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Effect of miR-10a on sepsis-induced liver injury in rats through TGF-β1/Smad signaling pathway

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Abstract. – OBJECTIVE: To observe the effect of the micro ribonucleic acid (miR)-10a on sepsis-induced liver injury in rats through the transforming growth factor-β1 (TGF-β1)/Smad signaling pathway.

MATERIALS AND METHODS: The rat model of sepsis was established *via* cecal ligation and puncture, in which miR-10a was overexpressed and silenced using liposome transfection. The rats were randomly divided into miR-10a mimics group (Mimics group, n=10) and miR-10a inhibitors group (Inhibitors group, n=10), and the sham operation group (Sham group, n=10) was also set up. The transfection efficiency of miR-10a in liver tissues in each group was detected *via* quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), the serum liver function indexes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were determined. Moreover, the content of the serum reactive oxygen species (ROS), glutathione (GSH), and GSH peroxidase (GSH-Px) was determined using enzyme-linked immunosorbent assay (ELISA). The content of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and myeloperoxidase (MPO) in liver tissues was detected, and the pathological changes in liver tissues were observed through hematoxylin-eosin (HE) staining. Finally, the expression levels of cytochrome P4502E1 (CYP2E1) and TGF-β1/Smad signaling pathway genes and proteins in liver tissues were detected *via* qRT-PCR and Western blotting.

RESULTS: The expression of miR-10a was significantly increased in Mimics group ($p<0.05$) and extremely low in the Inhibitors group ($p<0.05$). In Mimics group, the levels of serum AST, ALT, and LDH were significantly increased ($p<0.05$), the content of ROS, TNF-α, IL-6, and MPO was substantially increased ($p<0.05$), while that of GSH and GSH-Px notably declined ($p<0.05$). According to the HE staining results, the liver cells were orderly arranged in the Inhibitors group, and they were disorderly arranged with more inflammatory cells in the Mimics group. The results of the gene and protein assays showed that the expression levels of CYP2E1, TGF-β1, and Smad2 in Mimics group were markedly higher than those in the Sham group ($p<0.05$), while they displayed the opposite trends in the Inhibitors group ($p<0.05$).

CONCLUSIONS: Silencing miR-10a can inhibit the occurrence of sepsis-induced liver injury in rats by downregulating the TGF-β1/Smad pathway.

Key Words

MiR-10a, TGF-β1/Smad signaling pathway, Sepsis, Rats, Liver injury.

Introduction

Sepsis is a kind of severe systemic inflammatory response syndrome (SIRS), whose pathogenesis is closely related to the systemic inflammatory response network. When the immune system responds to infection, the inflammatory response may lead to dysfunction of vital organs, such as the lung, heart, kidney, and liver[1,2]. The liver plays a key role in the host defense mechanism, as well as an important organ that regulates the synthesis, metabolism, storage, and redistribution of carbohydrates, proteins, and lipids[3]. In addition, the liver also plays an important role in detoxification, and these toxic substances will cause various liver diseases, including alcoholic liver injury during the scavenging of the exogenous drugs and toxins[4]. When sepsis occurs, the liver can regulate the inflammatory process, and produce and release a large number of various cytokines, thus exerting an important effect of inflammatory signal transduction[5]. In sepsis, the inflammatory cytokines, including tumor necrosis factor (TNF) and interleukin-6 (IL-6), affect the onset of the inflammatory process[6]. It is reported that the incidence rate of sepsis complicated by liver injury, one of the most common complications of sepsis,

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is 34.7%, and it is an important predictor for the poor prognosis of patients with sepsis. According to a study on 206 ICU cases in France[7], the incidence rates of liver injury and liver failure are 46.6% and 6.3%, respectively, within the first 24 h in ICU. The liver is the main source of cytokines. In the early stage of sepsis, the body is in a pro-inflammatory status, and a large number of pro-inflammatory cytokines are released, such as TNF, IL-1, and IL-6. Pro-inflammatory cytokines can activate the cytokine cascade, lead to cytokine storm, and aggravate inflammatory and immune responses[8], thereby worsening the condition of the disease, which, therefore, is also one of the major causes of death in patients. Although there is a new understanding of the pathophysiology of sepsis, it is still difficult to radically treat sepsis complications, leading to a high mortality rate of sepsis. Therefore, searching for a kind of new, efficacious, and safe drug to prevent liver diseases is an area of concern, and the development of more efficacious and safer drugs to prevent and treat a sepsis-induced liver injury is of great value. Moreover, it is of great significance to study the mechanism of sepsis-induced liver injury for the clinical treatment of the liver injury.

Currently, many authors[9] have found that micro ribonucleic acids (miRNAs) are involved in cell differentiation, proliferation, and apoptosis. MiRNAs are a group of small non-coding RNAs with about 22 nucleotides in size, which can regulate the gene expression, and participate in the specific regulation of protein-coding and non-coding genes mainly by binding to the 3' untranslated region (3'-UTR) of the target messenger RNAs (mRNAs)[10]. According to previous reports, binding to the 3'-UTR of the target mRNA in a base-pairing way will lead to mRNA instability. Yang et al[11] have found that miRNAs can regulate various cellular processes, such as normal development and disease onset, and they are also considered to contribute to the development of many diseases. One-third of human genes may be regulated by miRNAs, and they play important roles in physiological homeostasis and health, as well as various diseases, including cell cycle, development, metabolism, and immune responses[12]. In addition, the role of miRNAs in the pathogenesis of different diseases has also been explored, and they have become important regulators of the gene expression in many diseases, whose regulatory network has attracted much attention in recent years[13]. There are some researches evidencing that the transforming growth factor-β1 (TGF-β1)/Smad signaling pathway is involved in the process of liver injury. Zhou et al[14] have found that miR-10a can inhibit the TGF-β1/Smad pathway. TGF-β1, the most potent factor in the TGF-β family, plays a crucial role, and it is a major factor involved in the initiation and maintenance of liver injury. Once activated, TGF-β will bind to TGF-β type-2 receptor and activate the downstream phosphorylated Smad2 and Smad3. After phosphorylation, Smad2, and Smad3 will form the transcriptional complex with Smad4, and such complex accumulates in the nucleus to regulate transcription[15,16]. However, the regulatory effect of miR-10a on TGF-β1/Smad and its effect on sepsis-induced liver injury are rarely studied, and its specific mechanism of action remains unclear.

In the present study, the effect of miR-10a on sepsis-induced liver injury in rats through the TGF-β1/Smad signaling pathway was explored. The classical rat model of sepsis was established, the biochemical indexes were detected, and the changes in the TGF-β1/Smad pathway genes and proteins in tissues were determined using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blotting, so as to reveal the effect of miR-10a on sepsis-induced liver injury in rats and provide an experimental basis for the subsequent research and development of new drugs.

Materials and Methods

Commonly Used Reagents and Instruments

The main reagents used were: tissue homogenizer and electrophoresis apparatus (Bio-Rad, Hercules, CA, USA), microplate reader (Thermo Fisher Scientific, Waltham, MA, USA), TRIzol (Invitrogen, Carlsbad, CA, USA) reagent, diethyl pyrocarbonate (DEPC)-treated water, SuperScript III RT kit, and SYBR qPCR Mix (Thermo Fischer Scientific, Waltham, MA, USA), radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China), loading buffer, protease inhibitor, and bicinchoninic acid (BCA) protein concentration assay kit (Biosharp, Hefei, China), TNF-α and IL-6 enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), β-actin and secondary antibodies (Boster Technology, Cambridge, MA, USA), primary antibodies (CST, Danvers, MA, USA), 2500 gel imager (Bio-Rad,

Hercules, CA, USA), and qPCR instrument (7900 Fast, Applied Biosystems, Foster City, CA, USA).

Animal Modeling and Grouping

After preoperative fasting for 12 h, the Sprague-Dawley rats weighing about 260 g and aged about 12 weeks old were intraperitoneally anesthetized with 3% pentobarbital sodium, the abdomen was routinely disinfected, and a 2 cm-long incision was made to expose the cecum. The distal cecum was separated from the large intestinal mesentery, half of which was ligated, with needle punctured in the middle. Then, the cecum was placed back into the abdominal cavity, and the abdomen was sutured, so as to establish the rat model of sepsis through cecal ligation and puncture (CLP). MiR-10a was transferred to adenovirus vectors using liposomes and transfected into rats, and the rats were divided into miR-10a inhibitors group (Inhibitors group), and miR-10a mimics group (Mimics group). The sham operation group (Sham group) was also set up, in which CLP was not performed, and the remaining operations were the same as those in the modeling. The experimental scheme was approved by the Laboratory Animal Ethics Committee. The rats in each group were fed for 2 weeks. After the trial period, the blood and liver tissue samples were collected from rats in each group, and one portion of tissues was stored in 4% paraformaldehyde for hematoxylin-eosin (HE) staining and the other one stored in a refrigerator at -80°C to detect the expression levels of genes and proteins.

Transfection Efficiency of MiR-10a in Liver Tissues in Each Group

MiR-10a was transfected into the rats using adenovirus, and the transfection efficiency of miR-10a in liver tissues was detected *via* qRT-PCR. The rats were anesthetized *via* intraperitoneal injection of pentobarbital sodium, and an appropriate number of liver tissues were carefully taken and smashed using a tissue homogenizer to detect the expression of miR-10a, so as to prepare for the subsequent study of the molecular mechanism of miR-10a in sepsis-induced liver injury.

Detection of Liver Function

After the trial period, the blood was routinely drawn from the caudal vein, placed at room temperature for 30 min, and centrifuged at 3500 g for 10 min. Then, the supernatant was collected to detect and observe the changes in the liver function indexes aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH), so as to further indicate the development of liver injury, hoping to provide an important reference for early diagnosis, predict the occurrence of disease, and prepare for subsequent experiments.

Detection of Serum Oxidative Stress Indexes in Each Group

After the trial period, the blood was routinely drawn from the caudal vein, placed at room temperature for 30 min, and centrifuged at 3000 g for 15 min. Then, the supernatant was collected to detect the levels of the reactive oxygen species (ROS), reduced glutathione (GSH), and GSH peroxidase (GSH-Px) using the ELISA kits according to the actual conditions and instructions. Finally, the absorbance in each group was detected using a microplate reader.

Detection of Inflammatory Factors in Liver Tissues in Each Group

After the rats were anesthetized and sacrificed, the liver tissues were harvested and washed with normal saline. Then, 0.2 g of liver tissues were taken, smashed using the homogenizer with tissue lysis buffer prepared already, and centrifuged at 2500 g for 15 min. Subsequently, the supernatant was collected to detect the changes in the levels of myeloperoxidase (MPO), IL-6, and TNF-α. Finally, the absorbance of indexes in each group was detected using the microplate reader, and the standard curves were plotted, based on which the changes in the content were analyzed according to the instructions.

HE Staining

The rats were sacrificed *via* dislocation at one time, and the liver was aseptically isolated and fixed with 4% paraformaldehyde at 4°C for 48 h. The tissues were washed with running water, dehydrated with ethanol at different concentrations, and embedded in paraffin. Then, they were routinely sliced into 4-5 μm-thick sections, deparaffinized, and hydrated with 95%, 90%, 80%, 75%, and 50% ethanol. Finally, the pathological changes in the liver tissues were observed under a light microscope.

QRT-PCR

The rats were sacrificed *via* dislocation at one time, and the liver was isolated, from which the total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into

complementary deoxyribonucleic acids (cDNAs) after the RNA purity and concentration were detected. The primer amplification was performed using the system (20 μL): 2 μL of cDNA, 10 μL of mix, 2 μL of primers, and 6 μL of ddH_2O, a total of 40 cycles. Then, the PCR amplification was performed under the following conditions: pre-denaturation at 95°C for 2 min, 94°C for 20 s, 60°C for 20 s, and 72°C for 30 s, for a total of 40 cycles. The primer sequences of the target genes and the internal reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed according to those in the GenBank (Table I). The expression levels of the target genes were detected *via* qRT-PCR. The mRNA expression level in liver tissues in each group was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

The liver tissues were cut into pieces, weighed, and added with radioimmunoprecipitation assay (RIPA) lysis buffer (100 mg: 1 mL) for tissue homogenization (Beyotime, Shanghai, China). The protein was extracted, and the protein concentration was calculated using the bicinchoninic acid (BCA) protein assay kit. Then, the Western blotting was performed as follows: the gel was prepared for protein loading and electrophoresis, and the protein was transferred onto a membrane, sealed, incubated with the primary antibody overnight, and incubated again with the secondary antibody for 1 h. The freshly prepared enhanced chemiluminescence (ECL) mixture was added, and the color was developed in a darkroom. The protein band was scanned and quantified using the Odyssey scanner, and the level of the protein to be detected was corrected using GAPDH. Finally, the Western blotting bands were quantified using Image Lab software. The protein expression in each group was calculated.

Table I. Primer sequences.

Target gene	Primer sequence (5'-3')
GAPDH	F: CAGTGCCAGCCTCGTCTCAT R: AGGGCCATCCACAGTCTTC
CYP2E1	F: CCAGTGGAGTCTACATTCT F: TTCATTCTGTGTTCTAACTGG
TGF-β1	F: TGT GGC TCC TAG TGT TGACG R: GCAGTTTGGACAGGATCTGG
Smad2	F: GCTTCTTGACGAGAGAGTCTACGG R: TACTAACACTGGTGGCAGCACTGG
MiR-10a	F: AATTCGACGAGGACGACAAGGAG R: CGAGTCACATCACATCCTTGTGCT

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp., Armonk, NY, USA) software was used for the processing of the raw experimental data, and the multiple comparisons were performed for the data. The experimental results obtained were expressed as mean ± standard deviation ($\overline{\chi}$±SD), and $p<0.05$ suggested the statistically significant difference. The bar graph was plotted using the GraphPad Prism 5.0 (La Jolla, CA, USA).

Results

Transfection Effect of MiR-10a in Each Group

To observe the transfection efficiency of miR-10a in each group, its expression level was detected. As shown in Figure 1, the expression of miR-10a was significantly increased in the Mimics group ($p<0.05$) and significantly decreased in the Inhibitors group ($p<0.05$), indicating that the transfection effect is evident and subsequent experiments can be performed.

Detection Results of Serum Liver Function Indexes

After the trial period, the blood was drawn from the caudal vein and centrifuged, and the supernatant was collected to detect the content of the biochemical indexes ALT, AST, and LDH. The results revealed that the content of LDH, ALT, and AST was substantially increased in the Mimics group ($p<0.05$), while it was remarkably decreased in the Inhibitors group ($p<0.05$) (Table II), indicating that liver injury occurs in sepsis rats.

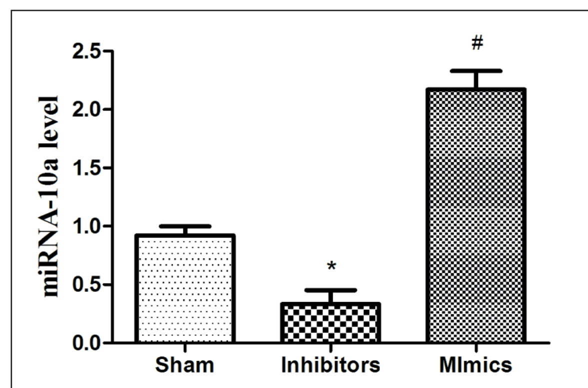

Figure 1. Transfection effect of miR-10a. The expression of miR-10a is increased in the Mimics group ($p<0.05$) and significantly decreased in the Inhibitors group ($p<0.05$). *$p<0.05$ *vs.* Sham group, #$p<0.05$ *vs.* Inhibitors group.

Table II. Content of ALT, AST, and LDH (U/L).

Group/Index	ALT	AST	LDH
Sham	8.3±0.78	46.8±1.5	8.3±0.3
Mimics	126.8±1.6[a]	159.1±2.7[a]	100.6±2.6[a]
Inhibitors	19.6±1.9[b]	69.5±1.8[b]	20.5±1.0[b]

Note: The content of LDH, ALT, and AST is notably increased in the Mimics group (p<0.05), while it is substantially decreased in the Inhibitors group (p<0.05). [a]p<0.05 *vs*. Sham group, [b]p<0.05 *vs*. Mimics group.

Table III. Content of oxidative stress indexes (U/L).

Group/Index	ROS	GSH	GSH-Px
Sham	6.3±0.8	104.7±1.5	135.3±0.2
Mimics	44.8±1.6[a]	33.1±2.7[a]	29.5±2.6a
Inhibitors	10.7±1.1[b]	92.7±1.6[b]	119.7±1.9[b]

Note: Mimics group has a notably increased level of oxidative index ROS (p<0.05) and remarkably decreased levels of antioxidant indexes GSH and GSH-Px (p<0.05), while the Inhibitors group has the opposite trends to those in the Mimics group (p<0.05). [a]p<0.05 *vs*. Sham group, [b]p<0.05 *vs*. Mimics group.

Detection Results of Serum Oxidative and Antioxidant Factors in Each Group

The levels of oxidative stress indexes in each group were measured through ELISA. As shown in Table III, compared with the Sham group, the Mimics group had a markedly increased level of oxidative index ROS (p<0.05) and notably decreased levels of antioxidant indexes GSH and GSH-Px (p<0.05), while the Inhibitors group had the opposite trends to those in the Mimics group (p<0.05).

Detection Results of Inflammatory Factors in Each Group

The levels of the inflammatory factors TNF-α, IL-6, and MPO were determined in this study. As shown in Table IV, the levels of TNF-α, IL-6, and MPO were evidently higher in the Mimics group than those in the other two groups, while they evidently declined in the Inhibitors group (p<0.05), which suggests the production of a large number of inflammatory factors in liver tissues in sepsis rats, further indicating the development of liver injury.

Table IV. Levels of inflammatory factors.

Group	IL-6 (mg/L)	TNF-α (fmol/mL)	MPO (U/mg)
Sham	61.1±4.1	42.7±3.0	3.5±1.2
Mimics	169.5±5.2[a]	96.9±2.3[a]	19.1±1.0[a]
Inhibitors	80.1±2.0[b]	53.0±4.2[b]	5.6±1.4[b]

Note: The levels of TNF-α, IL-6, and MPO are evidently higher in the Mimics group, while they evidently decline in the Inhibitors group. [a]p<0.05 *vs*. Sham group, [b]p<0.05 *vs*. Mimics group.

HE Staining

The morphological changes in the liver tissues in each group were detected using HE staining. The results showed that the cells were disorderly arranged with inflammatory cell infiltration in the Mimics group, while the cells had basically the normal structure and ordered arrangement with milder pathological changes in the Inhibitors group (Figure 2).

RT-PCR Results of Expressions of Key Molecules and Pathway-Related Genes

The expression levels of CYP2E1, TGF-β1, and Smad2 were remarkably higher in the Mimics group than those in the Sham group (p<0.05), while they showed the opposite trends in the Inhibitors group (p<0.05) (Figure 3), demonstrating that the overexpression of miR-10a promotes the occurrence of liver injury and increases the expression of the pathway genes.

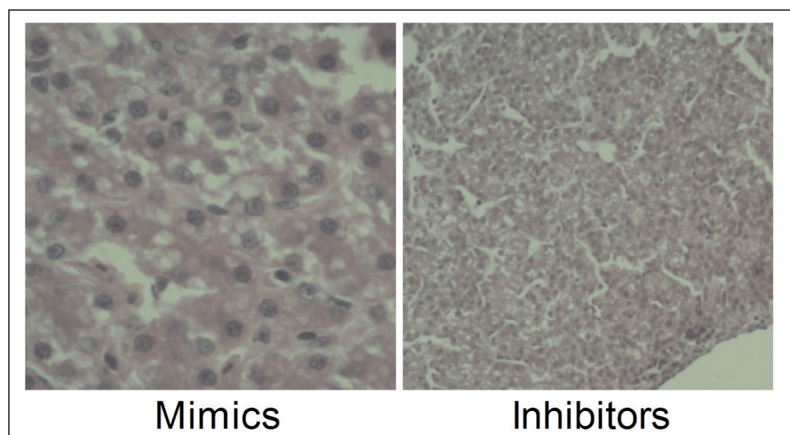

Figure 2. HE staining. The cells are disorderly arranged with inflammatory cell infiltration in the Mimics group (10×), while the cells have basically normal structure and ordered arrangement with mild pathological changes in the Inhibitors group (10×).

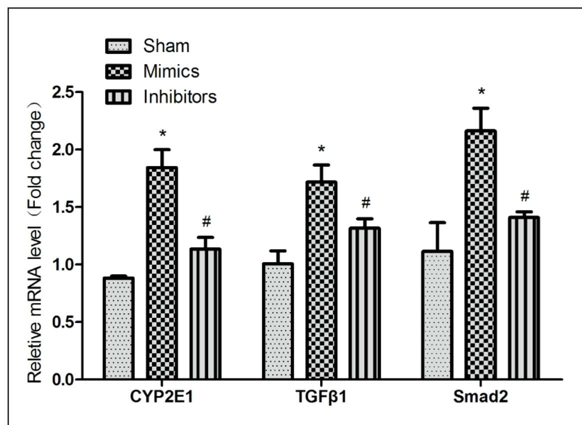

Figure 3. PCR results. The expression levels of CYP2E1, TGF-β1, and Smad2 are remarkably higher in the Mimics group than those in the Sham group ($p<0.05$), while they show the opposite trends in the Inhibitors group ($p<0.05$). *$p<0.05$ *vs.* Sham group, #$p<0.05$ *vs.* Mimics group.

Western Blotting Results

The expression levels of TGF-β1 and Smad2 were significantly increased in the Mimics group ($p<0.05$) and significantly declined in the Inhibitors group (Figure 4), indicating that the overexpression of miR-10a promotes the activation of TGF-β1/Smad2 signaling pathway and the occurrence of sepsis-induced liver injury.

Discussion

Sepsis is a severe inflammatory response syndrome that frequently occurs in clinic[17], and the liver is one of the most important foci of it. In sepsis, a large number of inflammatory mediators are released always accompanied by tissue and organ damage, and sepsis will develop into multiple organ dysfunction syndromes in severe cases, which is also one of the causes of a high mortality rate of sepsis[18,19]. Sepsis-induced liver injury is one of the most common complications of sepsis. In the present study, the effect of miR-10a on sepsis-induced liver injury in rats through the TGF-β1/Smad signaling pathway was explored, and the classical animal model of sepsis was established. The expression level of miR-10a was detected, and it was found that the expression of miR-10a was significantly increased in the Mimics group and remarkably decreased in the Inhibitors group, indicating that the transfection effect is evident and subsequent experiments can be performed. The liver biochemical indexes were also detected, and the results showed that the content of LDH, ALT, and AST was notably increased in the Mimics group, while it was substantially decreased in the Inhibitors group, indicating that liver injury occurs in sepsis rats. Oxidative stress may trigger the signaling pathway through mitochondrial toxicity, ultimately causing cell death. GSH-Px is a major peroxide detoxifying enzyme that catalyzes the intercellular reductive GSH, which is a hydrogen donor for the production of oxidized GSH. Moreover, GSH-Px scavenges H_2O_2 and catalyzes the reduction of peroxide in liver cells. GSH is an important ROS scavenger that can remove ROS by reducing the peroxide in the presence of GSH-Px[20]. In this work, the changes in the levels of the oxidative stress indexes in each group were measured through ELISA. The results manifested that Mimics group had a significantly increased level of oxidative index ROS and notably decreased levels of antioxidant indexes GSH and GSH-Px, while the Inhibitors group had the opposite trends to those in the Mimics group. The clinicopathological changes of sepsis mainly include the increased inflammatory

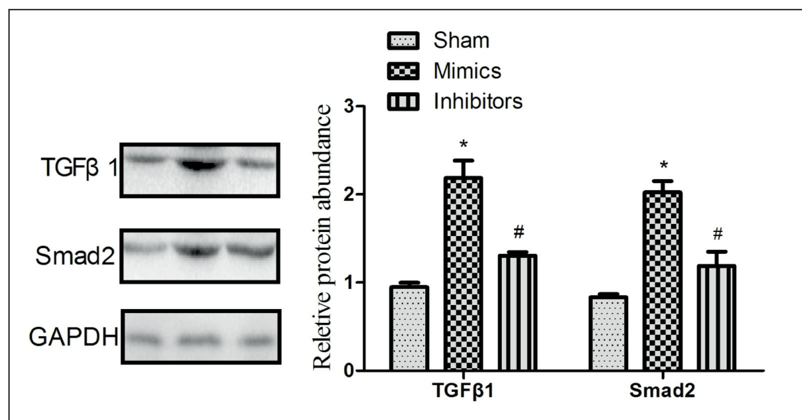

Figure 4. Expression levels of pathway proteins. The expression levels of TGF-β1 and Smad2 are significantly increased in the Mimics group ($p<0.05$) and significantly decline in the Inhibitors group. *$p<0.05$ *vs.* Sham group, #$p<0.05$ *vs.* Mimics group.

cytokines, and interaction between intestinal endotoxemia and inflammation, and the inflammatory factors TNF, IL-1, and IL-6 released due to sepsis can activate the downstream key transcription factor NF-κB[21,22]. In this study, the levels of the inflammatory factors TNF-α, IL-6, and MPO were determined, and it was found that they were significantly higher in the Mimics group than those in the other two groups, while they evidently declined in the Inhibitors group, which suggests the production of a large number of inflammatory factors in liver tissues in sepsis rats, further indicating the development of liver injury. In addition, according to the results of HE staining, the cells were arranged disorderly with inflammatory cell infiltration in the Mimics group, while the cells had basically the normal structure and ordered arrangement with milder pathological changes in the Inhibitors group, similar to previous investigations[23,24].

TGF-β1 has a regulatory effect on proliferation and apoptosis of liver cells, and it binds to the receptor after activation, further activating the phosphorylation of its downstream protein Smad[25]. After phosphorylation, Smad2 and Smad3 will form the transcriptional complex with Smad4, and such complex aggregates in the nucleus to regulate the transcription and playing an important role in liver injury. Some researchers have found that miRNAs can regulate various cellular processes such as normal development and disease onset, and they are also considered to contribute to the development of many diseases. Besides, miRNAs have become important regulators of gene expression in many diseases, whose regulatory network has attracted much attention in recent years. However, the regulatory effect of miR-10a on TGF-β1/Smad and its effect on sepsis-induced liver injury are rarely studied, and its specific mechanism of action remains unclear and needs further research. In this investigation, the gene detection revealed that the expression levels of CYP2E1, TGF-β1, and Smad2 were remarkably higher in the Mimics group than those in the Sham group, while they showed the opposite trends in the Inhibitors group, demonstrating that the overexpression of miR-10a promotes the occurrence of liver injury and increases the expression of the pathway genes. The protein detection manifested that the expression levels of TGF-β1 and Smad2 were significantly increased in the Mimics group and significantly declined in the Inhibitors group, indicating that the overexpression of miR-10a promotes the activation of TGF-β1/Smad2 signaling pathway and the occurrence of sepsis-induced liver injury, similar to the analyses of Lee et al[26] and Su et al[26,27]. To sum up, it was found through the above animal experiments that silencing miR-10a can inhibit the production of inflammatory factors, and suppress the development of oxidative stress, thereby resisting the sepsis-induced liver injury.

Conclusions

In this study, the silence of miR-10a may have a protective effect in sepsis-induced liver injury, inhibit the expressions of TNF, IL-6, and ROS, and suppress the further development of inflammation and oxidative stress in sepsis rats, which affects the development of sepsis-induced liver injury mainly by inhibiting the TGF-β1/Smad2 pathway. The present work can provide a new theoretical basis for the prevention and treatment of sepsis and new ideas for the development and research on new drugs. The cell researches can be further performed in the future.

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Conflict of Interests

The authors declare that they have no conflict of interests.

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