



# Evaluating the Effect of Silibinin on the Expression of Pannexin1 Gene During Hepatic Ischemia-Reperfusion

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## Abstract

**Objectives:** Liver ischemia-reperfusion (I/R) is the director's origin of damages in various clinical situations, especially surgery and transplantation. Inflammatory damages are critical because of the chronicity of I/R injuries (I/RI). The hepatoprotective and anti-inflammatory properties of silibinin have been reported in different studies. This study aimed to investigate the effect of Silibinin on the expression of the pannexin-1 (*Panx1*) gene during hepatic I/R.

**Materials and Methods:** In this case-control animal study, a total of 32 male Wistar rats (n=8 in each) were surveyed. The animals were randomly assigned into four equal groups as follows: Group 1 (Control): the rats underwent a midline laparotomy with normal saline injection; Group 2 (SILI): the rats received Silibinin (50 mg/kg) after laparotomy; Group 3 (I/R): the rats underwent I/R surgery and received normal saline; and Group 4 (I/R+SILI): the rats received silibinin before ischemia and directly following reperfusion. Blood and liver tissue samples were taken after three hours of reperfusion aftermath 1-hour ischemia to evaluate histological changes, gene expression, and serum markers of hepatic injury.

**Results:** While the serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the I/R group significantly increased compared to the control group ( $P<0.001$ ), they significantly decreased in the SILI+I/R group ( $P<0.001$ ). Silibinin ameliorated inflammatory impairments of liver tissue, such as neutrophil and macrophage infiltration and activation, hepatocyte degeneration and vacuolation, hepatic vascular endothelial damage, and sinusoid proliferation in the I/R group. The expression of the *Panx1* mRNA during I/R significantly increased compared to the control group ( $P<0.001$ ), but silibinin reduced the expression ( $P<0.001$ ).

**Conclusion:** We witnessed that silibinin reduced liver tissue damages during hepatic I/R. Correcting the expression of the *Panx1* gene during I/R is probably one of the mechanisms of anti-inflammatory effects of silibinin.

**Keywords:** Ischemia, Pannexin-1, Reperfusion, Silibinin

## Introduction

Ischemia-reperfusion (I/R) is the main cause of liver injury in pathological situations and the leading cause of acute liver failure. Damage caused by hepatic I/R occurs by different molecular mechanisms (1). Inflammation has a destructive role in I/R pathogenesis and is a principal factor in liver cell damage, immune cell activation, and liver inflammation enhancement. The adenosine 5'-triphosphate (ATP) discharge in the extracellular milieu is a critical factor in inflammatory processes and cell necrosis. Releasing of ATP and uridine-5'-triphosphate (UTP) by the apoptotic cells in the early steps of cell death work as signals for the uptake of monocytes, macrophages, and microglia (2). Conversely, the release of ATP into the extracellular space causes neuronal cell necrosis in ischemic situations (3) and tumor cell death during chemotherapy (4,5).

Pannexins (*Panx*) are known to be the primary

channels of extracellular ATP discharge. Among the three divisions of *Panx* family (*Panx1*, *Panx2*, and *Panx3*) (6), *Panx1* has been evaluated more commonly. *Panx1* channels are activated during pathological conditions by various signals including increased extracellular K<sup>+</sup> and intracellular Ca<sup>2+</sup> concentration, caspase-mediated cleavage, c-Jun N-terminal kinases (7,8), and Src family of tyrosine kinases (9). Some previous studies showed the role of *Panx* channel in inflammation and cell death associate with pathological conditions (6,10).

I/R damage has been an unresolved problem in medical surgery for many years. Therefore, studies should focus on inflammation and potential monitoring regimens to improve liver surgery outcomes. Silibinin is a natural plant polyphenol derived from *Silybum marianum* with high antioxidant properties (11-15) and anti-inflammatory characteristics. It improves histological liver tissue damages, such as cell death, inflammatory

Received 22 September 2021, Accepted 20 February 2022, Available online 27 July 2022

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## Key Messages

- ▶ Ischemia-reperfusion (I/R) is the main cause of liver injury.
- ▶ Silibinin protects hepatocytes against I/R damage.
- ▶ *Panx1* canal is involved in inflammation and cell death.
- ▶ Silibinin inhibits *Panx1* mRNA expression.

responses, and vascular impairment in I/R conditions (16,17). Accordingly, this study aimed to investigate the effect of silibinin on the expression of the *Panx1* gene as a stimulant of inflammatory pathways.

## Materials and Methods

### Animals and Grouping

In this interventional research, 32 male Wistar rats weighing  $250 \pm 20$  g were provided from the Laboratory Animal Research Center of Mazandaran University of Medical Sciences, Iran. The rats were stored in a room at  $23 \pm 2^\circ\text{C}$ , humidity  $55 \pm 5\%$ , 12-hour dark-light cycle, ventilation, water, and standard diet. The animals were randomly assigned into four equal groups as follows: Group 1 (Control): the rats underwent a midline laparotomy with normal saline injection; Group 2 (SILI): the rats received silibinin (50 mg/kg) after laparotomy; Group 3 (I/R): the rats underwent I/R surgery and received normal saline; and group 4 (I/R+SILI): the rats received silibinin before ischemia and directly following reperfusion (18).

### Surgical Procedure

Before the operation, the rats experienced a starvation period of 18 hours, but they had open access to water. The animals were anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg), intraperitoneally (IP). All surgeries were performed under sterile conditions between 2-8 PM to avoid time variables (19).

### Ischemia induction

A 2-cm long incision was made in the abdomen's midline below the sternum (laparotomy). After removing the fascia and cutting the abdominal rectus muscle, the liver appeared. Then, the connections linking the liver and peritoneal diaphragm were cut. The liver was removed from the peritoneal cavity with gentle pressure by both hands on the incision's sides. The left branches of the triad ports, including the portal vein, the hepatic artery, and the bile duct of the left and middle lobes, were blocked by a bulldog clamp for one hour, but the right and tail lobes had free blood flow to prevent intestinal congestion and clogging of the mesenteric arteries. This method allows for 30% perfusion. After 60 minutes of ischemia, the clamp was detached to restore blood flow. Throughout the ischemia, the liver was maintained moist by sterile gauze impregnated with normal saline to prevent dehydration. During this time, rats were re-anesthetized with ketamine (50 mg/kg) whenever necessary. After the ischemia time,

the clamp was slowly removed for reperfusion, the liver was shifted into the peritoneal cavity, and the cut site was sutured. Control animals were provided similarly, but no clamps were placed on their vessels (19).

### Silibinin Injection

Lyophilized silibinin with dihydrogen succinate formulation (Legalon) was obtained from Sigma (St. Louis, MO, USA). Due to its high solubility in water, this formulation was dissolved in normal saline and injected (50 mg/kg; 0.5 mL) once one hour before surgery and again immediately after reperfusion (IP) (19).

### Biochemical Analysis

After reperfusion, approximately 2 mL of blood was obtained from the inferior vena cava under general anesthesia and kept in a sterile glass tube for 30 minutes, and centrifuged at 3000 rpm for 10 minutes. The serum was then separated and kept in a 1.5 mL vial at  $-70^\circ\text{C}$  until the onset of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) biochemical analysis. Serum ALT and AST enzymes were measured by a biochemical autoanalyzer (BT-3000-plus, Biotechnica, Italy) using the Pars Azmoon test kit (Karaj, Iran).

### Tissue Collection and Examination

For pathological examination, 1 mm of liver tissue sections were taken from all animals' ischemic lobe and immediately washed in normal saline to remove blood. Next, the tissue sections were fixed and kept in 10% formalin at room temperature until further investigation. All tissue sections were washed with water, dehydrated with different alcohol grades (50-100%), cleaned with xylene, and finally placed inside molten paraffin to prepare tissue samples for hematoxylin and eosin (H&E) staining. Finally, the thin sections (3–5  $\mu\text{m}$ ) were incised with a microtome, stained by H&E, and studied under optical microscopy (20).

### RNA Extraction and Gene Expression

According to the manufacturer's protocol, the total RNA of all tissue samples was extracted using an RNeasy plus mini kit (Qiagen, Germany). The concentration and purity of the whole RNA were evaluated by NanoDrop spectrophotometer (Thermo Scientific, USA) based on the UV absorbance at 260 nm and 260/280 nm-ratio. Agarose gel 1% electrophoresis stained with SYBR Green was utilized to detect 18S and 28S ribosomal RNA bands and confirm the RNA. A cDNA synthesis kit (EURx, Poland) was employed to produce cDNA applying 1  $\mu\text{g}$  of total RNA per reaction. Finally, to determine the *Panx1* mRNA expression, real-time polymerase chain reaction (PCR) was performed in triplicate, and the beta-actin gene was used as an internal control. Each reaction included 12.5  $\mu\text{L}$  of SYBR Green PCR Master Mix reagent (EURx, Poland), 10 pM of specific primers (0.5  $\mu\text{L}$  of forward and 0.5  $\mu\text{L}$

of reverse), 2  $\mu$ L of cDNA (50 ng), and DD water up to 25  $\mu$ L volume. The PCR cycles were as follows: pre-treatment with Uracil-N-glycosylase (UNG) (50°C for 2 minutes), preliminary denaturation (95°C for 12 minutes), and 40 cycles (each at 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds). The nucleotide sequences of the primers are listed in Table 1.

### Statistical Analysis

REST-RG software was used to analyze the results of real-time PCR and SPSS 18 software was utilized to evaluate the data. The results were expressed as mean  $\pm$  standard error of the mean (mean  $\pm$  SEM). The mean change was compared and reported by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. A *P* value <0.05 was considered as statistically significant.

## Results

### Biochemical Results

There was no significant difference in the serum levels of AST and ALT enzymes between the control and SILI groups (*P* > 0.05). As Table 2 depicts, while the serum levels of ALT and AST enzymes were significantly higher in the I/R group than the control group (*P* < 0.001), the serum levels of the two enzymes were significantly lower in the I/R+SILI group than the I/R group (*P* < 0.001).

### Histology Results

The hepatic artery and bile duct branches were healthy in the portal space (Figure 1A). As Figure 1B shows, the hepatic lobules were entirely intact in different zones. Hepatic cords with high stainability, normal nuclei with evident nucleoli, and the minimal apoptotic vacuoles display the normal hepatic tissue in the portal space. Also, we observed healthy sinusoidal spaces with intact

endothelial cells lining and many Kupffer cells (Figures 1A, B).

In hepatic sections after 1-hour ischemia, hepatocytes with distinctive boundaries but full of clear apoptotic vacuoles were seen. Decreased stainability occurred due to the extensive destruction of mitochondria in the central vein's longitudinal section in zone III of the hepatic lobules (Figure 1C). The hepatic congestion and desquamation of endothelial cells of the sinusoidal wall were observed in zone III, and Signet ring cells appeared due to the vast devastation of cytoplasmic organelles apoptotic attachment vacuoles to each other. We also witnessed the accumulation of small lymphocytes in the sinusoidal space and elongated Kupffer cell emerged (Figure 1C).

The IP injection of silibinin impeded deleterious liver tissue changes compared to the ischemia insulted group (Figure 1D). The tissue structure components (bile ducts, Colangelo, longitudinal and cross-sections of portal vein branches with an intact endothelium) of the portal spaces were healthy at the periphery of a classical hepatic lobule. In general, unlike one-hour ischemic tissue sections (I/R group), the protective effect of silibinin significantly reduced the severity of liver tissue damages, especially in zones I and III of the classical hepatic lobules.

### Real-Time PCR Results

According to the results of real-time PCR, the mRNA levels of *Panx1* in control and SILI groups did not differ. As Figure 2 shows, the expression of *Panx1* was significantly higher in the I/R group compared to the control

**Table 1.** Primer Sequences

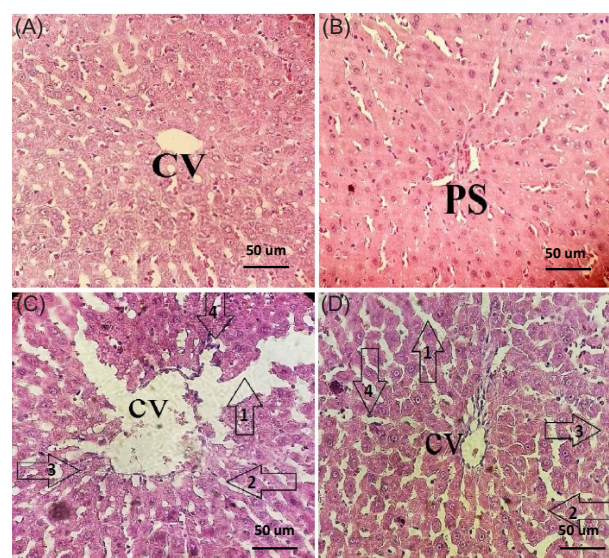
Gene	Primer sequence 5'→3'	Product Length (bp)
<i>Panx1</i>	Sense: 5' TCTACTTCTGCCACCTGGACAT -3'	212
	Antisense: 5'- GAAGGGCTTCCTAGTCCATACG -3'	
$\beta$ -actin	Sense : 5'-CCCATCTATGAGGGTTACGC-3'	149
	Antisense: 5'-TTTAATGTCACGCACGATTTC-3'	

**Table 2.** Effect of Silibinin on the Serum Levels of AST and ALT After Hepatic I/R

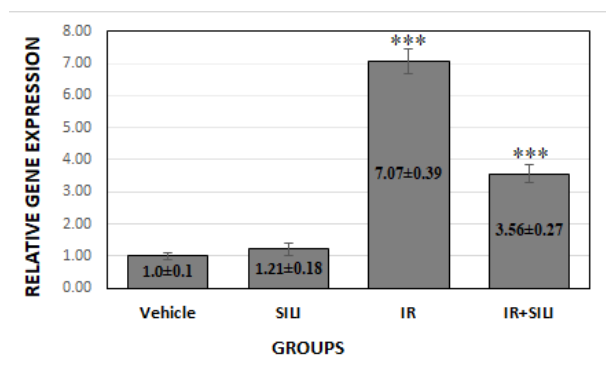
Group	AST	ALT
Control	133 $\pm$ 5	87 $\pm$ 5
I/R	1438 $\pm$ 75***	1236 $\pm$ 75***
SILI+I/R	735 $\pm$ 33***	582 $\pm$ 33***
SILI	136 $\pm$ 4.5	83 $\pm$ 4.5

SILI: Silibinin; I/R: ischemia/reperfusion; AST: aspartate aminotransferase; ALT: alanine aminotransferase.

The results were reported as mean  $\pm$  SEM. \*\*\* *P* < 0.001 and \*\*\* *P* < 0.001 show significant differences in the control and I/R groups, respectively.



**Figure 1.** Liver H&E Staining at  $\times$ 400 Magnification. A) Control group (normal saline-treated). The microscopic image shows a healthy tissue structure with no defect. B) SILI group. Intact triad port vessels and tissue structure are observed. C) IR group. Arrow 1: sinusoid dilation, arrow 2: high vacuolation, arrow 3: severe degeneration, and arrow 4: accumulation of inflammatory cells. D) SILI+IR group. Arrow 1: slight expansion of sinusoids, arrow 2: vacuolation, arrow 3: Mild degeneration, and arrow 4: neutrophil activation. CV: central vein, PS: portal space, SILI: Silibinin, IR: Ischemia-reperfusion.



**Figure 2.** Relative Panx1 Gene Expression. \*\*\* shows a significant difference compared to the control group (\*\*P<0.001). +++ shows a significant difference between I/R+SILI and I/R groups (\*\*P<0.001). SILI: Silibinin; I/R: ischemia/reperfusion; Panx1: pannexin1.

group ( $7.07 \pm 0.39$  and  $1 \pm 0.1$ , respectively) ( $P < 0.001$ ). However, there was a significant decrease in the *Panx1* gene expression in the I/R+SILI group ( $3.56 \pm 0.27$  and  $7.07 \pm 0.39$ , respectively) ( $P < 0.001$ ).

## Discussion

According to the results of this study, silibinin could significantly increase the *Panx1* gene expression and reduce hepatic impairment after I/R. Also, liver I/R prompted significant liver damages, resulting in reactive oxygen species (ROS) production and pro-inflammatory changes, such as the neutrophils infiltration and increased expression of inflammatory mediators such as TNF- $\alpha$  and IL-1 $\beta$  (2,21).

Several studies defined a novel mechanistic aspect of hepatic I/R damage mediated by *Panx1* channels. These channels play an essential role in the control of paracrine signaling and tissue homeostasis by regulating the extracellular transfer of substances such as ATP, cAMP, and calcium (22,23). Studies have demonstrated that *Panx1* channels are frequently involved in pathological conditions. Previous studies evaluated the crucial role of *Panx1* channel in kidney and lung damage during I/R (24,25). During vascular inflammation, stimulating the endothelial *Panx1* channel releases ATP into the extracellular space, leading to vascular permeability, leukocyte infiltration, and lung injury after I/R (24). In a retinal ischemia model, high *Panx1* activity caused cell membrane permeability, leading to metabolic and ionic imbalance and ischemic stresses (26). Many recent studies illustrated the vital role of *Panx1* in hepatic toxicity and injury, especially in the hepatocytes and Kupffer cells (22,27,28). Kim et al revealed that *Panx1* is needed for inflammasome initiation after liver I/R (29). The *in vivo* examinations showed that *Panx1* mRNA levels significantly increased in I/R insulted rats compared to control ones. Pelegrin et al indicated the role of *Panx1* in innate immunity and inflammasome stimulation, caspase1, and releasing of IL-1 $\beta$  and IL-18 (30-33). In a previous study, suppressing the expression of *Panx1*

inhibited the extracellular release of ATP (7). Taken together, *Panx1* can play a principal role in triggering inflammatory responses and tissue injury in pathological circumstances, and its inhibition may be an effective strategy for controlling cellular damages. Therefore, pharmacologic inhibition and genetic ablation of *Panx1* channels could introduce a novel strategy for protecting the liver against ischemic injury (34).

In this research, we detected the protecting effects of silibinin, as a flavonoid compound derived from *Silybum marianum*. The protective effects of this substance against liver injuries after I/R are well-documented (18,19). Regarding the hepatoprotective effects of silibinin, we observed that the serum levels of liver enzymes and histologic injuries after I/R dramatically decreased in the I/R+SILI rats. Comparison between I/R and I/R+SILI groups revealed that hepatocytes and sinusoidal endothelium were less injured, and leukocyte infiltration was not monitored in I/R+SILI rats. These results are in line with those reporting that silibinin protected liver tissue after I/R inflammatory injury (19,35).

## Conclusions

Silibinin can protect hepatocytes against I/R inflammatory damage by regulating the mRNA expression of *Panx1*, which results in maintaining liver tissue structure. This study demonstrated the inhibitory effect of silibinin on the *Panx1* mRNA expression in I/R rats. Although we could investigate different cellular pathways and genes disrupted in this pathological condition, we could not present more data due to laboratory equipment limitations. Techniques such as Western blotting are recommended to confirm the results. It is also suggested that the effect of silibinin on I/R-related pathways be investigated.

## Authors' Contribution

AKT and HM designed the article, HM and MSS performed laboratory tests, and ZN and FG edited the manuscript. PM and ER participated in performing steps and the writing. All authors read the final draft of the manuscript and approved its content.

## Conflict of Interests

Authors have no conflict of interest.

## Ethical Issues

The current study has been approved by the Research Committee of Mazandaran University of Medical Sciences, Mazandaran, Iran (Code: IR.Mazandaran.REC.13981007).

## Financial Support

This study has been financed by Mazandaran University of Medical Sciences, Mazandaran, Iran (Grant no: s/98/5/1007).

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