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Expression Pattern of MicroRNA-21 during the Liver Ischemia/Reperfusion

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ABSTRACT

Ischemia/reperfusion (I/R) injury in cadaveric liver transplantation is not avoidable. Liver I/R injury is an important phenomenon in hepatic damage. MicroRNA-21 (miR-21) plays an important role in I/R injury. The present study aimed to determine the expression pattern of miR-21 in liver I/R injury/recovery and its correlation with the immunologic transmission signals pathways in several days post-reperfusion.

In an animal model for I/R in the liver, 40 male Balb/c mice were divided into 3 groups. The animals were monitored for 3 and 24 hours, and also for 4, 7, 14, and 28 days post-reperfusion. Liver tissue damage was assessed by histopathology. The plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and total antioxidant capacity (TAC) levels were measured with enzymatic assays. MiR-21, programmed cell death 4 (PDCD4) mRNA, T-cell-restricted intracellular antigen 1 (TIA1) mRNA, and fas ligand (FASL) mRNA expression levels were measured; using reverse transcription-polymerase chain reaction (RT-PCR) at different times after the reperfusion in liver tissue and blood.

Histopathology and plasma ALT, AST, ALP, and TAC levels confirmed liver damage induced by I/R injury. MiR-21 increased by twofold in the liver tissue and on the inflammatory phase after 24 hours of reperfusion; it then continued to decrease up to day 7 post-reperfusion. Afterward, it continued to rise slightly up to day 14 post-reperfusion. This trend was in parallel with the recovery of the liver damage.

MiR-21 expression level in the liver and blood is a predictor of the extent of I/R injury.

Keywords: Ischemia; Liver; Mice; MicroRNA-21; Reperfusion

INTRODUCTION

A large number of people with end-stage hepatic failure are in desperate need of liver transplantation.[1] Ischemia/reperfusion (I/R) duration is one of the important subjects in transplantation success.

Therefore, a full understanding of I/R injury could significantly contribute to the reduction of post-

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transplantation complications and even transplant rejection.[2] I/R injury can ignite a cascade of physiological and biochemical events that ultimately results in cell death and the loss of the target organ. The underlying molecular basis of this phenomenon has been studied.[3]

MicroRNAs (miRNAs) are 21–23-nucleotide noncoding RNA segments with biological activities. Briefly, they should undergo three steps to be constructed pri-miRNAs, pre-miRNAs, and mature miRNAs. MiRNAs bind to the 3' untranslated region of target mRNAs and negatively regulate the gene expression.[4] They have a very conservative structure.[5] The operation of miRNAs in immunology is very challenging.[6] They affect hepatocyte proliferation during liver regeneration.[7]Nowadays, miRNAs are used as biomarkers.[8,9]

MiR-21 is a regulatory RNA that plays a crucial role in various biological activities and disease conditions including inflammation, cancer, and cardiovascular diseases.[10] MiR-21 is a multi-faceted RNA.[11] Much research has been done on the relationship between miR-21 and I/R injury in the heart, kidneys, and intestines.[12,13]MiR-21 correlates with hepatocellular carcinoma.[14] MiR-146a and miR-148a improve liver I/R injury.[15,16] Previous studies show that miR-21 has dual effects it may either increase or decrease the I/R injury in organs.[17] However, the exclusive effects of miR-21 on liver I/R injury is still unknown.

We conducted this study to evaluate the role of miR-21 in liver I/R injury in a murine animal model of 70% partial ischemia. Programmed cell death 4 (PDCD4) mRNA was selected because this marker has the final target for miR-21. Furthermore, T-cell-restricted intracellular antigen 1 (TIA1) and Fas ligand (FASL) were studied to confirm inflammation and apoptosis. TIA1mRNA was used as an auto-control for PDCD4 mRNA that positively regulates PDCD4 mRNA and assists in modulating the dynamic cytoplasmic PDCD4mRNA.[18]

MATERIALS AND METHODS

Animals

Forty male 26–30-g Balb/c mice, aged 8–10 weeks, were purchased from Shiraz University of Medical Sciences Animal Center (Shiraz, Iran). The mice had free access to water and food and were kept at a temperature of 22±2°C with 12:12 light-dark. All animal experiments were approved by the State Committee on Animal Ethics, Shiraz University, Shiraz, Iran (IACUC no: 4687/63). The recommendations of the European Council Directive (86/609/EC) of November 24, 1986, regarding the standards in the protection of animals used for experimental purposes, were also followed.

Animals were divided randomly into three groups the control, sham, and I/R test groups, each with 5 mice. The I/R test group followed by 3 hours, 24 hours, 4 days, 7 days, 14 days, and 28 days subgroups after reperfusion and 5 mice were evaluated at each time point. The sham group underwent an operation without vascular occlusion.

Chemicals

Xylazine, ketamine (Alfasan, Netherlands), hematoxylin (cat no.1159380025), eosin (cat no.1159350025), formalin, all purchased from Merck, Germany, and paraffin wax were used in this study.

Surgery Operation and Sampling

Animals were anesthetized by intraperitoneal injection of 10 mg/kg of xylazine and 100mg/kg ketamine.[19]Normothermia (37°C) was maintained with heating pads during the procedure.

Laparotomy was performed via a midline incision. Portal triads from left and median liver lobes were occluded by a small Yasargil vascular clamp (Geister, Germany). Through the 60-min obstruction of the hepatic artery and portal vein, partial ischemia was obtained in 70% of the livers. Time was recorded by a Hanhart stopwatch (Guetenbach, Germany).The abdominal cavity was hydrated by saline-moistened gauze. After 60-min of obstruction, the clamp was removed and the liver was reperfused, the abdominal cavity was closed by 05 silk sutures. The animals recovered and returned to a clean cage. After the end of reperfusion, animals were anesthetized and laparotomy was performed via a midline incision, whole blood samples were collected from the left ventricle and the liver was harvested.[20-23] For histopathology, the partial liver tissue was fixed in 10% formalin. The samples were stored at-80°C for further analysis.

Histopathological Analysis of Liver Injury

The specimen of the left lobes of the liver were embedded in paraffin wax, slices sectioned at 3-mm, and stained with hematoxylin and eosin; using a

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standard approach. The parenchymal histological changes, which were evaluated by sinusoidal congestion, cell infiltration, cytoplasmic vacuolization, and necrosis were scored based on a 0–4 scale. Microscopic liver injury was confirmed by histopathology observation and Suzuki's score classification method (Figure 1).[24] Images were captured; using an Olympus CX23 microscope with an A14 Nikon DSLR camera (Tokyo, Japan).

Serum Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and Alkaline Phosphatase (ALP) Measurement

Serum ALT, AST, and ALP were measured using commercial kits (cat no. 1400019, 1400018, and 1400002) (Pars Azmoon Co, Iran), as well as a biochemical autoanalyzer (Cobas Mira B, USA).

The total antioxidant capacity (TAC) was measured by a commercial kit (cat no. ZB-TAC-48A, Zell Bio, Germany). Colorimetry of the color product of the chromogenic substrate (tetramethylbenzidine) was measured by a spectrophotometer (Biotek, Netherlands) at a wavelength of 450 nm.

Extraction of mRNA and Quantitative Real-time Polymerase Chain Reaction (RT-PCR)

RNA was extracted from the liver tissue and the whole blood with the RNX-Plus kit (Cinna Gen, Iran), according to the manufacturer. cDNA was synthesized by Primer Script reverse transcriptase (TaKaRa, Japan). The primer sequences were designed by the intron inclusion method, using Allele ID software (PREMIER Biosoft, USA) (Table 1). MiR-21 primer was designed as a universal stem-loop (Universal Reverse). Primers synthesized by Eurofins Genomics (Eurofins Genomics, Germany). RT-PCR was performed using the protocol setup; pre-denaturation at 95°C for 3 min for 1 cycle; followed by 40 cycles of amplification at 95°C for 15 sec, and at 62°C for 35 sec; and 1 cycle of melting at 95°C for 15 sec, at 62°C for 1 min, and at 95°C for 15 sec (Applied Biosystem, USA). The expression levels of miR-21, PDCD4 mRNA, TIA-1 mRNA, and FASL mRNA were measured and first normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (as an internal control) and compared with the sham-operation group using the $2^{-\Delta\Delta Ct}$ method.

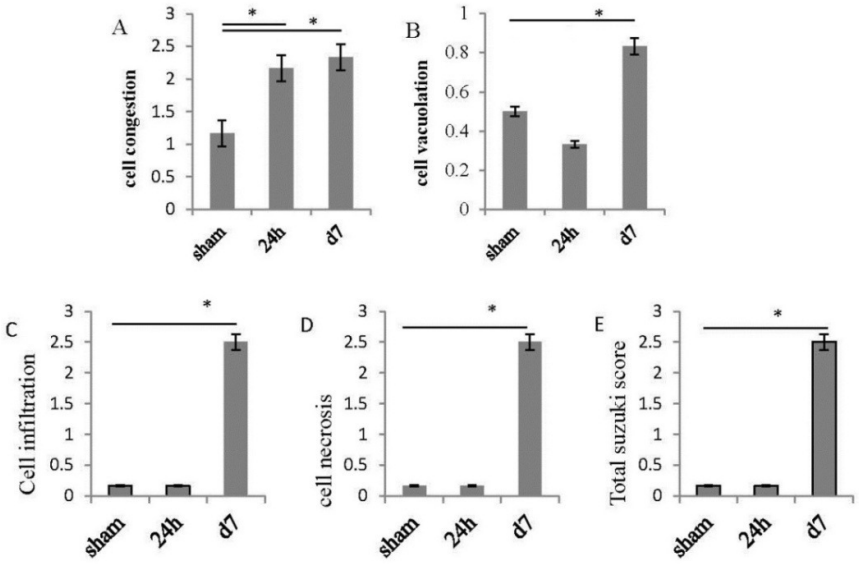

Figure 1. (A) liver cell congestion, (B) liver cell vacuolation, (C) liver cell infiltration, (D) liver cell necrosis, (E) Total Suzuki score. Factors were measured at Sham, 24 hours, and 7 days after the reperfusion. Statistical analysis; using the one-sample Wilcoxon signed-rank test and the Mann-Whitney U test. Scoring by Suzuki approach. Data represent the mean (±SD). Error bars indicate±SD. *=sign of (p<0.05).

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Statistical Analysis

Data were analyzed with SPSS for Windows ver16. Suzuki's score classification was analyzed with a one-sample Wilcoxon signed-rank test. ALT, AST, ALP, and TAC were analyzed with one-sample Student's t-test and one-way ANOVA. RT-PCR data were analyzed with the Kruskal-Wallis test, Spearman's test, and the Mann-Whitney U test. Data are shown as Mean±SEM. A p-value<0.05 was considered statistically significant.

RESULTS

Histopathology

Liver parenchymal tissue assessment showed cell congestion, vacuolization, infiltration of inflammatory cells, and necrosis maximized onday7 (Figure 2). This was followed by a cease in fibrosis of the hepatocytes on day 28. Signs of liver parenchymal tissue improvement were observed afterward. The results were compared with the sham operation group. Histopathology assessment revealed acute hepatocytes' response to I/R.

No significant change between the control group and sham-operation was observed.

MiR-21, PDCD4 mRNA, TIA1 mRNA, FASL mRNA RT-PCR in the Liver and Blood

MiR-21 levels increased in the liver (p<0.01) but decreased in the blood (p=0.0126) 24 hours after the reperfusion. On day 4, liver miR-21 levels decreased (p<0.05) while blood miR-21 levels increased (p<0.05) (Figures 4A and B).

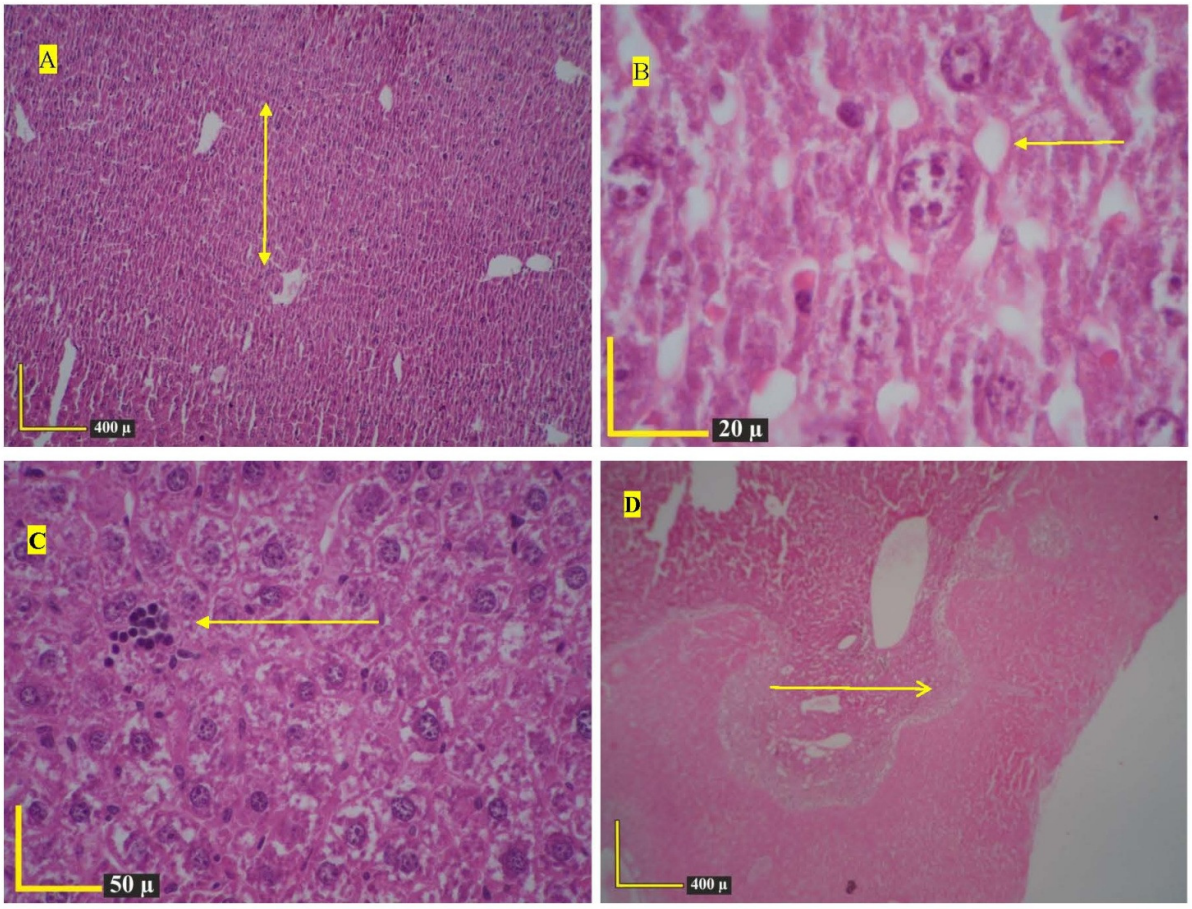

Figure 2. Microscopic histology, (A) cell congestion (×10), (B) cell vacuolation (×100), (C) Cell infiltration (×40), (D) cell necrosis (×4).

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Table 1. PCR Primers

Gene / miR	Sequences (5' - 3')	Product, bp	Tm°C	Length	Melt°C
miR-21	TAGCTTATCAGACTGATGTTGA	101	58	22	75.82
miRU6	CTCGCTTCGGCAGCACA	103	58	17	78.43
PDCD4-R	CAGCTCGAGCCTGTACACAA		58	20	
PDCD4-F	CCTGAGCTAGCCTTGGACAC	139	58	20	82.87
TIA1-R	TTCCAAATGGTGCAAACGCT		59	20	
TIA1-F	ACGAGCAATCATTTCCATGTGT	88	59	22	81.39
FASL-R	TAAATGGGCCACACTCCTCG		59	20	
FASL-F	ACTCCGTGAGTTCACCAACC	109	59	20	81.33
GAPDH-R	AGGGAAATCGTGCGTGAC		58	18	
GAPDH-F	AACGACCCCTTCATTGAC	119	58	18	83.62

R: reveres, F: forward

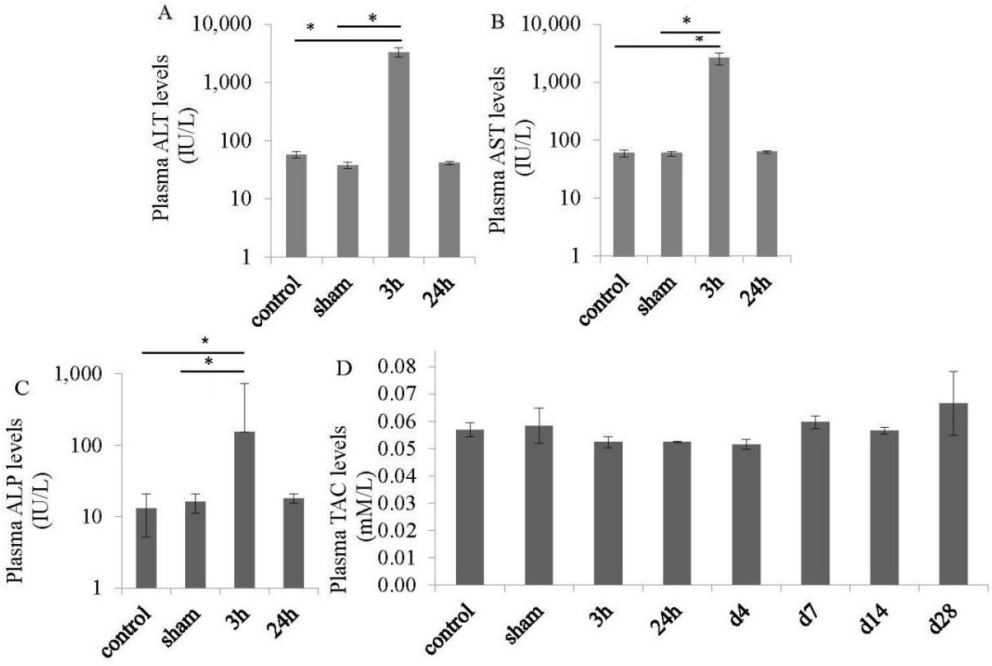

Figure 3. (A) A comparison of Alanine aminotransferase (ALT), (B) aspartate aminotransferase (AST), (C) alkaline phosphatase, levels between control, sham, 3h, and 24h operated groups, Photometric assay. (D) Total antioxidant capacity (TAC) levels between control, sham, and 3h, 24h, d4, d7, d14, and d28 operated groups. Colorimetric assay. 5 Balb/c mice in each group. Statistical analysis; using the one-sample Student's t-test and one-way ANOVA. Data represent the mean (±SD). Error bars indicate±SD. *=p<0.05. Plasma TAC levels showed no significant change in all groups and subgroups indicating no oxidative stress occurred between operated groups and the sham-operation group and post-ischemic phase in blood.

Levels of PDCD4 mRNA, TIA1 mRNA, FASL mRNA changed similarly over time; they increased in both the liver (p<0.01) and blood (p<0.05) on day 4 post-reperfusion; decreased in the liver (p<0.01) and blood (p<0.05), on days 7, and increased in both the liver (p<0.01) and blood (p<0.01) on day 14 (Figure 4).

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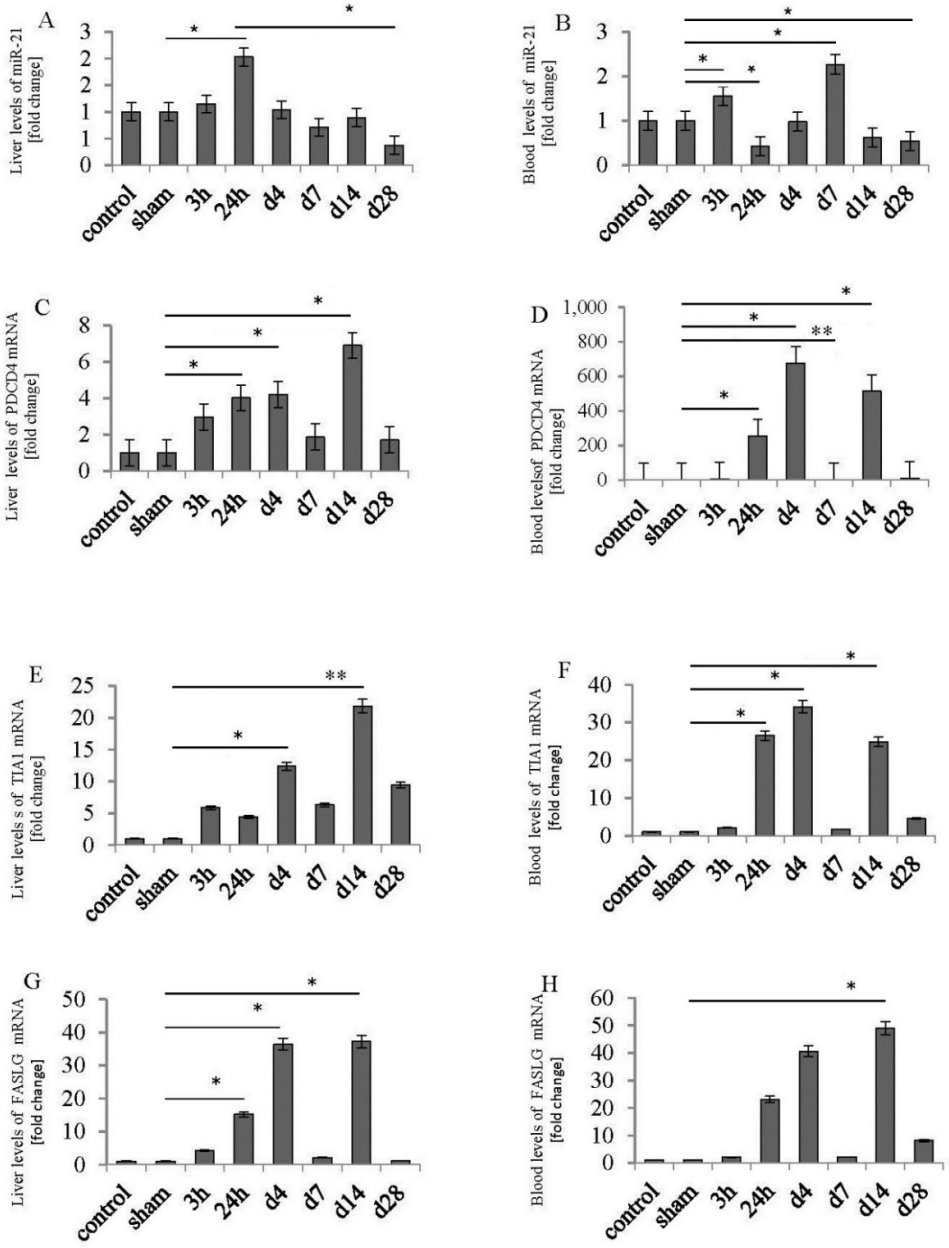

Figure 4. The evaluation of expression of programmed cell death 4 (PDCD4), T-cell intracellular antigen-1 (TIA-1), Fas ligand (FASL), miR-21 (MicroRNA-21). (A) Liver levels of miR-21, (B) Blood levels of miR-21, (C) Liver levels of PDCD4 mRNA, (D) Blood levels of PDCD4 mRNA, (E) Liver levels of TIA1 mRNA, (F) Blood levels of TIA1 mRNA, (G) Liver levels of FASL mRNA, (H) Blood levels of FASL mRNA. Ischemia was induced for 60 minutes. PDCD4, TIA-1, FASL, and miR-21 were measured between control, sham, and 3 h, 24 h, d 4, d7, d14, and d28 operated groups after the reperfusion. Real-time Polymerase Chain Reaction. Five Balb/c mice in each group. Statistical analysis; using the Kruskal-Wallis test and Spearman's test. Data represent the mean (±SD) of the fold change. Error bars indicate±SD. $^{*}p<0.05$, $^{**}p<0.01$.

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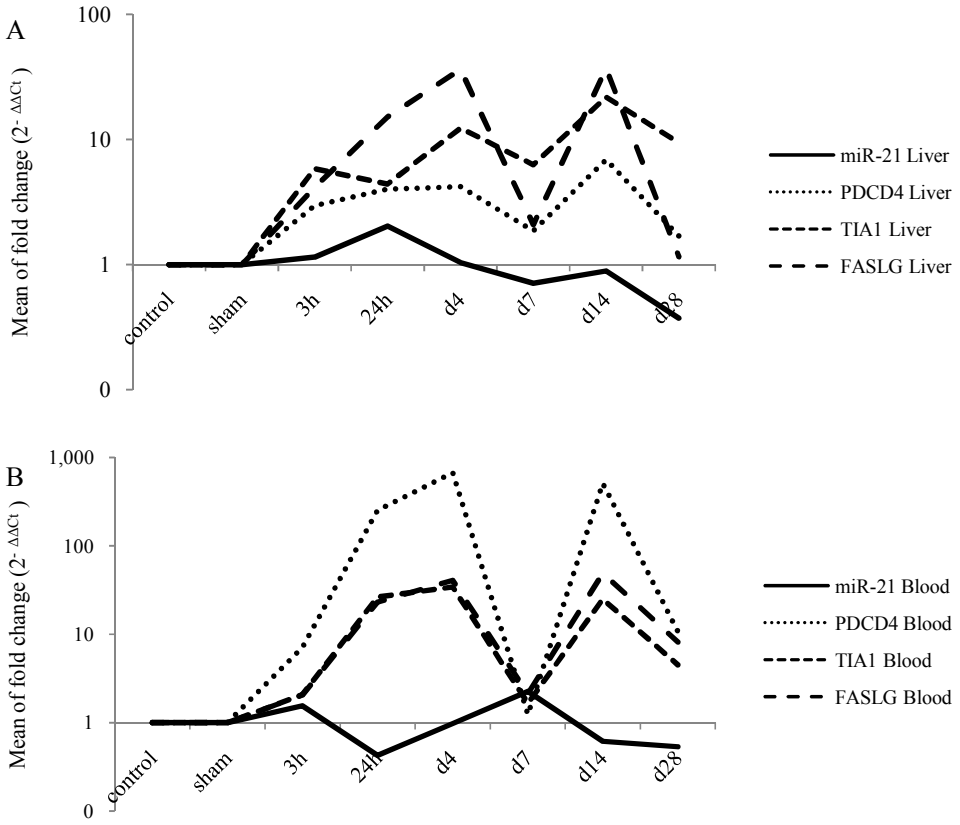

Figure 5. (A) The serial day evaluation of MicroRNA-21 (miR-21), Programmed cell death 4 (PDCD4), T-cell intracellular antigen-1 (TIA-1), and Fas ligand (FASL) expression level in the liver. Continuous relative levels on serial days showed that in the reperfusion phase, miR-21 had an opposite polar pattern of the other apoptotic mRNA; PDCD4, TIA-1, and FASL in 24 hours and day 4, but on day 7, day 14, and day 28 the polarity shifted to a harmonized pattern (B) The serial day evaluation of miR-21, PDCD4, TIA-1, and FASL expression level in the Blood. Ischemia was induced for 60 minutes. Targets were followed between control, sham, and 3h, 24h, d4, d7, d14, and d28 operated groups after the reperfusion. Five Balb/c mice in each group. Scale log based 10.

Liver miR-21, PDCD4 mRNA, TIA1 mRNA, and FASL mRNA

Continuous relative levels on serial days showed that in the reperfusion phase, miR-21 had an opposite polar pattern of the other apoptotic mRNA; PDCD4, TIA-1, and FASL in 24 hours and day 4, but on day 7, day 14, and day 28 the polarity shifted to a harmonized pattern (Figure 5A).

Blood miR-21, PDCD4 mRNA, TIA1 mRNA, and FASL mRNA

Continuous relative levels on serial days showed that in the reperfusion phase, miR-21 had an opposite direction pattern of other apoptotic mRNAs; PDCD4, TIA-1, and FASL (Figure 5B).

DISCUSSION

Histopathology findings showed that hepatic injury and plasma ALT, AST, and ALP levels increased immediately after the I/R injury, which together reflected cellular damage, similar to the findings of Suzuki's experiment. Plasma TAC levels showed no significant change in all groups and subgroups indicating no oxidative stress occurred between operated groups and the sham-operation group and post-ischemic phase in blood. To confirm the findings, three major markers, PDCD4 mRNA, FASL mRNA, and TIA1 mRNA levels, were measured. The results showed that the mRNA expression levels of FASL mRNA and TAI1 mRNA changed similarly. The

changes were in parallel with the measured miR-21 and PDCD4 mRNA levels. The results showed a significant difference between the liver and blood PDCD4 mRNA levels. Similar patterns observed in the temporal change of TIA1 mRNA levels and PDCD4 mRNA in blood confirmed the validity of the methods used in this study.

The role of miR-21 in renal I/R injury has been well documented.[25,26] In kidney I/R injury, miR-21 regulates two pathways: the damage phase and the protection/repair phase. The damage phase includes inflammation and fibrosis, while the protection/repair phase consists of angiogenesis and reduced apoptosis.[18] Hu et al, believe that miR-21 has a significant maintenance function in renal I/R injury.[27] Xu et al, investigated four microRNAs (miR-23a, miR-326, miR-346_MM1, and mmu-miR-370) in the liver I/R injury in mice and recommended further studies on the miRNAs in hepatic I/R.[28] Mean while, the role of miR-21 in the liver ischemia has not been fully studied.[17] Accordingly, at this stage, we tried to create a perspective of miR-21 in the liver I/R.

Datta et al, in a literature review, documented that I/R protocols were published in 16 papers. The least reperfusion period was 30 min; the I/R outcomes were measured at a maximum of 4 days after the ischemia.[29] In our study, the outcome was measured on 3 and 24 hours, and days 4, 7, 17, 28 after the ischemia. We were looking for the relationship between liver injury/recovery and the level of miR-21 damage. Twenty-four hours after hepatic reperfusion of an ischemic liver, miR-21 had a significant overexpression in the liver tissue and blood; PDCD4 mRNA, TIA1 mRNA, FASL mRNA levels significantly increased; and all these changes paralleled with the inflammatory phase. The trends showed a decreasing pattern for miR-21 from 24 hours to 28 days and the final recovery of the liver injury. The upregulation of miR-21 might prompt its protective role in the initial stage of I/R through a reduction pathway similar to that of apoptosis. The results showed an inflammatory phase during which miR-21 increased and a recovery phase where miR-21 decreased.

Xu et al, revealed that miR-21 has a dual effect in I/R injury.[17] In the acute ischemic phase, depletion of ATP causes the accumulation of reactive oxygen species in the hepatocytes.[30] Protein kinase C and regulatory T cells are activated. In the delayed ischemic phase, through an unknown mechanism, hypoxia-inducible factor-1 α (HIF1α) is up-regulated;[25] HIF1α regulates transcription factor for miR-21.[13,31,32] MiR-21 is the target gene for PDCD4.[33] Meng et al, show that miR-21 has a modulator role in hepatocellular carcinoma. This effect occurs through phosphatase and tensin homolog (PTEN).[34] MiR-21, through the inhibition of MyD88, leads to the excitation of NF-κB, which causes inflammation.[35] MiR-21 is also upregulated during the process of T cell differentiation.[36] Sheedy et al,[37] report a negative correlation between PDCD4 and the miR-21 that has a role in the inflammatory phase; we observed similar findings. MiR-21 induces hepatic stellate cells via PTEN/Akt signaling.[38] Liver fibrosis has a relationship with the miR-21 level of expression.[39] MiR-21 has also a crucial role in promoting cellular growth.[40] Our findings confirmed this subject.

Events occurring during liver I/R injury have direct and indirect effects on the kidney, intestine, pancreas, lungs, and myocardium. These effects include micro-vascular, endothelial, apoptotic, and transcription factors.[41] The effects of miR-21 on the I/R of various organs have been studied.[12,13]

Serum miR-21 level is higher in patients with hepatitis B than in healthy subjects.[42] The level is also an independent predictor for recurrence of hepatocellular carcinoma.[43] When these patients undergo liver transplantation, because of an I/R event, the level of miR-21 increases. The difference between these two types of increments in miR-21 caused by hepatocellular carcinoma or I/R is still unknown.

Studying biomarkers is a challenging topic for research aimed at identifying clinically effective treatments, diagnoses, and prognoses for many liver diseases.[44] MiRNAs are potential biomarkers.[45] miR-320 is considered a renal I/R biomarker.[9] Future research will determine whether miR-21 can join the liver I/R biomarker.

Portion expression with western blotting as limitations was not assayed.

In conclusion, the evaluation of the expression level of miR-21/PDCD4 signaling axis during the liver I/R injury/recovery showed significant overexpression of miR-21 and PDCD4 mRNA in the liver 24 hours after the reperfusion. The increased miR-21 expression would result in I/R injury/recovery in the liver.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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