ± 3.73*,$	211.80 ± 1.96	220.80 ± 3.26*,$	229.50 ± 3.71*,$
Liver index	0.0223 ± 0.0013	0.0329 ± 0.0015#	0.0272 ± 0.0013*,$	0.033 ± 0.0017	0.0303 ± 0.0008*,$	0.0253 ± 0.0014*,$
AST (IU/L)	81.86 ± 12.71	300.00 ± 14.57#	118.90 ± 13.59*,$	306.40 ± 12.66	231.30 ± 9.091*,$	160.30 ± 12.63*,$
ALT (IU/L)	33.47 ± 5.28	146.90 ± 3.80#	49.23 ± 4.48*,$	142.10 ± 6.14	94.77 ± 7.06*,$	62.50 ± 6.11*,$
SOD (U/mg of protein)	6.72 ± 0.47	2.14 ± 0.32#	5.44 ± 0.41*,$	2.80 ± 0.33	3.93 ± 0.49*,$	5.83 ± 0.47*,$
GSH (μg/mg of protein)	14.43 ± 0.83	5.63 ± 0.79#	14.08 ± 0.66*,$	6.55 ± 0.46	10.75 ± 0.68*,$	12.09 ± 0.59*,$
MDA (nM/mg of protein)	0.20 ± 0.03	0.80 ± 0.03#	0.36 ± 0.04*,$	0.71 ± 0.03	0.55 ± 0.03*,$	0.39 ± 0.04*,$
NO (μg/mg of protein)	0.18 ± 0.03	0.88 ± 0.04#	0.23 ± 0.03*,$	0.73 ± 0.07	0.42 ± 0.04*,$	0.25 ± 0.02*,$

Data were represented as mean ± SEM ($n = 6$). Data were analyzed by one-way ANOVA, followed by Tukey's multiple range test. Figures in parentheses indicate oral dose in mg/kg.
IRI: ischemia-reperfusion Injury; TQ (30): thymoquinone (30 mg/kg) treated; P (5): pinitol (5 mg/kg) treated; P (10): pinitol (10 mg/kg) treated; P (20): pinitol (20 mg/kg) treated rats; AST: aspartate transaminase; ALT: alanine aminotransferase; SOD: superoxide dismutase; GSH: glutathione; MDA: malondialdehyde; NO: nitric oxide.
#$P < 0.05$ as compared with sham group.
*$P < 0.05$ as compared with IRI control group.
$$P < 0.05$ as compared thymoquinone with pinitol.

Table 2. Effect of pinitol treatment on IRI-induced alterations in hepatic mitochondrial complex, TNF-α, IL-1β, and IL-6 levels in rats.

Parameters	Treatment					
	Sham	IRI control	TQ (30)	P (5)	P (10)	P (20)
Complex I (nmole of NADH oxidized/min/mg protein)	33.57 ± 3.49	6.86 ± 3.61#	30.59 ± 3.31*,$	10.01 ± 2.88	16.85 ± 2.75*,$	27.71 ± 3.10*,$
Complex II (mmole/mg protein)	14.62 ± 0.76	4.34 ± 0.76#	11.92 ± 0.83*,$	4.71 ± 0.68	9.03 ± 0.70*,$	11.30 ± 0.53*,$
MTT assay (OD at 540 nm)	0.45 ± 0.04	0.16 ± 0.02#	0.44 ± 0.02*,$	0.22 ± 0.03	0.31 ± 0.03*,$	0.39 ± 0.04*,$
Complex-IV (nmol cyto-C oxidized/min/mg protein)	6193.00 ± 329.9	880.50 ± 251.50#	5312.00 ± 339.10*,$	1561.00 ± 226.60	3183.00 ± 159.20*,$	4550.00 ± 286.40*,$
TNF-α (pg/mg of protein)	0.22 ± 0.01	1.56 ± 0.15#	0.34 ± 0.03*,$	1.21 ± 0.13	0.79 ± 0.06*,$	0.44 ± 0.03*,$
IL-1β (pg/mg of protein)	0.02 ± 0.01	0.28 ± 0.03#	0.05 ± 0.01*,$	0.22 ± 0.02	0.12 ± 0.01*,$	0.07 ± 0.01*,$
IL-6 (pg/mg of protein)	0.14 ± 0.01	0.56 ± 0.05#	0.18 ± 0.02*,$	0.49 ± 0.05	0.31 ± 0.02*,$	0.22 ± 0.01*,$

Data were represented as Mean ± SEM ($n = 4$) and analyzed by one-way ANOVA followed by Tukey's multiple range test. Figures in parentheses indicate oral dose in mg/kg.
IRI: ischemia-reperfusion injury; NADH: nicotinamide adenine dinucleotide; TQ (30): thymoquinone (30 mg/kg) treated; P (5): pinitol (5 mg/kg) treated; P (10): pinitol (10 mg/kg) treated; P (20): pinitol (20 mg/kg) treated rats; TNF-α: tumor necrosis factor-alpha; ILs: interleukins.
#$P < 0.05$ as compared with sham group.
*$P < 0.05$ as compared with IRI control group.
$$P < 0.05$ as compared thymoquinone with pinitol.

Effect of IRI-induced alterations in hepatic pro-inflammatory cytokines levels in rats

The levels of hepatic pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) were significantly ($P < 0.05$) elevated in the control group after hepatic IRI as compared to the sham group. Compared to the IRI control group, TQ pre-treatment effectively ($P < 0.05$) reduced hepatic TNF-α, IL-1β, and IL-6 levels. Pinitol (10 and 20 mg/kg) administration also showed significant ($P < 0.05$) inhibition of hepatic IRI-induced elevated hepatic pro-inflammatory cytokines levels as compared to the IRI control group. Elevated levels of hepatic pro-inflammatory cytokines were more significantly ($P < 0.05$) inhibited by TQ treatment compared to pinitol treatment (Table 2).

Effect of IRI-induced alterations in hepatic apoptosis in rats

Induction of IRI resulted in significant ($P < 0.05$) apoptosis reflected by elevated caspase-3, -9, and -12 protein expressions and apoptotic cells in the IRI control group compared to the sham group. Compared with the IRI control group, TQ treatment showed a significant ($P < 0.05$) reduction of caspase-3, -9, and -12 protein expressions and apoptotic cells. Pinitol (10 and 20 mg/kg) treatment also significantly ameliorated IRI-induced apoptosis compared to the IRI control group. However, the TQ group showed a more effective reduction in IRI-induced apoptosis than pinitol treatment (Figure 1).

Effect of IRI-induced alterations in hepatic GRP78 and CHOP protein, and mRNA expressions in rats

The hepatic GRP78 and CHOP protein and mRNA expressions were up-regulated significantly ($P < 0.05$) in the IRI control group compared to the sham group. TQ administration significantly ($P < 0.05$) inhibited IRI-induced elevated hepatic GRP78 and CHOP protein and mRNA expressions compared to the IRI control group. Administration of pinitol (10 and 20 mg/kg) also prominently down-regulated hepatic GRP78 and CHOP protein and mRNA expressions compared to the IRI control group. However, pinitol treatment showed less significant ($P < 0.05$) amelioration in hepatic GRP78 and CHOP protein and mRNA expressions compared to the TQ group (Figure 2).

Effect of IRI-induced alterations in hepatic AFT4, AFT6α, XBP-1, ERK-1/2, and p38 protein expressions in rats

There was a significant ($P < 0.05$) increase in the hepatic AFT4, AFT6α, and XBP-1 protein expressions, whereas hepatic ERK-1/2 and p38 protein expressions markedly ($P < 0.05$) decreased in the IRI control group as compared to the sham group. Administration of pinitol (10 and 20 mg/kg) efficiently attenuated these IRI-induced modifications in hepatic AFT4, AFT6α, XBP-1, ERK-1/2, and p38 protein expressions compared to the IRI control group. TQ treatment also significantly ($P < 0.05$) decreased hepatic AFT4, AFT6α, and XBP-1 protein expressions and prominently ($P < 0.05$) increased hepatic ERK-1/2 and p38 protein expressions as compared to the IRI control group. Inhibition in IRI-induced modifications in hepatic AFT4, AFT6α, XBP-1, ERK-1/2, and p38 protein expressions was more significant ($P < 0.05$) in the TQ group as compared to pinitol treatment (Figure 3).

Effect of IRI-induced alterations in hepatic histopathology of rats

IRI induces histological aberration in hepatic tissue of the IRI control group, evident by a significant ($P < 0.05$) increase in Suzuki score (Figure 4a) as compared to a sham group (Figure 4b). When compared with the IRI control group, TQ administration showed a significant ($P < 0.05$) reduction in Suzuki score (Figure 4c). Pinitol (10 and 20 mg/kg) treatment also markedly ($P < 0.05$) inhibited IRI-induced histological aberration reflected by reduced Suzuki score (Figure 4d and e) as compared to the IRI control group (Figure 4f).

Effect of IRI-induced alterations in hepatic ultrastructure of rats

Transmission electron microscopy of liver tissue from the sham group showed the presence of normal vesicular cytoplasm, nuclear membrane, and mitochondria within the endoplasmic reticulum (Figure 5a). However, liver tissue from the IRI control group showed distorted vesicular cytoplasm, thickened nuclear membrane, electron-dense mitochondria, accumulation of autophagosomes, and rough endoplasmic reticulum (Figure 5b). However, administration of TQ and Pinitol showed marked

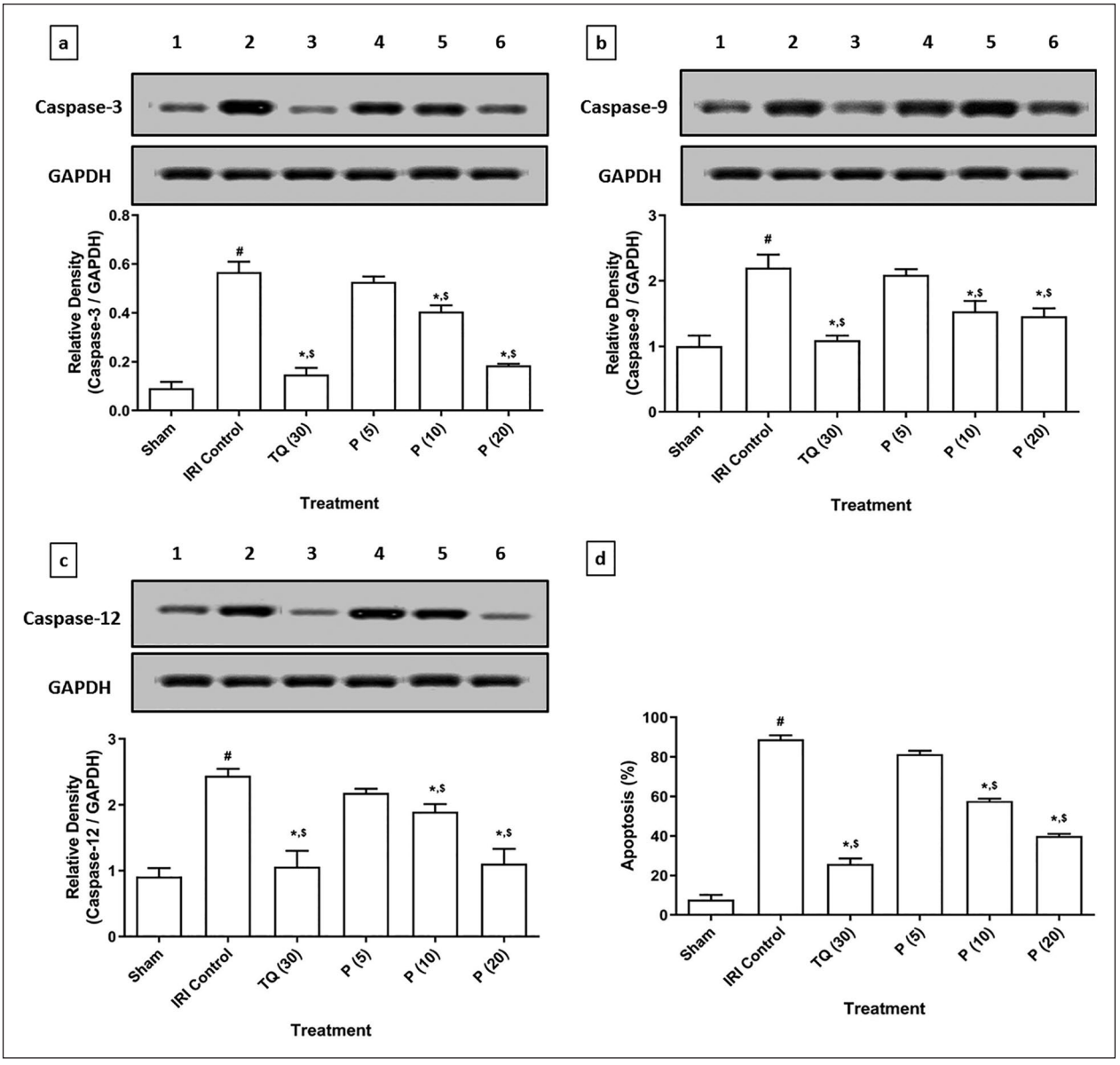

Figure 1. Effect of pinitol treatment on IRI-induced alterations in hepatic caspase-3 (a), caspase-9 (b), caspase-12 (c) protein expression, and apoptosis (d) in rats.

Data were represented as Mean ± SEM ($n = 4$) and analyzed by one-way ANOVA followed by Tukey's multiple range test. Figures in parentheses indicate oral dose in mg/kg.

IRI: ischemia-reperfusion injury; TQ (30): thymoquinone (30 mg/kg) treated; P (5): pinitol (5 mg/kg) treated; P (10): pinitol (10 mg/kg) treated; P (20): pinitol (20 mg/kg) treated rats.

$^{\#}P < 0.05$ as compared with sham group.

$^{*}P < 0.05$ as compared with IRI control group.

$^{\$}P < 0.05$ as compared thymoquinone with pinitol.

attenuation of IRI-induced ultrastructural alterations in hepatic tissue (Figure 5c and d).

Discussion

Hepatic IRI is a major clinical problem associated with patients who undergo liver surgery, transplantation, and circulatory shock.[28,29] Studies demonstrate that IRI-induced insult, which stimulates the generation of ROS, the release of inflammatory cytokines, microvascular modification, and induction of apoptosis, results in hepatocellular dysfunction.[29,30] Furthermore, treatment options are very limited for the clinical management of hepatic IRI. Thus, many researchers have investigated the antiapoptotic potential of various therapeutic moieties for the

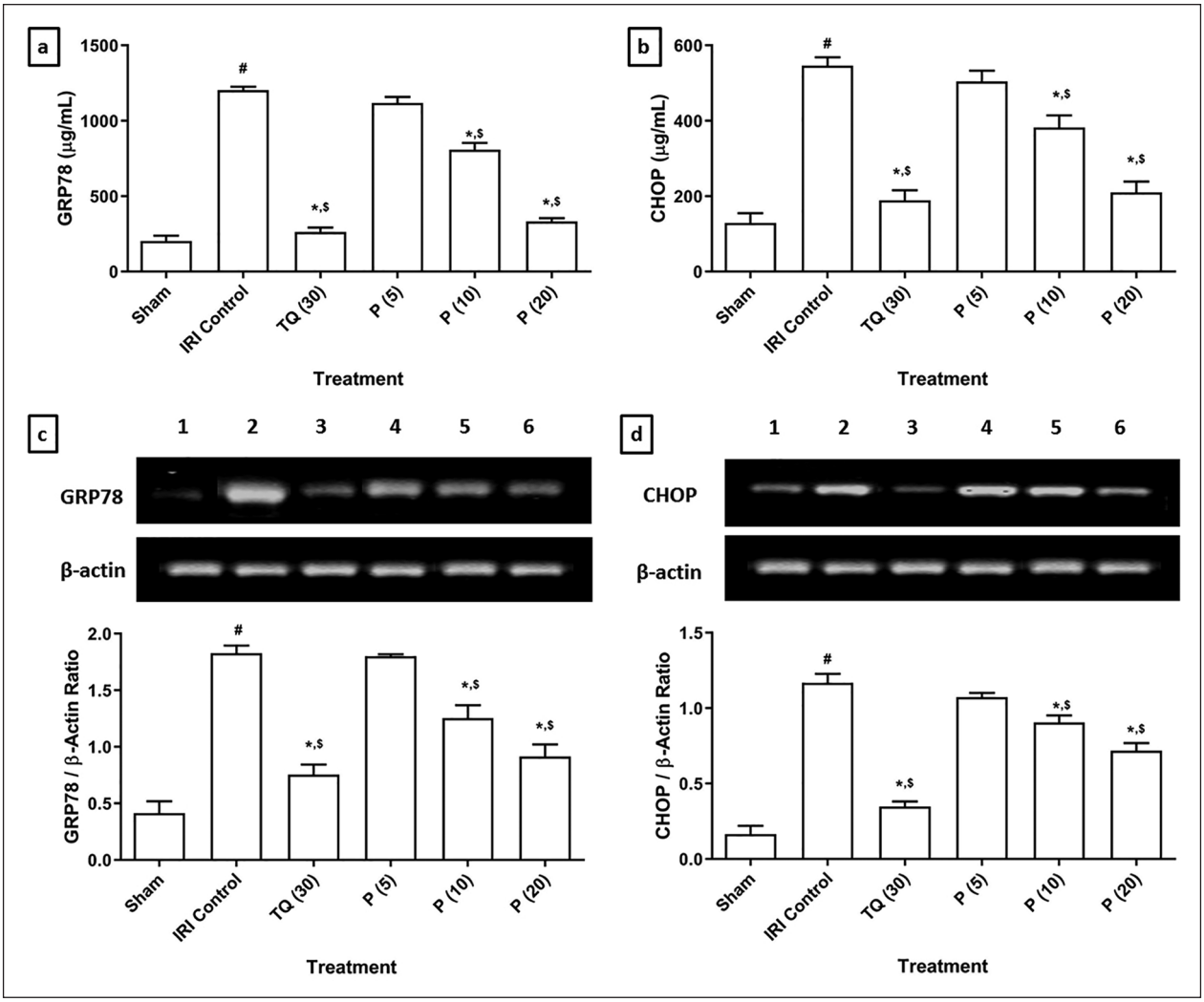

Figure 2. Effect of pinitol treatment on IRI-induced alterations in hepatic GRP78 (a) and CHOP (b) protein levels as well as GRP78 (c) and CHOP (d) mRNA expressions in rats.

Data were represented as Mean ± SEM ($n = 4$) and analyzed by one-way ANOVA followed by Tukey's multiple range test. Figures in parentheses indicate oral dose in mg/kg.

IRI: ischemia-reperfusion injury; TQ (30): thymoquinone (30 mg/kg) treated; P (5): pinitol (5 mg/kg) treated; P (10): pinitol (10 mg/kg) treated; P (20): pinitol (20 mg/kg) treated rats; GRP78: ER chaperone 78-kDa glucose-regulated/binding immunoglobulin protein; CHOP: CCAAT/enhancer-binding protein homologous protein.

#$P < 0.05$ as compared with sham group.

*$P < 0.05$ as compared with IRI control group.

$$P < 0.05$ as compared thymoquinone with pinitol.

treatment of hepatic IRI. Pinitol has been reported for its anti-inflammatory, antioxidant, and antiapoptotic potential.[12,14,15] Thus, in the current study, we have evaluated the potential of pinitol against ER stress-mediated apoptosis during hepatic IRI. The results demonstrated that pre-treatment with pinitol inhibited IRI-induced oxidative stress (SOD, GSH, MDA and NO), pro-inflammatory cytokines (TNF-α and ILs), ER stress (GRP78, CHOP, AFT-4, and AFT6α), mitochondrial damage, and apoptosis (Caspase-3, -9, and -12), thus improving histological and ultrastructural derangements to ameliorate hepatic damage.

Inflammation plays a central role in the induction and maintenance of ER stress during the pathophysiology of hepatic IRI.[10,28] Reperfusion causes activation of Kupffer cells (KCs) to release various pro-inflammatory cytokines such as TNF-α and ILs.[31] These cytokines inaugurate inflammatory response, resulting in leukocytes recruitment and its infiltration. Furthermore, TNF-α stimulates the release of various apoptotic proteins and

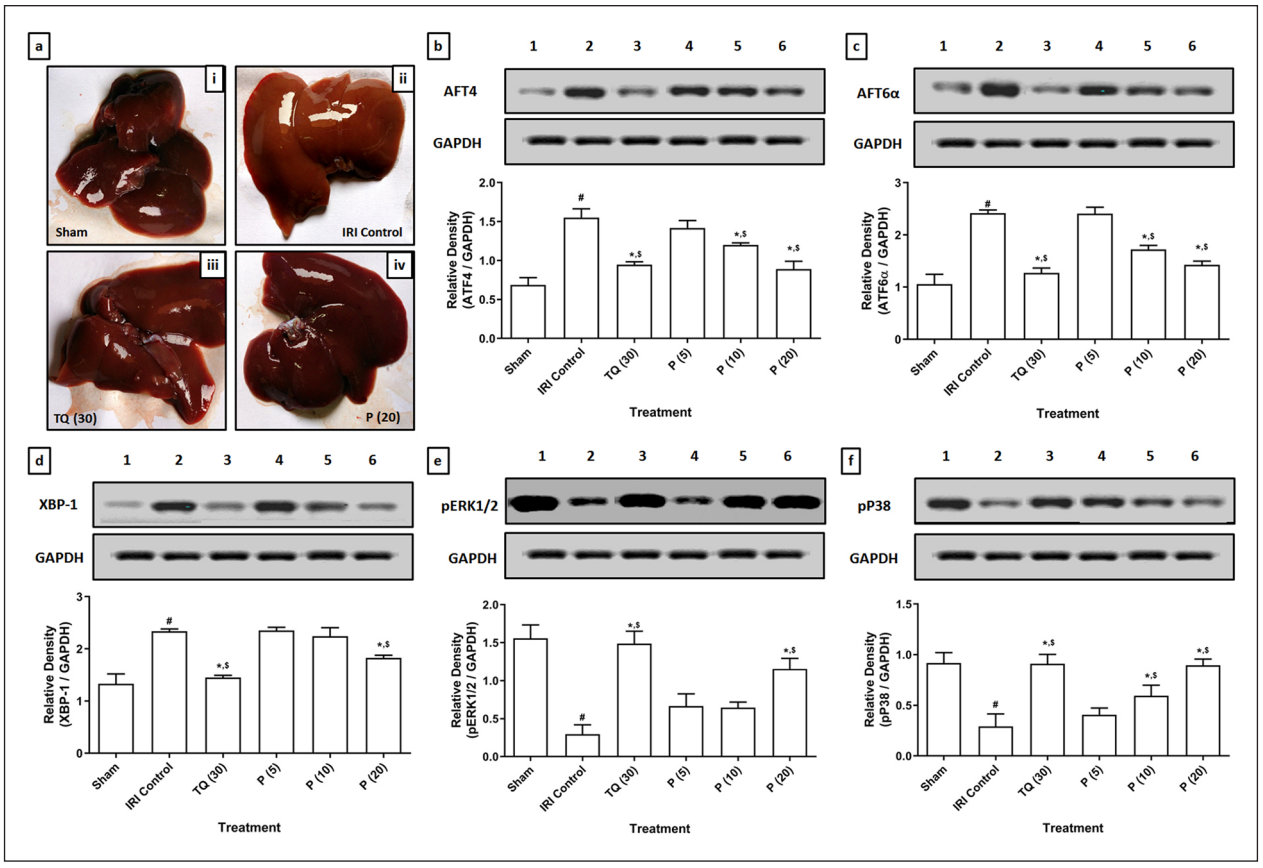

Figure 3. Effect of pinitol treatment on IRI-induced alterations in hepatic morphology (a), AFT4 (b), AFT6α (c), XBP-1 (d), ERK-1/2 (e), and p38 (f) protein expressions in rats. Photographs of liver tissue from sham (i), Ischemia-reperfusion Injury control (ii), Thymoquinone (30 mg/kg) treated (iii) and Pinitol (20 mg/kg) treated (iv) rats.

Data were represented as Mean ± SEM ($n = 4$) and analyzed by one-way ANOVA followed by Tukey's multiple range test. Figures in parentheses indicate oral dose in mg/kg.

IRI: ischemia-reperfusion injury; TQ (30): thymoquinone (30 mg/kg) treated; P (5): pinitol (5 mg/kg) treated; P (10): pinitol (10 mg/kg) treated; P (20): pinitol (20 mg/kg) treated rats; AFT4: activating transcription factor 4; AFT6α: activating transcription factor 6 alpha; XBP-1: X-box binding protein 1; ERK-1/2: Extracellular signal-regulated kinase-1/2.

$^{\#}P < 0.05$ as compared with sham group.

$^{*}P < 0.05$ as compared with IRI control group.

$^{\$}P < 0.05$ as compared thymoquinone with pinitol.

Cytochrome-C in cellular cytosol, which activates the phase of apoptotic degradation. Whereas interleukins are thought to play a vital role in elevated ROS production. Several studies have documented the direct relation of the elevated inflammatory response of TNF-α and ILs with hepatic damage.[31,32] The results of the present investigation support the findings of previous investigators where hepatic IRI is associated with elevated pro-inflammatory cytokines levels (TNF-α, IL-1β, and ILs). However, administration of pinitol inhibited IRI-induced elevated inflammatory response via attenuation of cytokine levels to reduce the hepatic damage. Zheng et al. (2017) also documented the anti-inflammatory efficacy of pinitol via inhibition of TNF-α and ILs during adjuvant-induced arthritis,[16] and the results of the present investigation corroborate the same.

A researcher documented hepatocellular apoptosis as an important cause of IRI.[1,33] During hepatic I/R, apoptosis is induced in almost 40–60% of hepatocytes and 50–70% of endothelial cells, and caspase-3 plays a vital role in the induction of this apoptosis.[34] ER stress-induced in mitochondria released cytochrome C to cell cytosol, which further activated caspase-3 and caspase-9.[33,34] ER, stress-induced activation of caspase-3 dissociates procaspase-12 from the ER membrane. Dissociation of procaspase-12 resulted in its activation, which further initiated a downstream pathway where TNF-α

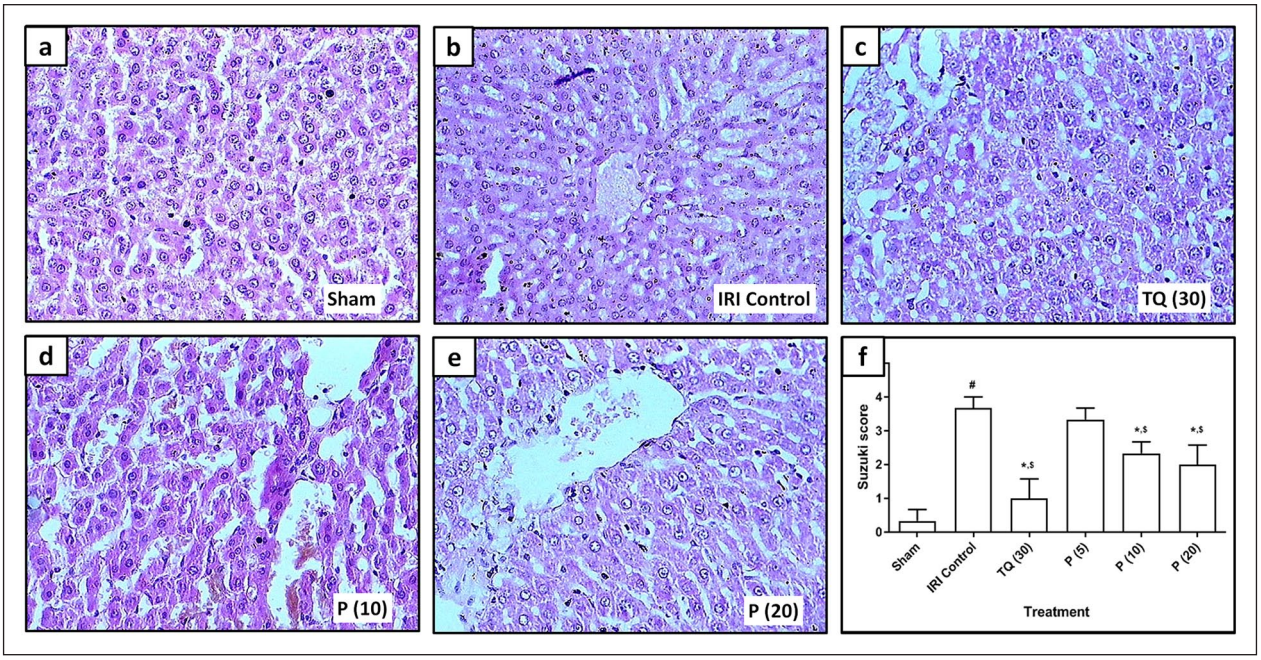

Figure 4. Effect of pinitol treatment on IRI-induced alterations in hepatic histopathology in rats. Photomicrograph of sections of hepatic tissue from sham (a), IRI control (b), Thymoquinone (30 mg/kg) treated (c), Pinitol (10 mg/kg) treated (d), and Pinitol (20 mg/kg) treated (e) rats (H&E stain). Quantitative representation of Suzuki score (f).
Data were expressed as mean ± SEM ($n = 3$), and one-way ANOVA followed by the Kruskal–Wallis test was applied for post hoc analysis.
IRI: ischemia-reperfusion injury; TQ (30): thymoquinone (30 mg/kg) treated; P (5): pinitol (5 mg/kg) treated; P (10): pinitol (10 mg/kg) treated; P (20): pinitol (20 mg/kg) treated rats.
#$P < 0.05$ as compared with sham group.
*$P < 0.05$ as compared with IRI control group.
$$P < 0.05$ as compared thymoquinone with pinitol.

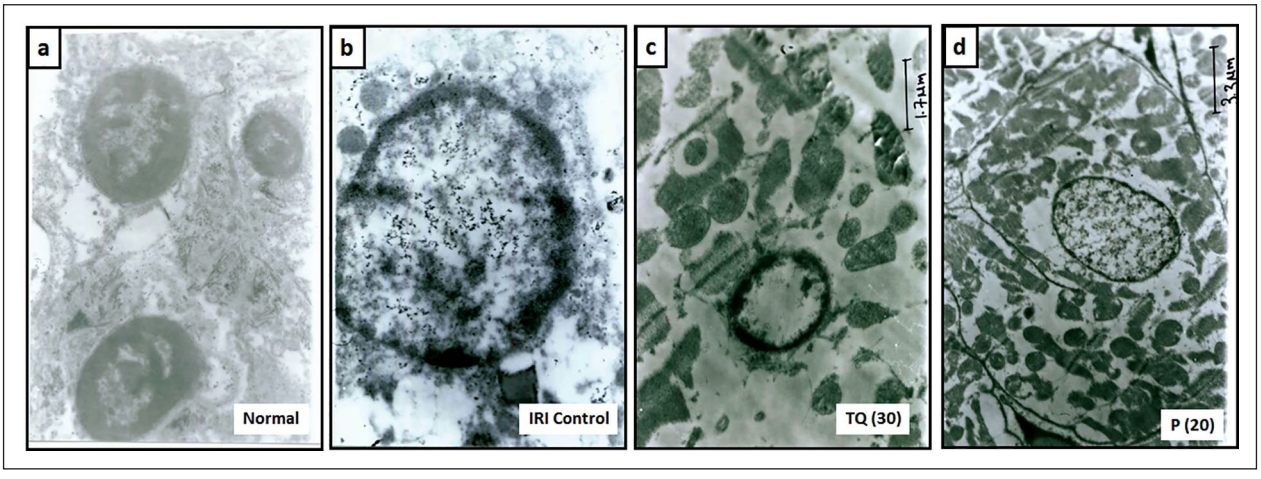

Figure 5. Effect pinitol treatment on IRI-induced alterations in hepatic ultrastructure in rats. Photomicrographs of sections of hepatic tissue from sham (11580 X) (a), IRI control (19300 X) (b), Thymoquinone (30 mg/kg) treated (11580 X) (c), and Pinitol (20 mg/kg) treated (5790 X) (d) rats.

promoted the binding of caspase-3 and caspase-12, leading to apoptosis.[9] The induction of caspase-dependent apoptosis has been well supported by previous researchers where caspase-12-deficient mice failed to induce ER stress-related apoptosis.[9,33] In the present study, hepatic IRI also showed significant induction of caspase-dependent apoptosis, further evident by flow cytometric analysis where apoptotic cell populations were seen to be significantly increased. Interestingly, the antiapoptotic

potential of pinitol has been well studied by various researchers.[14,35] The data of the present investigation also showed that pinitol protected against ischemia-induced apoptosis in hepatocytes which are in line with previous researchers.[14,35]

It has been well documented that ER stress initiated unfolded protein response (UPR) facilitates cell survival and apoptosis.[11] Thus, GRP78, a main molecular chaperone, plays a regulatory role in the induction and maintenance of ER homeostasis.[10] Under normal physiological conditions, GRP78 remains in an inactive state via the formation of its complex with inositol requiring 1 (IRE1) and protein kinase RNA-like ER kinase (PERK).[36] However, under ER stressful conditions, phosphorylation of IRE1a and PERK causes activation of GRP78 from its complex, which further combines with unfolded proteins to initiate protein folding.[37] Furthermore, CHOP is another pro-apoptosis transcription factor for the induction of ER stress through downstream targets of AFT-4, AFT6α, and XBP-1 pathway in UPR.[38] CHOP has an ability to inhibit the activation of Bcl-2, which is an important regulator of apoptosis.[10,38] Thus, studies have demonstrated that AFT4-CHOP mediated activation of GRP78 is a vital apoptotic pathway for ER stress during hepatic IRI.[10,36,37] In this study, up-regulated mRNA and protein analysis of GRP78, and CHOP from IRI control group suggested activation of ER stress during hepatic IRI. However, pinitol down-regulated expression of GRP78 and CHOP, suggesting its antiapoptotic potential.

Evidence suggests that unfolded protein response during ER stress is initiated to maintain homeostasis mediated by various signaling proteins such as IRE1, PERK, and AFT6.[37] Stress-induced phosphorylation and dislocation of IRE1a from GRP78 results in its activation, which further produces a potent transcription factor XBP-1.[38] The activation of XBP-1 induces upregulation of UPR via its direct binding to their related promoters. Similarly, ER stress-induced phosphorylation and PERK activation induce activation of AFT4, which regulates various UPR target genes responsible for oxidative stress and regulation of CHOP expression.[39] Meanwhile, phosphorylation mediated release of GRP78 also initiates translocation of AFT6α to the nucleus where its activated form further up-regulates the expression of various chaperone genes such as GRP78 and CHOP.[40] Conversely, p38 is essential for the inherent cellular responses against external stress, and a body of evidence reported that activation of p38 offers protection against stress-induced apoptosis.[35] Our data revealed that the IRI control group was associated with elevated AFT4, AFT6α, and XBP-1 expressions suggesting induction of ER stress after hepatic reperfusion. Results are in line with previous research findings that highlighted ER stress after hepatic IRI.[39,40] These molecular analyses are consistent with histopathological and ultrastructural findings where induction of ER stress is reflected in the presence of electron-dense mitochondria with the rough endoplasmic reticulum. However, administration of pinitol protects ER from ischemic reperfusion damage via its inhibition of ERK1/2 and p38 phosphorylation at GRP78, thus diminishing elevated AFT4, AFT6α, and XBP-1 response.

Pinitol is a cyclic polyol compound widely found in various food constituents, including soy, alfalfa, and pinewood. Pinitol has a history of traditional medicinal use across geographical barriers, including China, Sri Lanka, India, and European countries, to manage an array of disorders.[13,41] Clinical findings suggested that pinitol is a therapeutic moiety with potent antioxidant property and has proven its efficacy against non-alcoholic fatty liver disorder and Type 2 diabetes mellitus.[12,13] A large body of experimental studies supported its hepatoprotective potential.[17–19] Thus, based on the available evidence and findings from the present investigation, pinitol can be considered as a potential therapeutic moiety for further clinical development during the management of hepatic IRI.

Thymoquinone (2-isopropyl-5-methylbenzo-1, 4-quinone), a major phytoconstituents from *Nigella sativa* seeds, has been well documented for its array of pharmacological potential. A recent systematic review demonstrated its potential against hepatic IRI via activation of P38 and ERK pathway as well as inhibition of mitochondrial damage, oxidative stress, nitric oxide signaling, ER stress, and apoptosis.[10,42–45] Thus, in the present investigation, thymoquinone serves as a positive control to determine and compare the mechanism of action of D-Pinitol against hepatic IRI. The results of the present investigation also in accordance with findings of previous researchers where thymoquinone inhibited elevated oxidative stress, pro-inflammatory cytokines, ER stress, mitochondrial damage,

and apoptosis to ameliorate IRI-induced hepatic damage.[10,42] Moreover, the protective effect of thymoquinone is more pronounced against attenuation of IRI-induced hepatic injury as compared to D-Pinitol. However, thymoquinone may interact with few medications such as warfarin and beta-blockers (like metoprolol) processed through the cytochrome P450 pathway.[46] Thus, future research can be considered where a reduced dose of thymoquinone combined with D-Pinitol could target against hepatic IRI.

However, the present investigation has several limitations that we need to consider. First, the findings of the present study based on various pathological pathways in the experimental animal model may not be completely applicable for clinical pathways. Interestingly, a recent study established a similarity of major mRNA involved in hepatic IRI during experimental and clinical settings.[47] Secondly, the ischemic preconditioning by using D-Pinitol cannot be considered as a routine treatment option for the hepatic infraction as this event is not preplanned thus, this preconditioning can utilize for controlled elective situations. Thirdly, although the D-Pinitol showed promising potential against warm IRI during hepatic transplant, these results cannot be extended to prevent organ damage during cold storage. Nevertheless, there are only a fraction of patients undergoing orthotopic liver transplantation. Fourthly, Doppler ultrasound is an advanced technique that is generally recommended to determine the hepatic ischemia. However, due to the limitation of the state of the art of the existing facility, the present investigation could not determine the Doppler ultrasound. Lastly, the effect of D-Pinitol alone on the various parameters has not been evaluated in the separate group as its broad margin of safety has been well established in experimental and clinical settings.[48]

Conclusion

The findings of the present investigation suggested that pinitol attenuated ischemia-reperfusion induced hepatic damage in experimental rats. Pinitol offered protection against ER stress-mediated phosphorylation of ERK1/2 and p38, thereby inhibiting AFT4-CHOP/GRP78 signaling response and inducing caspase-3 induced hepatocellular apoptosis during hepatic ischemia-reperfusion insults.

Author contributions

The authors declare that all data were generated in-house and that no paper mill was used. LY: concepts, design, manuscript preparation, manuscript editing, and manuscript review. HL: experimental studies, data acquisition, manuscript editing, and manuscript review. XL: concepts, design, statistical analysis, and manuscript review. YL: manuscript editing and manuscript review.

Declaration of conflicting interests

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

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work has been funded by a Basic research project of Natural Science of Shaanxi Province (No. 2020JQ-998 and No. 2021JQ-938).

Ethics approval

Ethical approval for this study was obtained from the Institutional Animal Ethics Committee of Second Affiliated Hospital of Chongqing Medical University, China (CQMU-efy-2020021 and No. 2021JQ-938).

Animal welfare

The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation. The experimental was performed in accordance with the guidelines of the National Institute of Health Guide for Care and Use of Laboratory Animals and was approved by the Animal Ethics and Use.

ORCID iD

Yongyong Li https://orcid.org/0000-0001-6225-1866

Data availability

The raw data underlying this article will be shared at reasonable request to the corresponding author.

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