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Original Research Article

Effect of MiR-423-5p expression on the severity of lipopolysaccharide-induced acute liver injury, inflammatory response and immune function in mice

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Abstract

Purpose: *To investigate the biological functions of miR-423-5p in a mouse model with lipopolysaccharide (LPS)-induced acute liver injury.*
Methods: *Sixty male C57BL/6 mice were randomized into control group (n = 20), LPS group (n = 20) and LPS + miR-423-5p mimic group (n = 20). The levels of pro-inflammatory cytokines and apoptosis-associated proteins in the liver tissues were determined. Finally, the survival of mice was recorded within 7 days after the injection of LPS.*
Results: *Overexpression of miR-423-5p improved the liver function of septic mice, and decreased the content of ALT, AST and LDH. Furthermore, overexpression of miR-423-5p also lowered the levels of the oxidative stress markers 4-HNE and MDA, and raised the content of anti-oxidative enzymes SOD and GSH-Px in the liver tissues of septic mice ($p < 0.05$). The levels of inflammation and apoptosis in the liver of mice in LPS + miR-423-4p mimic group were lower than those in the LPS group ($p < 0.05$). The overexpression of miR-432-5p increased CD4+ and CD8+ T cells in the spleen of septic mice ($p < 0.05$), thereby reducing immunosuppression.*
Conclusion: *The expression of miR-423-5p is low in septic mice with liver injury, but its overexpression alleviates acute liver injury and inflammatory responses, and also enhances immune function in mice. Therefore, it has the potential to serve as a targeted drug for the management of liver injury.*

Keywords: *Liver injury, Lipopolysaccharide, miR-423-5p, Inflammation, Immunosuppression*

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INTRODUCTION

Sepsis is a bacterial-induced, life-threatening infection, accompanied by severe inflammatory responses and the dysfunction of multiple organs, including the heart, liver and kidneys [1]. Gram-negative bacteria-produced endotoxins can induce acute liver injury and even acute liver failure, so that the patients become casualties of hepatic encephalopathy, coagulation dysfunction, and multi-organ failure [2]. In fact, bacterial infection does not greatly damage the liver initially, but the secondary uncontrollable systemic inflammatory response syndrome (SIRS) and immunosuppression are the leading causes of multi-organ failure and death in septic patients [3, 4]. However, the development and progression mechanisms of sepsis-induced liver

injury remain elusive now. So it is important to further examine the molecular mechanism and therapeutic target of sepsis-induced liver injury for the forthcoming prevention and treatment of sepsis.

Micro ribonucleic acids (miRNAs) are a group of 20-24 nt-long single-stranded non-coding RNAs with regulatory function in eukaryotes [5]. MiRNAs can bind to the specific target gene to modulate the expressions of multiple life genes, playing a pivotal role in cell proliferation, differentiation, invasion, apoptosis and other behaviors [6, 7]. Large numbers of clinical and basic studies have revealed that miRNAs are important players in liver diseases, especially liver injury. For example, miR-143-3p regulates the phosphorylation of TAK1 in order to attenuate the progression of liver fibrosis in autoimmune hepatitis [8]. Besides, miR-20 in the umbilical cord mesenchymal stem cell exosomes regulates autophagy and apoptosis to relieve liver ischemia-reperfusion injury [9].

In the present study, the expression of miR-423-5p in the lipopolysaccharide (LPS)-induced acute liver injury in mice was first observed, and the potential mechanism of miR-423-5p in alleviating acute liver injury was further explored, in ordder to provide a certain basis for the clinical treatment of acute liver injury in the future.

EXPERIMENTAL

Animal grouping and modeling

A total of sixty 6 - 8 weeks old male C57B/6 mice weighing (21.55 ± 3.18) g were allocated into Control group (n = 20), LPS-induced acute liver injury group (LPS group, n = 20) and LPS-induced acute liver injury + miR-423-5p overexpression group (LPS + miR-423-5p mimic group, n = 20) using a random number table. The mice in LPS group and LPS + miR-423-5p mimic group were intraperitoneally injected with LPS at 5 mg/kg to establish the sepsis-induced liver injury model. Before operation, the mice in LPS + miR-423-5p mimic group were injected with miR-432-5p mimic *via* the caudal vein once daily for 1 week, while those in Control and LPS groups were injected with an equal volume of normal saline. At 12 h after the injection of LPS, blood was drawn from the orbital vein, and the liver of mice in each group was sampled and weighed to calculate liver index. This study was approved by the Animal Ethics Committee of Northwest Women's and Children's Hospital Animal Center, and followed international guidelines for animal studies.

Determination of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH)

After the eyeballs of mice were removed in each group, anticoagulant blood was collected, and serum was isolated. Then, the levels of ALT, AST and LDH in each group were measured using an AU5800 automatic biochemical analyzer (Beckman Coulter, Miami, FL, USA).

Hematoxylin-eosin staining (H&E) staining

The liver of mice in each group was fixed in 10 % formalin overnight, dehydrated and embedded into paraffin blocks. The liver tissues were then sliced into 5 μm-thick sections, mounted on glass slides, baked dry and stained. According to the instructions, the resulting sections were soaked successively in xylene, graded concentrations of alcohol, and hematoxylin, and sealed in resin. After being dried, the morphology of the liver tissues were observed under an optical microscope.

Determination of pro-inflammatory cytokine expression in liver tissues by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were first extracted from the fresh frozen liver tissues of mice using TRIzol™ kit (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNAs in each group of samples were determined using a spectrophotometer. When the concentration and purity were qualified, 2 μg of RNAs from each sample are reversely transcribed into complementary deoxyribonucleic acids (cDNA). The cDNAs of each sample were then subjected to RT-PCR in a system comprised of 2.5 μL of 10× buffer, 1 μL of cDNAs, 0.5 μL of both 20 μmol/L forward and reverse primers, 10 μL of LightCycler® 480 SYBR Green I Master (2 ×) and 5.5 μL of ddH_2O. The RT-PCR amplification system was the same for all samples. The primers used are shown in Table 1.

Immunohistochemical staining

The prepared liver tissue sections were baked in an oven at 60 °C for 30 min, de-paraffinized in xylene for 5 min × 3 times, and then were dehydrated using 100 %, 95 % and 70 % alcohol. Subsequently, the activity of endogenous peroxidases was repressed by 3 % hydrogen peroxide methanol, and the tissues were sealed using goat serum for 1 h.

Table 1: Primer sequences used in RT-PCR

Target gene		Primer sequence
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Forward	5'-GACATGCCGCCTGGAGACAACCC-3'
	Reverse	5'-AGCCCAGGATGCCCTTTAGTGGCCA-3'
Interleukin (IL)-1β	Forward	5'-CGATGCGATGTCCAAAGTCGAGTG-3'
	Reverse	5'-GCATGTCAGTACGACGTGCTCGCTC-3'
IL-6	Forward	5'-ACGTAGCTGATATGGTCGTAGTGACC-3'
	Reverse	5'-CTAGTCGTAGTGATGGGTCGTAGTGTA-3'
Tumor necrosis factor-α (TNF-α)	Forward	5'-ACACGTAGCTACGTGGATTACGTGTAC-3'
	Reverse	5'-ACTTGATCGTGACTCGTCGATCGTG-3'
MiR-423-5p	Forward	5'-TGTAGCTCTAGGATCGATGAATTGTGT-3'
	Reverse	5'-AACTAGTCGTAGCTAGCTCGTAGGTAG-3'

The sections were incubated with the antibodies against 4-hydroxynonenal (HNE) and cluster of differentiation 45 (CD45) at 4°C overnight and then washed by PBS on a shaking table for 4 times. Following incubation with the secondary antibodies, diaminobenzidine staining was performed.

Western blotting

The fresh frozen liver tissues of mice in each group were taken out from the refrigerator at -80°C, then cut into pieces and fully ground using a grinder. Subsequently, the tissues were ultrasonicated, and the lysate was centrifuged. The supernatant was then aspirated and aliquoted into Eppendorf (EP) tubes. Protein concentration was measured using the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Proteins from all samples were diluted to the same constant concentration, aliquoted, and preserved in the refrigerator at -80 °C. The extracted total proteins were then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the resulting proteins in the gels were transferred onto cellulose acetate/polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), incubated with the corresponding primary antibodies at 4°C overnight, and then with the goat anti-rabbit secondary antibodies in the dark for 1 h. Finally, the protein bands were scanned with an Odyssey (Seattle, WA, USA) scanner and quantified.

Determination of $CD4^+$ and $CD8^+$ cells in the spleen via flow cytometry

The spleen of the mice was first cut into small bit, added to Hank's balanced salt solution, and digested with collagenase D and DNase I in a thermostatic incubator at 37 °C for 30 min. Subsequently, the erythrocytes in the spleen were lysed using erythrocyte lysate, and the resulting erythrocyte suspension was filtered through a cell filter with a pore diameter of 70 μm, and centrifuged at 300 g and 4 °C for 5 min, followed by the collection of lymphocytes. After washing, fluorescence-activated cell sorting was immediately performed as follows: Single lymphocytes were first incubated with FcR blocker (Miltenyi Biotec GmbH, Germany) for 10 min and then with the FITC-labeled anti-CD4 antibody and anti-CD8 antibody, was conjugated for 20 min. Finally, the washed cells were evaluated using FACSCalibur (BD, Detroit, MI, USA), and the data were analyzed using FlowJo 7.6.

Determination of oxidative stress markers

A total of 100 μL liver tissue homogenate (10 %) was taken. Then, according to the instructions on the kit the proteins of each sample were quantified using Coomassie brilliant blue staining. The levels of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were measured according to the protocols.

Statistical analysis

All data were analyzed using Statistical Package for Social Sciences (SPSS) 22.0 software (IBM, Armonk, NY, USA), and the measurement data are presented using mean ± standard deviation. The data were compared between two groups using *t*-test, and the differences were considered statistically significant at $p < 0.05$.

RESULTS

Changes in miR-423-5p expression in mouse liver tissues after LPS induction

According to the RT-PCR results (Figure 1), the expression level of miR-423-5p in the liver tissues of mice in LPS group was obviously lower than that in the control group ($p < 0.05$), and about 0.41 times more that in the control group. Compared with the LPS group, the expression of miR-423-5p evidently rose in the liver tissues of mice in the LPS + miR-423-5p mimic group ($p < 0.05$), suggesting that overexpression in the miR-

423-5p mouse model was established successfully.

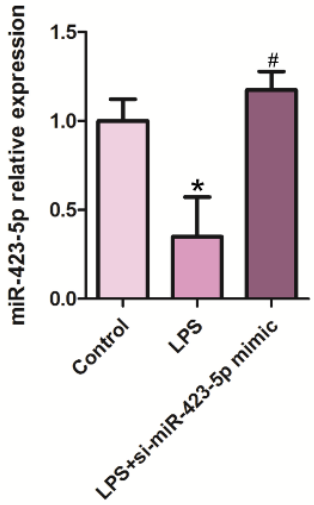

Figure 1: Changes in miR-423-5p expression in the liver tissues of mice after induction with LPS. * $p < 0.05$ *vs.* Control group, # $p < 0.05$ *vs.* LPS group

Effect of miR-423-5p overexpression on acute liver injury in mice

After the overexpression of miR-423-5p, the septic mice exhibited alleviated the destruction of the hepatic lobule structure, and hepatocellular degeneration and swelling, without ballooning degeneration, and the liver index of mice declined (Figure 2 A). It was further discovered that the overexpression of miR-423-5p decreased the levels of ALT, AST and LDH in the liver tissues of septic mice (Figure 2 B).

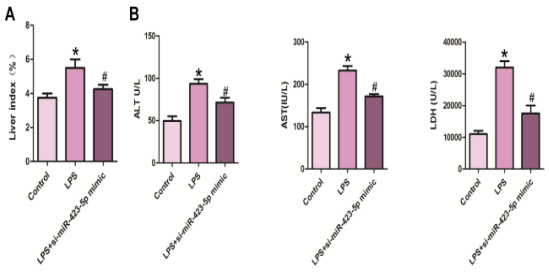

Figure 2: Effect of miR-423-5p overexpression on acute liver injury in mice. (A) Overexpression of miR-423-5p decreased the liver index; (B) overexpression of miR-423-5p distinctly decreased the levels of ALT, AST and LDH in the liver tissues of septic mice

Effect of miR-423-5p overexpression on oxidative stress in the liver of mice with acute liver injury

The overexpression of miR-423-5p decreased the expression of the lipid peroxidation product 4-HNE in the liver tissues of septic mice (Figure 3 A) ($p < 0.05$). Besides, the LPS + miR-423-5p mimic group had a lower level of MDA ($p < 0.05$), and higher levels of anti-oxidative enzymes SOD and GSH-Px ($p < 0.05$) in the liver tissues of mice than the LPS group (Figure 3 B).

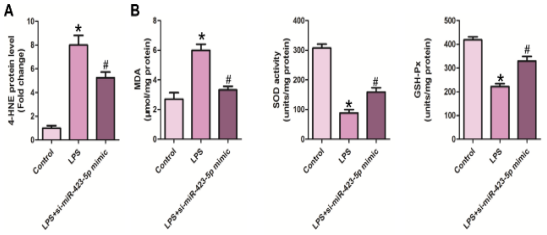

Figure 3: Effect of miR-423-5p overexpression on oxidative stress in the liver of mice with acute liver injury. (A) Overexpression of miR-423-5p obviously decreased the expression of the lipid peroxidation product 4-HNE in the liver tissues of septic mice; (B) overexpression of miR-423-5p markedly reduced level of MDA, and increased levels of SOD and GSH-Px in the liver tissues of septic mice

Effect of miR-423-5p overexpression on the liver inflammation in mice with acute liver injury

After the overexpression of miR-423-5p, the number of CD45-positive cells (leukocytes) in the liver of septic mice decreased ($p < 0.05$) (Figure 4 A). As shown by the RT-PCR, the levels of pro-inflammatory cytokines IL-1, IL-6 and TNF-α in the LPS + miR-423-5p mimic group were lower than those in LPS group (Figure 4 B) ($p < 0.05$).

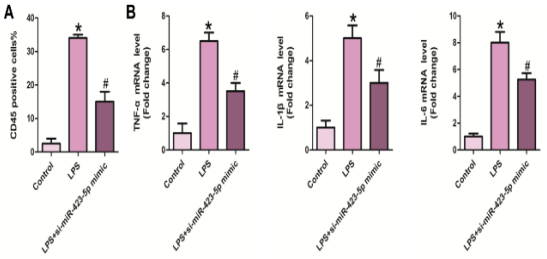

Figure 4: Effect of miR-423-5p overexpression on the liver inflammation of mice with acute liver injury. (A) Overexpression of miR-423-5p significantly reduced the number of CD45-positive cells (leukocytes) in the liver of septic mice; (B) overexpression of miR-423-5p markedly decreased levels of pro-inflammatory cytokines IL-1, IL-6 and TNF-α. * $p < 0.05$ *vs.* Control group, # $p < 0.05$ *vs.* LPS group)

Effect of miR-423-5p overexpression on liver cell apoptosis in mice with acute liver injury

According to the Western blotting results (Figure 5), the overexpression of miR-423-5p up-regulated the expression of anti-apoptotic protein

Bcl-2 ($p < 0.05$), and inhibited the expression of pro-apoptotic protein Bax ($p < 0.05$) in the liver tissues of septic mice.

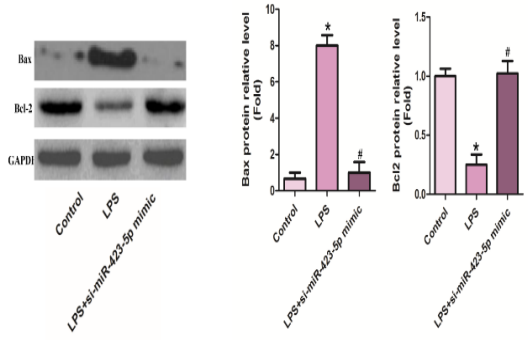

Figure 5: Effect of miR-423-5p overexpression on liver cell apoptosis. $^{*}P$ <0.05 *vs.* control group, $^{\#}p$ <0.05, control group *vs.* LPS group

Effect of miR-423-5p overexpression on immune function in mice with acute liver injury

The levels of $CD4^+$ and $CD8^+$ T cells in the spleen of mice in each group were measured. It was discovered that the levels of $CD4^+$ and $CD8^+$ T cells in the spleen of mice decreased after LPS stimulation ($p < 0.05$), and the overexpression of miR-423-5p raised their levels, potentiating the immune function of mice ($p < 0.05$) (Figure 6).

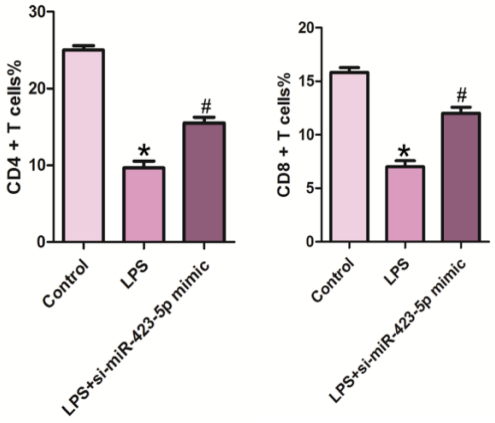

Figure 6: Effect of miR-423-5p overexpression on immune function in mice with acute liver injury. $^{*}p < 0.05$, *vs.* control group; $^{\#}p < 0.05$, *vs.* LPS group

Effect of miR-423-5p overexpression on the 7-day survival of mice with acute liver injury

After LPS stimulation, the 7-day survival of mice declined ($p < 0.05$), after the overexpression of miR-423-5p ($p < 0.05$) (Figure 7).

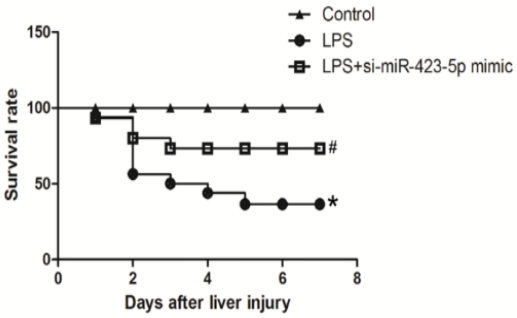

Figure 7: Effect of miR-423-5p overexpression on the 7-day survival rate of mice with acute liver injury: Control group: control, LPS group: LPS-induced acute liver injury in mice, and LPS + miR-423-5p mimic group: LPS-induced acute liver injury in mice + miR-423-5p overexpression. $^{*}P < 0.05$, *vs.* control group; $^{\#}p < 0.05$ *vs.* LPS group

DISCUSSION

Sepsis, one of the most common diseases in the Intensive Care Unit, is caused by massive endotoxins produced by Gram-negative bacteria in the body. Endotoxins induce systemic inflammation through the main component of LPS, which can activate various receptors on the cell membrane and trigger the innate immune response [10]. However, long-standing massive LPS can induce systemic inflammation and immunosuppression in the body. Sepsis-induced SIRS causes multi-organ failure in organisms, and the liver is one of the main target organs that are attacked by LPS [11,12]. The patients with sepsis complicated by acute liver injury have a remarkably higher morbidity rate than those with sepsis alone [13]. Therefore, it is of great significance to further explore whether sepsis is the molecular mechanism in liver injury for the maintenance of normal organ functions and raising the survival in septic patients.

MiRNAs are a class of about 20 - 24 nt-long endogenous small-molecule non-coding RNAs that can regulate the expression of various genes at the post-transcriptional level in organisms. Specifically, miRNAs binded to the 3'UTR of the target gene to inhibit the translation of the corresponding proteins [14]. As one member of the miRNA family, miR-423-5p is implicated in the development and progression of various human diseases. For example, the expression of miR-423-5p is obviously increased in glioma, and it acts as a potential oncogene by targeting ING-4. Besides, the overexpression of miR-423-5p increases the phosphorylation levels of Akt, ERK1/2 and other important signaling molecules, and drives the formation of glioma neurospheres, thereby weakening the chemosensitivity of glioma cells to temozolomide as well [15]. In the

mouse model of lupus nephritis, the expression levels of miR-663a and miR-423-5p in the renal tissues substantially rise and the expression of TNFAIP3 interacting protein 2 (TNIP2) is prominently inhibited. The further study revealed that miR-663a and miR-423-5p can target TNIP2 to activate the NF-κB signaling pathway to participate in the progression of lupus nephritis [16].

The current study showed that the expression level of miR-423-5p decreased in the liver tissues of mice at 12 h after the injection of LPS. The mice were further injected with miR-423-5p mimic *via* the caudal vein to overexpress miR-423-5p, and the results showed that the up-regulation of miR-423-5p alleviated the pathological injury of the liver, decreased the liver index, and repressed the expressions of ALT, AST and LDH in septic mice, ultimately improving the survival of mice. Sepsis triggers a series of complicated immune responses, accompanied by pro-inflammatory and anti-inflammatory responses with time.

Based on the findings of the present study, the expression levels of the pro-inflammatory genes IL-1β, IL-6 and TNF-α were elevated in the liver tissues of mice and the levels of $CD4^+$ and $CD8^+$ cells in the spleen evidently decreased at 12 h after the injection of LPS, suggesting that the immune function of mice was weakened at this time. Conversely, after the overexpression of miR-423-5p, the levels of inflammatory factors and inflammatory cell infiltration in the liver tissues of mice were lowered, and immunosuppression was also reduced, indicating that miR-423-5p has a protective effect on the liver of septic mice probably by resisting inflammation and enhancing immunity.

Previous studies have reported that excessive oxidative stress in liver cells in sepsis cases caused cell injury and even death, and it serves as the primary mechanism in acute liver injury and even liver failure [17,18]. In this study, the expression of lipid peroxide MDA increased in the liver at 12 h after the injection of LPS. Therefore, it can be inferred that LPS stimulation severely damaged liver cell membranes and organelles with membrane structure. However, miR-423-5p inhibited oxidative stress in liver cells, which is also a mechanism by which miR-423-5p protects the liver.

CONCLUSION

MiR-423-5p is down-regulated in sepsis-induced liver injury in mice. Overexpression of miR-423-5p protects against LPS-induced acute liver injury by inhibiting inflammatory responses and improving t immune function. Thus, MiR-423-5p has a potential frole in the management of acute liver injury.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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