

# D-Pinitol Protected Against Endoplasmic Reticulum Stress and Apoptosis in Hepatic Ischemia-Reperfusion Injury Via Modulation of AFT4-CHOP/GRP78 and Caspase-3 Signaling Pathways

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## Research

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# Abstract

*Background:* Hepatic ischemia-reperfusion injury (IRI) is a major unavoidable clinical problem often occurs during various liver surgery and transplantation. D-Pinitol, a cyclic polyol, showed its hepatoprotective efficacy in clinical and experimental settings.

*Aim:* To determine the potential and possible mechanism of pinitol against ER stress regulation-mediated hepatic IRI in experimental rats.

*Materials and methods:* Male SD rats were pre-treated with pinitol for 21 days and then subjected to 60 min. of partial hepatic ischemia followed by 24 h. of reperfusion. Various parameters were evaluated, including liver function tests, inflammatory release, endoplasmic reticulum (ER) stress, apoptosis, and structural modifications.

*Results:* Pre-treatment with pinitol (10 and 20 mg/kg) effectively protected IRI-induced hepatic damage reflected by attenuation of elevated AST, ALT, oxidative stress (SOD, GSH, MDA and NO) and pro-inflammatory cytokines (TNF- $\alpha$  and IL's) release. Interestingly, western blot and ELISA analysis suggested that pinitol significantly down-regulated the expression of ER stress apoptotic markers, namely GRP78, CHOP, AFT-4, AFT-6 $\alpha$ , XBP-1, and caspase-3, 9 and 12. Additionally, pinitol pre-treatment improved mitochondrial function and phosphorylation of ERK1/2 and P38. Pinitol markedly protected IRI-induced hepatic apoptosis determined by flow cytometry. The hepatic histological and ultrastructural aberration induced by IRI was effectively protected by pinitol.

*Conclusion:* Findings of the present investigation suggested that pinitol offered protection against ER stress-mediated phosphorylation of ERK1/2 and p38, thereby inhibited AFT4-CHOP/GRP78 signaling response and induction of caspase-3 induced hepatocellular apoptosis during hepatic ischemia-reperfusion insults.

## 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 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, the hepatic ischemia-reperfusion is considered one of the most challenging elements during hepatic surgery, which 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 [1].

Over the past several decades, a researcher has investigated and explored various molecular pathways to understand the mechanisms behind the induction of endoplasmic reticulum (ER) stress-associated hepatic damage during the IRI [3]. Number of evidence suggests that hepatic IRI involved complex physiological processes wherein the initial phase, activation of the Kupffer cell causes release of reactive oxygen species (ROS), which followed by releases of pro-inflammatory cytokines and apoptotic factor in later phase leading to induction of endoplasmic reticulum (ER) stress [3, 4]. Additionally, increased intracellular calcium overload and neutrophil infiltration though to play an essential role in IRI-induced ER stress. Researcher documented that ER stress caused disruption of ER homeostasis which further leads to the accumulation of unfolded and misfolded proteins in its lumen [5]. Thus, to restore the ER homeostasis, unfolded protein response (UPR) is activated to overcome the defect of protein-folding [4, 5]. However, due to insufficiency of UPR to ameliorate stress, an additional caspase depends mitochondrial apoptosis pathway activated which leads to programmed cell death. Recently, glucose-regulated protein 78 (GRP78) has been documented as important regulator of accumulated unfolded proteins which contributed significantly to inhibit ER stress responses [6]. IRI-induced activation of GRP78 leads to increased expression of activating transcription factor (AFT)-4 and CCAAT/enhancer-binding protein homologous protein (CHOP) which further promotes oxidative stress, inflammation and apoptosis during hepatic IRI [5, 6]. Thus, numerous researchers have implemented cellular apoptosis inhibition to alleviate ER stress as one of the critical strategies for the clinical management of hepatic IRI [6, 7].

Despite significant pharmaceutical industry development, the safe and effective treatment option for clinical management of hepatic IRI remains challenging. Moreover, various surgical techniques have been evaluated during hepatic reperfusion however, none could prevent mortality associated with IRI [2, 5]. Thus, there is a need of an alternative options for prevention and improvement of patient survival during hepatic IR insult. Several researcher used pre-conditioning strategy by implementing various moieties such as lipoic acid and isoflurane to protect against hepatic IRI [7, 8]. These agents shown to inhibit the elevated postsurgical levels of aspartate transferase and alanine transferase thus improved the outcome in patient with hepatic IR. Furthermore, intravenous administration of N-acetylcysteine in patients undergoing liver transplantation showed improved liver function [9, 10]. However, mechanism for this protective effect is not yet full elucidated and clinical outcomes about the effect of these treatment options against hepatic warm IRI are still ambivalent [11]. Thus, 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 [6, 12, 13]. However, partial warm hepatic ischemia induced by occlusion of the hepatic artery and the portal vein is well established and widely used animal model which mimics most of the clinical feature of human hepatic IRI [12, 14]. In the present investigation we have also implemented this animal model to evaluate potential of D-Pinitol against hepatic IRI.

Pinitol (3-O-methyl-chiro-inositol) is a cyclic polyol that has been shown its clinical potential in patients with the non-alcoholic fatty liver disorder (NAFLD) [15] and Types 2 Diabetes Mellitus [16]. In a 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 to exert its hepatoprotective potential [15]. The antidiabetic effect of pinitol was mediated by a significant diminution in generation of ROS in Type 2 Diabetes Mellitus patients during 12-week, double-blind randomized trial [16]. Its wide range of pharmacological activities includes antihyperlipidemic, hepatoprotective, cardioprotective, antidiabetic, anti-inflammatory, antioxidant, and anticancer [17, 18]. Inhibitory potential of pinitol against various pro-inflammatory cytokines including TNF- $\alpha$  (Tumour Necrosis Factor-alpha), and ILs (Interleukins) has been significantly implemented for its antiarthritic potential [19]. Furthermore, pinitol exerts its antiapoptotic potential via modulation of expression of Bcl-2, and Bcl-xL *in-vitro* [17]. The hepatoprotective effect of pinitol against D-galactosamine induced [20], high-fat diet-induced [21], and streptozotocin-induced [22] hepatotoxicities have been well established. However, the potential of pinitol against IRI is still remains unknown. Thus, the present investigation was undertaken to determine the effect of pinitol along with its possible mechanism behind the regulation of 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- $\alpha$ , IL-1 $\beta$ , 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), AFT6 $\alpha$  (Activating transcription factor-6 $\alpha$ ), 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 one week in housed at the institute animal house at standard laboratory conditions, i.e., a temperature of 24 $\pm$ 1 $^{\circ}$ C, the relative humidity of 45–55% and 12:12 h light/dark cycle. Animals had free access to standard chaw pelleted food and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee of Second Affiliated Hospital of Chongqing Medical University, China.

### Induction of hepatic IRI

Rats were anesthetized with an intraperitoneal (i.p.) injection of pentobarbital (5%), and then they were subjected to surgery as described previously [6, 14]. Briefly, a partial warm hepatic ischemia model (70% of liver mass) was induced by occlusion of the hepatic artery and the portal vein (hepatic pedicle) with a microvascular clamp for 60 min. After removal of the clamps, reperfusion was initiated for next 24 hr.

# 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 solution (Dimethyl sulfoxide), 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 [18, 19].

## 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 levels of serum Aspartate transaminase (AST) and alanine aminotransferase (ALT) were measured by using reagent assay kits (Accurex Biomedical Pvt. Ltd., Mumbai, India) with an ultraviolet-visible spectrophotometer.

## Biochemical estimation:

### Tissue homogenate preparation, estimation of oxidative stress and pro-inflammatory markers

All animals were sacrificed at the end of the study, i.e., on the 22<sup>nd</sup> day, the liver was immediately isolated. Tissue homogenates were prepared with 0.1 M Tris-HCl buffer (pH 7.4), and supernatant of homogenates was employed to estimate superoxide dismutase (SOD), reduced glutathione (GSH), lipid peroxidation

(MDA, i.e., Malondialdehyde content), and nitric oxide (NO content) as described previously [23, 24]. Another portion of aliquot was used for estimation of hepatic pro-inflammatory markers using rat-specific TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 enzyme-linked immunosorbent assay (ELISA) Kit (Bethyl Laboratories Inc., Montgomery, TX, USA).

## Mitochondrial enzymes estimation:

Liver mitochondrial isolation was performed according to a previously described method [25]. Mitochondrial complex-I activity was measured spectrophotometrically according to a previously described method [26]. Mitochondrial Complex-II activity Succinate dehydrogenase (SDH) was measured spectrophotometrically according to an already described method [27]. Mitochondrial redox activity (Complex-III) i.e., 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 [28]. Mitochondrial complex-IV (Cytochrome oxidase assay) activity was assayed in liver mitochondria according to the previously described method [29].

## Reverse Transcriptase PCR

The mRNA expressions of GRP78, CHOP, and  $\beta$ -actin were analyzed in liver tissue using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) according to the method described elsewhere [30]. PCR was performed using 1 X forward and reverse primers, and 2.5 U Taq polymerase (MP Biomedicals India Private Limited). Amplification of  $\beta$ -actin served as a control for sample loading and integrity.

## Western blot procedure

The protein expressions of caspase-3, caspase-9, caspase-12, AFT4, AFT6 $\alpha$ , XBP-1, ERK-1/2, P38, and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) were estimated in liver tissue according to the method described elsewhere [31]. Briefly, liver tissue was dissected and sonicated in Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Inc.). The lysates were centrifuged at 10,000 x 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  $\mu$ g) 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, AFT6 $\alpha$ , 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. 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 determined as previously described [32]. 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, grinded 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 and 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.

## Histological and electron microscopic analysis

Histopathological analysis of liver tissue was carried out using hematoxylin and eosin (H&E) stain under a light microscope whereas, the liver ultrastructural studies were performed under a transmission electron microscope according to the method described previously [33].

## Statistical analysis

GraphPad Prism (GraphPad, San Diego, CA) was used to perform data analysis. Data are expressed as mean ± standard error mean (SEM) and analyzed by using One-Way ANOVA followed by Tukey's multiple range post hoc analysis (for 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 of 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).

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 (gm)	234.00 ± 3.73	220.70 ± 2.36 <sup>#</sup>	233.70 ± 3.73 <sup>*,§</sup>	211.80 ± 1.96	220.80 ± 3.26 <sup>*,§</sup>	229.50 ± 3.71 <sup>*,§</sup>
Liver Index	0.0223 ± 0.0013	0.0329 ± 0.0015 <sup>#</sup>	0.0272 ± 0.0013 <sup>*,§</sup>	0.033 ± 0.0017	0.0303 ± 0.0008 <sup>*,§</sup>	0.0253 ± 0.0014 <sup>*,§</sup>
AST (IU/L)	81.86 ± 12.71	300.00 ± 14.57 <sup>#</sup>	118.90 ± 13.59 <sup>*,§</sup>	306.40 ± 12.66	231.30 ± 9.091 <sup>*,§</sup>	160.30 ± 12.63 <sup>*,§</sup>
ALT (IU/L)	33.47 ± 5.28	146.90 ± 3.80 <sup>#</sup>	49.23 ± 4.48 <sup>*,§</sup>	142.10 ± 6.14	94.77 ± 7.06 <sup>*,§</sup>	62.50 ± 6.11 <sup>*,§</sup>
SOD (U /mg of protein)	6.72 ± 0.47	2.14 ± 0.32 <sup>#</sup>	5.44 ± 0.41 <sup>*,§</sup>	2.80 ± 0.33	3.93 ± 0.49 <sup>*,§</sup>	5.83 ± 0.47 <sup>*,§</sup>
GSH (µg/mg of protein)	14.43 ± 0.83	5.63 ± 0.79 <sup>#</sup>	14.08 ± 0.66 <sup>*,§</sup>	6.55 ± 0.46	10.75 ± 0.68 <sup>*,§</sup>	12.09 ± 0.59 <sup>*,§</sup>
MDA (nM/ mg of protein)	0.20 ± 0.03	0.80 ± 0.03 <sup>#</sup>	0.36 ± 0.04 <sup>*,§</sup>	0.71 ± 0.03	0.55 ± 0.03 <sup>*,§</sup>	0.39 ± 0.04 <sup>*,§</sup>
NO (µg/ml)	124.80 ± 11.88	236.60 ± 15.47 <sup>#</sup>	141.60 ± 13.50 <sup>*,§</sup>	229.90 ± 10.32	172.30 ± 12.36 <sup>*,§</sup>	145.00 ± 13.65 <sup>*,§</sup>

Data were represented as Mean ± SEM. <sup>#</sup> $p < 0.05$  as compared with sham group, <sup>\*</sup> $p < 0.05$  as compared with IRI control group. 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 mice; AST: Aspartate transaminase; ALT: alanine aminotransferase; SOD: superoxide dismutase, GSH: glutathione, MDA: malondialdehyde; NO: nitric oxide.

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 were decreased significantly ( $p < 0.05$ ), whereas hepatic MDA and NO levels were 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 were 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 2

Effect of pinitol treatment on IRI-induced alterations in hepatic mitochondrial complex, TNF- $\alpha$ , IL-1 $\beta$ , 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 $\pm$ 3.49	6.86 $\pm$ 3.61 <sup>#</sup>	30.59 $\pm$ 3.31 <sup>*,§</sup>	10.01 $\pm$ 2.88	16.85 $\pm$ 2.75 <sup>*,§</sup>	27.71 $\pm$ 3.10 <sup>*,§</sup>
Complex II (mmole/mg protein)	14.62 $\pm$ 0.76	4.34 $\pm$ 0.76 <sup>#</sup>	11.92 $\pm$ 0.83 <sup>*,§</sup>	4.71 $\pm$ 0.68	9.03 $\pm$ 0.70 <sup>*,§</sup>	11.30 $\pm$ 0.53 <sup>*,§</sup>
MTT assay (OD at 540 nm)	0.45 $\pm$ 0.04	0.16 $\pm$ 0.02 <sup>#</sup>	0.44 $\pm$ 0.02 <sup>*,§</sup>	0.22 $\pm$ 0.03	0.31 $\pm$ 0.03 <sup>*,§</sup>	0.39 $\pm$ 0.04 <sup>*,§</sup>
Complex-IV (nmol cyto-C oxidized/min/mg protein)	6193.00 $\pm$ 329.9	880.50 $\pm$ 251.50 <sup>#</sup>	5312.00 $\pm$ 339.10 <sup>*,§</sup>	1561.00 $\pm$ 226.60	3183.00 $\pm$ 159.20 <sup>*,§</sup>	4550.00 $\pm$ 286.40 <sup>*,§</sup>
TNF- $\alpha$ (pg/ml)	156.30 $\pm$ 11.29	406.30 $\pm$ 14.06 <sup>#</sup>	211.40 $\pm$ 13.43 <sup>*,§</sup>	378.10 $\pm$ 17.12	324.50 $\pm$ 11.87 <sup>*,§</sup>	256.20 $\pm$ 12.25 <sup>*,§</sup>
IL-1 $\beta$ (pg/ml)	15.58 $\pm$ 2.95	72.11 $\pm$ 1.69 <sup>#</sup>	29.25 $\pm$ 2.21 <sup>*,§</sup>	68.03 $\pm$ 1.89	49.36 $\pm$ 2.49 <sup>*,§</sup>	41.62 $\pm$ 2.38
IL-6 (pg/ml)	101.40 $\pm$ 5.59	147.90 $\pm$ 5.12 <sup>#</sup>	113.40 $\pm$ 9.34 <sup>*,§</sup>	153.40 $\pm$ 6.25	130.10 $\pm$ 7.88 <sup>*,§</sup>	127.50 $\pm$ 7.18 <sup>*,§</sup>

Data were represented as Mean  $\pm$  SEM. # $p < 0.05$  as compared with sham group, \* $p < 0.05$  as compared with IRI control group. 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 mice; TNF- $\alpha$ : Tumour Necrosis Factor-alpha; ILs: Interleukins.

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

The levels of hepatic pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , 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- $\alpha$ , IL-1 $\beta$ , 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 (Fig. 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 (Fig. 2).

## ***Effect of IRI-induced alterations in hepatic AFT4, AFT6 $\alpha$ , XBP-1, ERK-1/2 and P38 protein expressions in rats***

There was a significant ( $p < 0.05$ ) increased in the hepatic AFT4, AFT6 $\alpha$ , and XBP-1 protein expressions, whereas hepatic ERK-1/2 and P38 protein expressions were 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, AFT6 $\alpha$ , 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, AFT6 $\alpha$ , 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, AFT6 $\alpha$ , XBP-1, ERK-1/2, and P38 protein expressions was more significant ( $p < 0.05$ ) in the TQ group as compared to pinitol treatment (Fig. 3).

## **Effect of IRI-induced alterations in hepatic histopathology of rats**

IRI induces histological aberration in hepatic tissue of the IRI control group, evidence by a significant ( $p < 0.05$ ) increased in Suzuki score (Fig. 4A) as compared to a sham group (Fig. 4B). When compared with the IRI control group, TQ administration showed a significant ( $p < 0.05$ ) reduction in Suzuki score (Fig. 4C). Pinitol (10 and 20 mg/kg) treatment also markedly ( $p < 0.05$ ) inhibited IRI-induced histological aberration reflected by reduced Suzuki score (Fig. 4D and 4E) as compared to the IRI control group. (Fig. 4G)

## **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 with the endoplasmic reticulum (Fig. 5A). However, liver tissue from the IRI control group showed distorted vesicular cytoplasm, thicken nuclear membrane, electron-dense mitochondria, accumulation of autophagosomes, and rough endoplasmic reticulum (Fig. 5B). However, administration of TQ and Pinitol showed marked attenuation of IRI-induced ultrastructural alterations in hepatic tissue (Fig. 5C and 5D).

## **Discussion**

Hepatic IRI is a major clinical problem associated with the patient who undergoes liver surgery, transplantation, and circulatory shock [3, 5]. Studies demonstrate IRI-induced insult, which stimulates the generation of ROS, release of inflammatory cytokines, microvascular modification, and induction apoptosis, resulting in hepatocellular dysfunction [4, 5]. Furthermore, treatment options are very limited for the clinical management of hepatic IRI. Thus, many researcher have investigated the anti-apoptotic potential of various therapeutic moieties for the treatment of hepatic IRI [4, 5]. Pinitol has been reported for its anti-inflammatory, antioxidant, and antiapoptotic potential [15, 17, 18]; thus, we have evaluated the potential of pinitol against ER in the current study 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- $\alpha$  and IL's), elevated ER stress (GRP78, CHOP, AFT-4, and AFT6 $\alpha$ ), mitochondrial damage and apoptosis (Caspase-3, -9 and -12) thus improved histological and ultrastructural derangements to ameliorate hepatic damage (Graphical abstract).

It has been well established that elevated circulatory AST and ALT levels are the hallmark of hepatic dysfunction [34]. Induction of ischemic reperfusion causes disturbance in the permeability of the hepatocellular membrane, which is further responsible for the leakage of hepatic enzymes (AST and ALT) into circulation, suggesting hepatocellular damage [2, 5]. This notion was further confirmed by the histopathological changes which reflected the sinusoidal congestion along with hepatic cell necrosis. Additionally, alteration in liver index also provides insights to disease state, thus revealing the severity of hepatic damage induced by ischemic reperfusion [5]. In the present investigation, administration of pinitol showed significant amelioration of IR induced hepatic damage revealed by decrease in levels of circulatory hepatic enzymes along with attenuation of histological aberrations. The present results are in accordance with the findings of previously clinical evidence where pinitol treatment normalization of activity of liver enzymes NAFLD patients [15].

The researcher has well established the linkage between IR injury and elevated ROS [3, 6]. The dysregulation of redox homeostasis in the ER tissue membrane resulted in ROS accumulation, further aggravating the oxidative stress in ER [12, 35]. Number of antioxidants including SOD, GSH and catalase has been serves as a first line of defense against this oxidative stress. SOD has an ability to convert the harmful superoxides into hydrogen peroxide to suppress it vicious effect [36–38]. Thus, SOD is an important enzyme that plays a vital role in eliminating harmful free radicals [39–41]. GSH is another important antioxidant which helps to strengthen endothelial intracellular resistance against oxidative damage [42–44]. Furthermore, the elevated production of free radical during IRI increases lipid peroxidation levels, which causes significant cellular damage [14]. Malonaldehyde is considered an important measure for lipid peroxidase induced cellular damage [45–47]. During IRI, the first line cellular defense system is impaired, resulting in hydrogen peroxide generation, divided into free radicals viz., hydrogen, and oxygen, which further damages the number of biological moieties, including proteins, amino acids, and nucleic acids [48–50]. The results of present investigation showed that pinitol treatment significantly inhibited elevated levels of ROS hepatic IRI, which was reflected by elevated levels of SOD and GSH along with diminished MDA and NO levels. The present study results are consistent with findings of previous researchers where administration of pinitol exerts its antioxidant potential via inhibition of elevated oxidative stress to ameliorate hepatotoxicity clinically [15].

Inflammation plays a central dogma role in the induction and maintenance of ER stress during the pathophysiology of hepatic IRI [3, 6]. Reperfusion causes activation of Kupffer cells (KCs) to release various pro-inflammatory cytokines such as TNF- $\alpha$  and ILs [3, 5, 6]. These cytokines inaugurates inflammatory response results in leukocytes recruitment and its infiltration. Furthermore, TNF- $\alpha$  stimulate the release of various apoptotic proteins and Cytochrome-C in cellular cytosol which activates the phase of apoptotic degradation [35, 51]. Whereas, interleukins thought to play a vital role in elevated ROS production [52–54]. Number of studies have well documented the direct relation of elevated inflammatory response of TNF- $\alpha$  and ILs with hepatic damage [55, 56]. The results of present investigation supports the findings of previous investigators where hepatic IRI is associated with elevated pro-inflammatory cytokines levels (TNF- $\alpha$ , IL-1 $\beta$  and IL's). However, administration of pinitol inhibited IRI-induced elevated inflammatory response via attention of cytokines levels to reduce the hepatic damage. Zheng et al.

(2017) also documented the antiinflammatory efficacy of pinitol via inhibition of TNF- $\alpha$ , and IL's during adjuvant-induced arthritic [19], and the results of the present investigation corroborate with the findings of previous investigator [19].

The researcher documented hepatocellular apoptosis as an important cause of IRI [1, 35]. During hepatic I/R, apoptosis has been induced in almost 40–60% of hepatocytes and 50–70% of endothelial cells and caspase-3 play a vital role in induction of this apoptosis [5]. ER stress-induced in mitochondria caused release of cytochrome C to cell cytosol which further activates caspase-3 and caspase-9 [35]. ER, stress-induced activation of caspase-3 dissociates procaspase-12 from the ER membrane [57, 58]. Dissociation of procaspase-12 results in its activation which in turn initiated a downstream pathway where TNF- $\alpha$  promote the binding of caspase-3 and caspase-12 leading to apoptosis [12, 59]. The induction of caspase-dependent apoptosis has been well supported by previous researcher where caspase-12-deficient mice failed to induce ER stress-related apoptosis [35]. 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 significantly increased. Interestingly, the antiapoptotic potential of pinitol has been well studied by various researchers [17, 60]. The data of present investigation also showed that pinitol protected against ischemia-induced apoptosis in hepatocytes which is in line with previous researchers [17, 60].

It has been well documented that ER stress initiated unfolded protein response (UPR) to facilitate cell survival and apoptosis [14]. Thus, Glucose-regulated protein 78 (GRP78), a main molecular chaperone plays a regulatory role in the induction and maintenance of ER homeostasis [6]. 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). However, under ER stressful conditions, phosphorylation of IRE1a and PERK cause activation of GRP78 from its complex which further combines with unfolded proteins to initiate the protein folding [61]. Furthermore, CCAAT/enhancer-binding protein homologous protein (CHOP) is another pro-apoptosis transcription factor for the induction of ER stress through downstream targets of AFT-4, AFT6 $\alpha$ , and XBP-1 pathway in UPR [62]. CHOP has an ability to inhibit the activation of Bcl-2, which is important regulator of apoptosis [6, 62]. Thus, accumulated studies have demonstrated that AFT4-CHOP mediated activation of GRP78 is vital apoptotic pathway for ER stress during hepatic IRI [6, 61]. 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 anti-apoptotic 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 [61]. Stress-induced phosphorylation and dislocation of IRE1a from GRP78 results in its activation which further produces a potent transcription factor XBP1 [62]. 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 involved regulating various UPR target genes responsible for oxidative stress and regulation of CHOP expression [63]. Meanwhile, phosphorylation mediated release of GRP78

also initiated translocation of AFT6 $\alpha$  to the nucleus where its activated form further up-regulates the expression of various chaperone genes such as GRP78 and CHOP [64]. Conversely, p38 is essential for the inherent cellular responses against external stress and body of evidence reported that activation of p38 offers protection against stress-induced apoptosis [60]. Our data revealed that the IRI control group associated with elevated AFT4, AFT6 $\alpha$ , and XBP-1 expressions suggested induction of ER stress after hepatic reperfusion. Results are in line with previous research findings that highlighted ER stress after hepatic IRI [63, 64]. These molecular analysis are consistent with histopathological and ultrastructural findings where induction of ER stress is reflected presence of electron-dense mitochondria with 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, diminished elevated AFT4, AFT6 $\alpha$  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 various geographies including China, Sri Lanka, India, and European countries, to manage an array of disorders [16, 65]. Clinical findings suggested that pinitol is a therapeutic moiety with potent antioxidant property and proven its efficacy against non-alcoholic fatty liver disorder and Type 2 Diabetes Mellitus [15, 16]. A large body of experimental studies supported its hepatoprotective potential [20–22]. Thus, based on the available evidence and findings from present investigation, pinitol can be considered as potential moiety for further clinical development during management of hepatic IRI.

## Conclusion

Taken together, 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 inhibited AFT4-CHOP/GRP78 signaling response and induction of caspase-3 induced hepatocellular apoptosis during hepatic ischemia-reperfusion insults.

## Abbreviations

ALT: Alanine aminotransferase; AST: Aspartate transaminase; AFT: Activating transcription factor; CHOP: CCAAT/enhancer-binding protein homologous protein; ERK-1/2: Extracellular signal-regulated kinase-1/2; ER: Endoplasmic Reticulum; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GSH: Glutathione; GRP78: ER chaperone 78-kDa glucose-regulated/binding immunoglobulin protein; IL's: Interleukins; IRI: Ischemia-reperfusion Injury; MDA: Malondialdehyde; NADH: Nicotinamide adenine dinucleotide; NO: Nitric oxide; PERK: protein kinase RNA-like ER kinase; ROS: Reactive Oxygen Species; RT-PCR: Reverse transcription-polymerase chain reaction; SOD: Superoxide dismutase; TNF- $\alpha$ : Tumour Necrosis Factor-alpha; TQ: Thymoquinone; UPR: Unfolded Protein Response; XBP-1: X-box binding protein 1.

## Declarations

## **Ethical Approval**

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 and was approved by the Animal Ethics and Use.

## **Consent to Participate**

Not applicable

## **Consent to Publish**

Not applicable

## **Data Availability**

The raw data underlying this article is available in supplementary data. The additional data will be shared on reasonable request to the corresponding author.

## **Competing Interests**

The authors declare that they have no conflicts of interest to disclose.

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## **Author's contribution**

The authors declare that all data were generated in-house and that no paper mill was used.

LY: Concepts, Design, Manuscript preparation, Manuscript editing, Manuscript review

HL: Experimental studies, Data acquisition, Manuscript editing, Manuscript review

XL: Concepts, Design, Statistical analysis, Manuscript review

YL: Manuscript editing, Manuscript review

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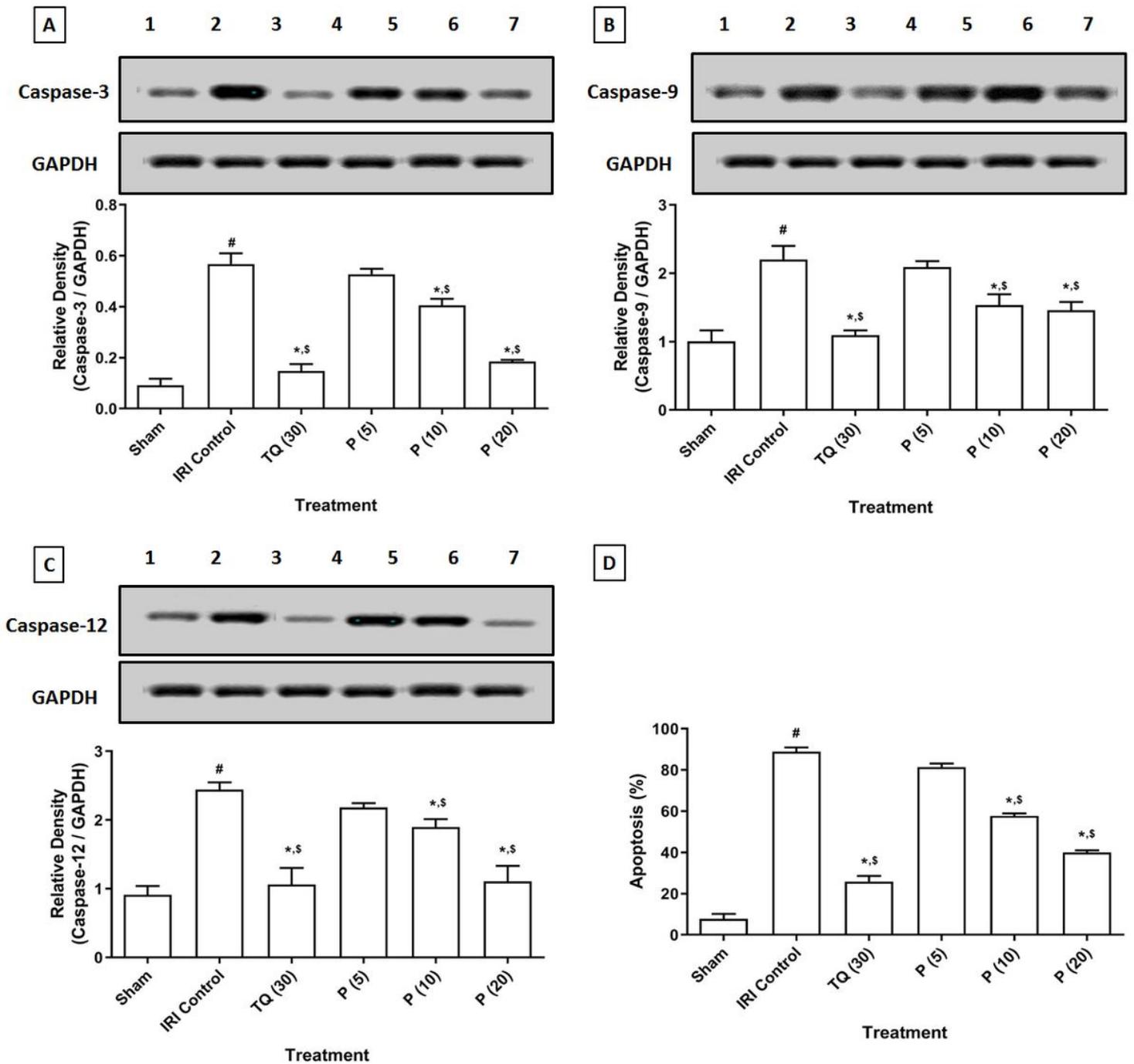
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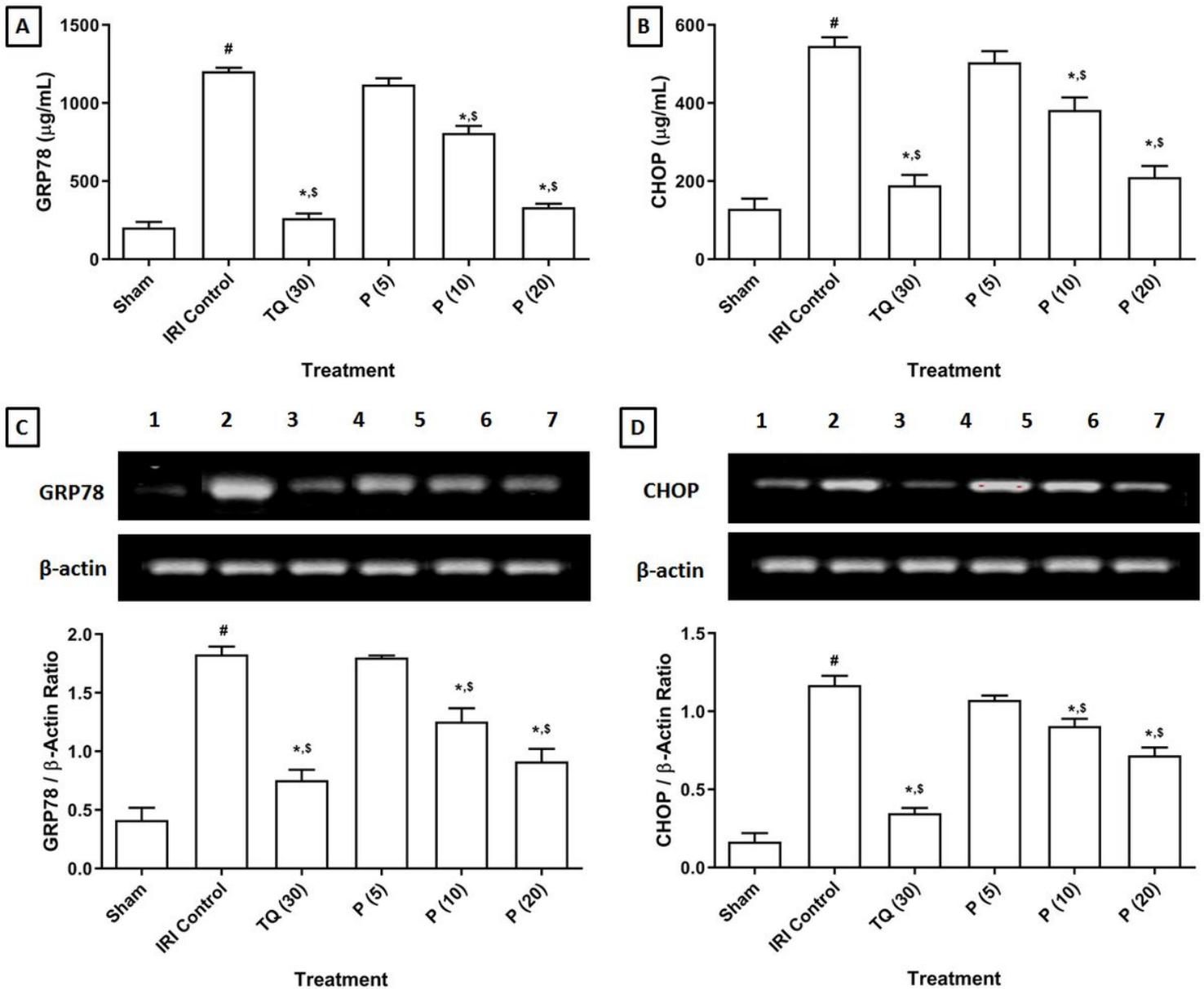
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## Figures



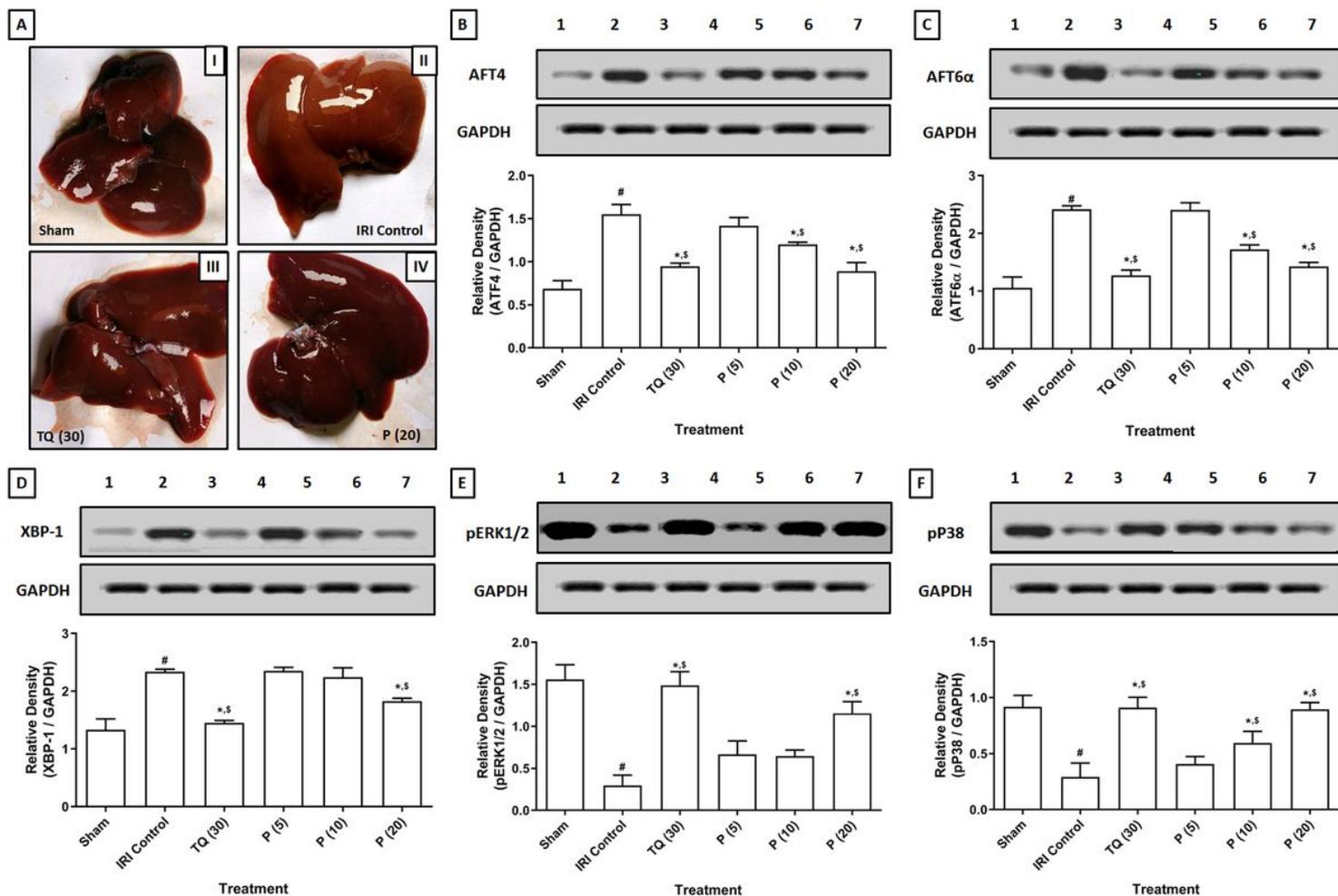
**Figure 1**

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  $\pm$  SEM. # $p < 0.05$  as compared with sham group, \* $p < 0.05$  as compared with IRI control group. 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 mice.



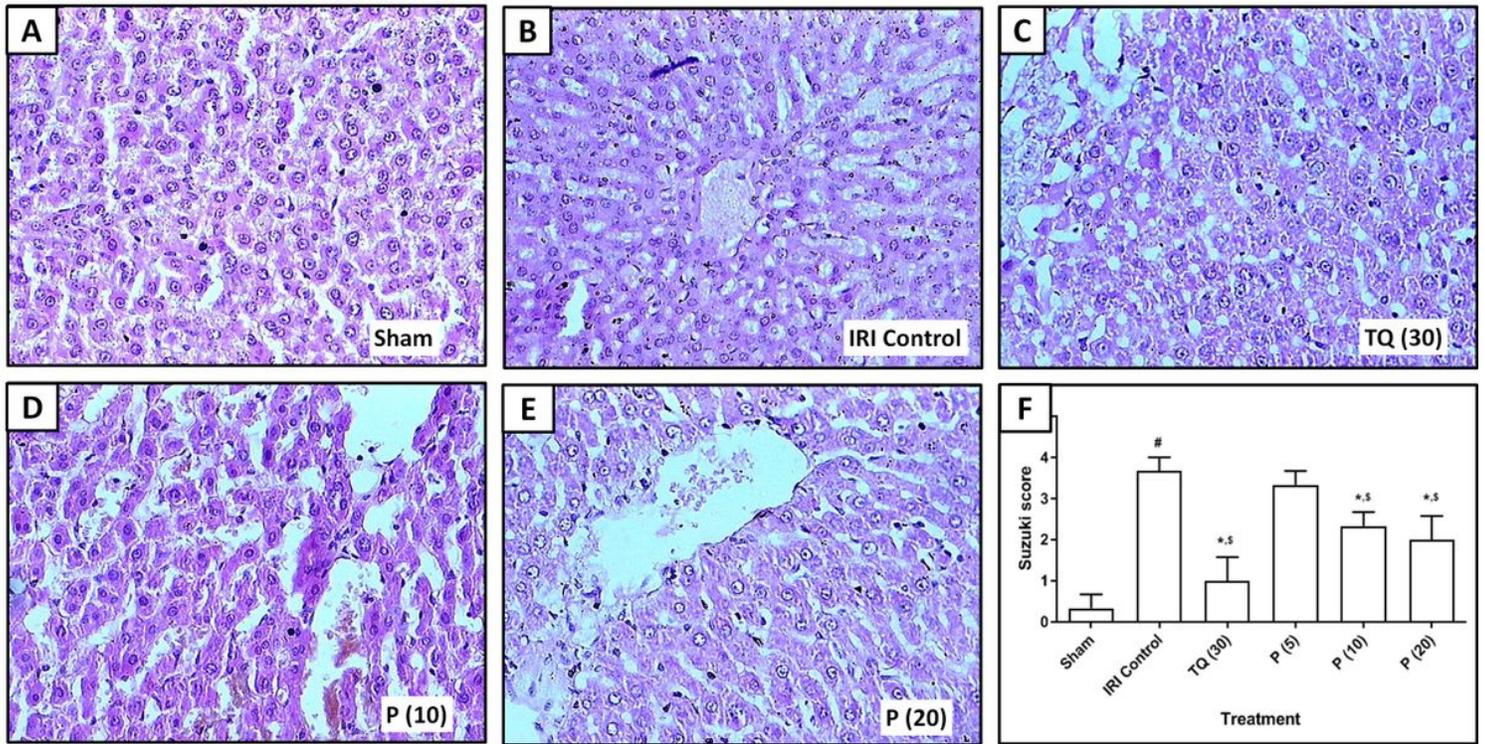
**Figure 2**

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  $\pm$  SEM. # $p < 0.05$  as compared with sham group, \* $p < 0.05$  as compared with IRI control group. 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 mice; GRP78: ER chaperone 78-kDa glucose-regulated/binding immunoglobulin protein; CHOP: CCAAT/enhancer-binding protein homologous protein.



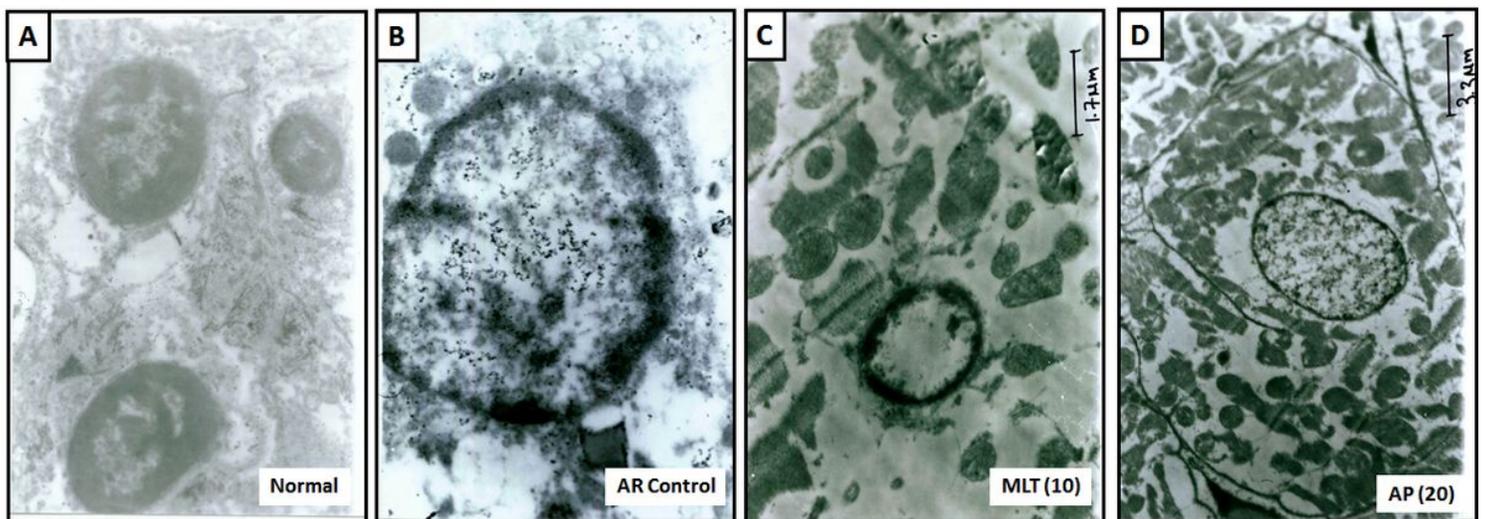
**Figure 3**

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. Data were represented as Mean ± SEM. # $p < 0.05$  as compared with sham group, \* $p < 0.05$  as compared with IRI control group. 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 mice; 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.



**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) mice (H&E stain). The quantitative representation of Suzuki score (F). Data were expressed as mean  $\pm$  SEM (n=3), and one-way ANOVA followed by the Kruskal-Wallis test was applied for post hoc analysis. #p < 0.05 as compared with sham group, \*p < 0.05 as compared with IRI control group. 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 mice.



**Figure 5**

Effect pinitol treatment on IRI-induced alterations in hepatic ultrastructure in rats. Photomicrographs of sections of hepatic tissue from sham (A), IRI control (B), Thymoquinone (30 mg/kg) treated (C) and Pinitol (20 mg/kg) treated rats.