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LncRNA NEAT1 alleviates sepsis-induced myocardial injury by regulating the TLR2/NF-κB signaling pathway

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Abstract. – OBJECTIVE: To investigate the effect of long non-coding ribonucleic acid nuclear paraspeckle assembly transcript 1 (lncRNA NEAT1) on lipopolysaccharide (LPS)-induced myocardial injury in mice and the underlying mechanism. This study aims to provide some references for the prevention and treatment of sepsis-induced myocardial injury.

MATERIALS AND METHODS: According to the random number table, 60 male C57 mice were divided into the Sham group (n=20), LPS group (n=20) and LPS + NEAT1 small interfering ribonucleic acid (siRNA) group (n=20). Sepsis-induced myocardial injury model in mice was established by intraperitoneal injection of LPS (10 mg/kg), and the NEAT1 knockout model was established by tail vein injection of NEAT1 siRNAs. After 12 h, the cardiac function of mice in each group was detected *via* the two-dimensional ultrasound; ejection fraction [EF (%)] and fraction shortening [FS (%)] were recorded. Hematoxylin and eosin (H&E) staining was conducted to evaluate the pathological changes in the heart tissues in each group. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was used to detect the apoptotic levels of myocardial cells and fibroblasts in each group. In addition, the expression level of the oxidative stress marker 4-hydroxynonena (4-HNE) and the positive proportions of cluster of differentiation 45 (CD45) and CD68 in the mouse heart of three groups were detected *via* immunohistochemical staining. Moreover, the messenger RNA (mRNA) expression levels of inflammatory indicators [interleukin-1 (IL-1), IL-6, monocyte chemotactic protein 1 (MCP-1) and tumor necrosis factor-alpha (TNF-α)] in mouse serum of the three groups were examined by enzyme-linked immunosorbent assay (ELISA). Finally, the effects of NEAT1 siRNAs on the Toll-like receptor 2 (TLR2)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway were detected by Western blotting.

RESULTS: ENEAT1 knockdown could significantly improve ischemia/reperfusion (I/R)-induced cardiac insufficiency in rats, and increase EF (%) and FS (%) (p<0.05). Besides, NEAT1 knockdown remarkably inhibited the LPS-induced myocardial injury. Compared with the LPS group, LPS + NEAT 1 siRNA group has more orderly arranged cardiac myofilament, a lower degree of degradation and necrosis, and significantly reduced cell edema. TUNEL staining showed that NEAT1 knockdown markedly reduced LPS-induced apoptosis of cardiac cells (p<0.05). Immunohistochemical results revealed that NEAT1 knockdown could remarkably reverse LPS-induced elevation of the myocardial 4-HNE expression and decrease the oxidative stress in the heart (p<0.05). At the same time, CD45+ and CD68+ cells were reduced after NEAT1 knockdown in myocardial tissues (p<0.05). Reverse Transcription-Polymerase Chain Reaction (RT-PCR) showed that the mRNA levels of inflammatory indicators in LPS + NEAT1 siRNA group were lower than that in the LPS group (p<0.05). According to Western blotting results, NEAT1 siRNAs could significantly downregulate the protein expressions of TLR2 and p-p65.

CONCLUSIONS: NEAT1 knockdown can improve LPS-induced myocardial injury in mice by inhibiting the TLR2/NF-κB signaling pathway. LncRNA NEAT1 is expected to be a potential target for clinical treatment of the sepsis-induced myocardial injury.

Key Words

Sepsis-induced myocardial injury, LncRNA NEAT1, TLR2.

Introduction

Sepsis is a serious inflammatory disease caused by bacterial infection, which is characterized by systemic inflammatory response syndrome (SIRS) and often induced by bleeding, trauma and abdominal surgery[1,2]. Sepsis is considered to be the main cause of death in the intensive care unit due to its susceptibility to SIRS and multiple organ dysfunction (MOD)[3]. Lipopolysaccharide (LPS) is the most important

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cause of sepsis. It enhances the inflammatory response by activating inflammatory cells, which in turn induces the synthesis and release of a large number of pro-inflammatory factors, eventually leading to severe sepsis inflammatory responses[4]. Cardiac dysfunction is a common and serious complication of sepsis and it is closely related to the mortality rate of sepsis patients. Compared with sepsis patients without cardiac dysfunction, the mortality rate of patients accompanied by cardiac dysfunction significantly increases[5,6]. Therefore, it is of great importance to clarify the pathogenesis of sepsis-induced myocardial injury for its future prevention and treatment.

Long non-coding ribonucleic acids (lncRNAs) are long-chain RNA molecules whose transcription length exceeds 200 nucleotides[7]. Although lncRNAs cannot encode corresponding proteins, they can regulate the expressions of corresponding target genes at post-transcriptional or epigenetic level, and ultimately affect disease occurrence and development[8,9]. As a member of the lncRNA family, lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) plays an important role in many diseases, including tumors, cardiovascular diseases and endocrine diseases. For example, in colorectal cancer, lncRNA NEAT1 can regulate the proliferation and apoptosis of cancer cells and ultimately affect the prognosis of patients[10]. Furthermore, down-regulated lncRNA NEAT1 can inhibit the mTOR/S6K1 signaling pathway, thus reducing the non-alcoholic fatty liver injury[11]. However, the role of lncRNA NEAT1 in sepsis-induced myocardial injury has not been reported yet.

In this study, NEAT1 knockdown mouse model was established by tail vein injection of NEAT1 siRNAs, and sepsis-induced myocardial injury mouse model was established using LPS. The role and mechanism of lncRNA NEAT1 in the development of the sepsis-induced myocardial injury were discussed.

Materials and Methods

Grouping and Processing of Experimental Animals

A total of 60 male C57 mice weighing (25.61±1.46) g were divided into 3 groups using a random number table, including the Sham group (*n*=20), LPS group (*n*=20) and LPS + NEAT1 small interfering ribonucleic acid (siRNA) group (*n*=20). There were no significant differences in age and weight among the three groups of mice. Mice in the LPS + NEAT1 siRNA group were injected with 2 mL/kg NEAT1 siRNAs through tail veins. After 3 days, 10 mg/kg LPS was injected intraperitoneally into each group of mice. After 12 h, the cardiac function of each group of mice was detected by ultrasound and the heart was taken and stored at -80°C for later use. This study was approved by the Animal Ethics Committee of Qingdao Municipal Hospital Animal Center.

Echocardiography Detection

To detect the cardiac function of each group of mice, MyLab 30 CV ultrasound system (Esaote, S.P.A, Genoa, Italy) and 10-MHz linear ultrasonic transducers were used to detect the echocardiogram of rats in each group. After shaving of the hair in the anterior thoracic region of mice and anesthesia, mice were placed on a heating plate at 37°C with the left side facing up. Parameters including ejection fraction [EF (%)], fraction shortening [FS (%)] and heart rate were detected.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Detection

(1) TRIzol assay (Invitrogen, Carlsbad, CA, USA) was adopted to extract the total RNA from myocardial tissues of mice in each group, and the concentration and purity of the RNA were detected by an ultraviolet spectrophotometer. The absorbance $(A)_{260}/A_{280}$=1.8-2.0 was considered qualified. (2) The qualified RNAs were synthesized into complementary deoxyribose nucleic acids (cDNAs) through RT and stored in a refrigerator at 80°C. (3) Reverse Transcription-Polymerase Chain Reaction (RT-PCR) system: 2.5 μL of 10 × Buffer, 2 μL of cDNAs, 0.25 μL of forward primers (20 μmol/L), 0.25 μL of reverse primers (20 μmol/L), 0.5 μL of dNTPs (10 mmol/L), 0.5 μL of Taq enzymes (2×10^6 U/L) and 19 μL of ddH_2O. The amplification systems of RT-PCR were the same. The primer sequences of each index in RT-PCR were shown in Table I.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Staining

Myocardial tissues were sectioned, baked in an oven at 60°C for 30 min, dewaxed with xylene (5 min × 3 times) and dehydrated with 100%, 95% and 70% ethanol, respectively, with each dehydration 3 times. Then, the sections were

Table I. Primer sequences of each index in RT-PCR.

Target gene		Primer sequence
GAPDH	Forward	5'-GAGCTCAGCTCGCCTGGAGAAAC-3'
	Reverse	5'-TGCTGATCGTAGCCCTTTAGT-3'
NEAT1	Forward	5'-TGCTGCCTTTTCTGTTCCTT-3'
	Reverse	5'-CTAGCTAGCTTTGGGTAGGGAAGT-3'
Interleukin-1 (IL-1)	Forward	5'-GTCCAACCCGTAAGGT-3'
	Reverse	5'-CGCTGCTGAGTCGATGCTAGCT-3'
IL-6	Forward	5'-ACGTAGCTAGCTAGTCGGTATG-3'
	Reverse	5'-TCGTAGCTTGGCTAGTCGATCG-3'
Interleukin-1 (MCP-1)	Forward	5'-ACTAGTCGATAGCTAGTCGAGCA-3'
	Reverse	5'-CCGATGCTACTAGCTAGCTAGC-3'
Tumor necrosis factor-alpha (TNF-α)	Forward	5'-TTGTGTTAGCTTAGCCCGATCGTA-3'
	Reverse	5'-CCGTCGTAAAAGCTAGTCGATC-3'

incubated with protein kinase K for half an hour. After washing with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA), the TdT and Luciferase-labeled dUTP were added. After reaction for 1 h at 37°C, the specific antibody labeled with horseradish peroxidase (HRP) was added for incubation again in an incubator for 1 h (37°C). Subsequently, the sections were incubated with diaminobenzidine (DAB; Solarbio, Beijing, China) at room temperature for 10 min. After the nucleus was stained with hematoxylin, cells were photographed and counted under an optical microscope.

Western Blotting

After heart tissues of each group of mice were fully grounded in lysis buffer, they were ultrasonically lysed, centrifuged to extract supernatant, and successively subpacked into Eppendorf (EP; Eppendorf, Hamburg, Germany) tubes. The protein concentration was measured *via* the bicinchoninic acid (BCA) assay (Pierce, Waltham, MA, USA) using the ultraviolet spectrophotometry, and the protein volume of all samples was set constant to equal concentration. After subpackaging, the sections were placed in the refrigerator at -80°C. The total protein was loaded on the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the protein in the gel was transferred to cellulose acetate polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland), incubated in the primary antibody at 4°C overnight, and the goat anti-rabbit secondary antibody for 1 h in the dark. Protein bands were scanned and quantified using an Odyssey membrane sweeper, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference.

Immunohistochemical Staining

Myocardial tissue sections were baked in an oven at 60°C for 30 min and then dewaxed with xylene (5 min × 3 times), followed by dehydration with 100%, 95% and 70% ethanol, respectively, with each dehydration 3 times. The endogenous peroxidase activity was blocked by 3% hydrogen peroxide methanol, and then the tissues were sealed with sheep serum for 1 h. Antibodies against 4-hydroxynonena (4-HNE), cluster of differentiation 45 (CD45) and CD68 were diluted at 1:200 PBS and incubated with sections at 4°C overnight, followed by washing with PBS 4 times in a shaker. After the second antibody was added, the color was developed with diaminobenzidine. Five fields of view were randomly selected from each sample, followed by photographing under 200× and 400× optical microscopes.

Statistical Analysis

All the data were analyzed by Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). Measurement data were expressed as mean ± standard deviation, and the comparisons of intergroup data were conducted using the *t*-test. $p<0.05$ showed that the difference was statistically significant.

Results

Expression Level of LncRNA NEAT1 in Mouse Myocardial Tissues

Firstly, the expression level of NEAT1 in mouse heart of three groups was detected by RT-PCR. The results revealed that the expression level of lncRNA NEAT1 in mouse myocardial tissues of the LPS group was significantly upreg-

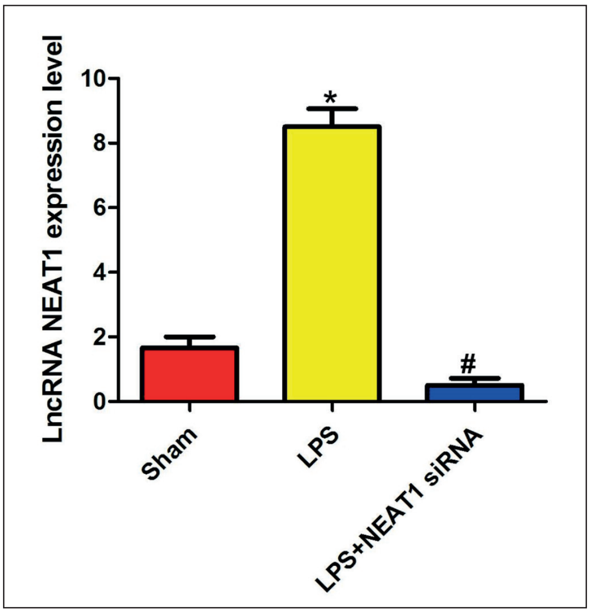


Figure 1. Expression level of lncRNA NEAT1 in myocardial tissues of mice in the three groups. Sham group: control group, LPS group: sepsis-induced myocardial injury group, and LPS + NEAT1 siRNA group: NEAT1 knockdown group. *$p<0.05$ *vs.* Sham group, and $^{\#}p<0.05$ vs. LPS group.

ulated than that of the Sham group ($p<0.05$). Nevertheless, the injection of NEAT1 siRNAs into mice notably decreased the expression of lncRNA NEAT1 in the myocardial tissues ($p<0.05$), proving that the NEAT1 knockdown mouse model was successfully induced (Figure 1).

Cardiac Function Level in Mice

As shown in Figure 2, ECG results manifested that there was no statistical difference in the heart rate among the three groups of mice. Compared with those in the Sham group, the mouse heart was enlarged and the heart wall became thinner in the LPS group. After injection of NEAT1 siRNAs in LPS-induced mice, pathological lesions in the heart structure was markedly improved. Furthermore, the levels of FS (%) and EF (%) in each group of mice were detected. NEAT1 siRNAs evidently reversed the abnormal decreases in FS (%) and EF (%) caused by LPS induction ($p<0.05$). The above results indicated that knockdown of NEAT1 improved cardiac function in septic mice.

Knockdown of NEAT1 Improved LPS-Induced Myocardial Pathological Injury in Mice

To evaluate pathological lesions in the myocardial cell microstructure, hematoxylin and eosin (H&E) staining was performed. According to the results, myocardial cells in LPS group showed

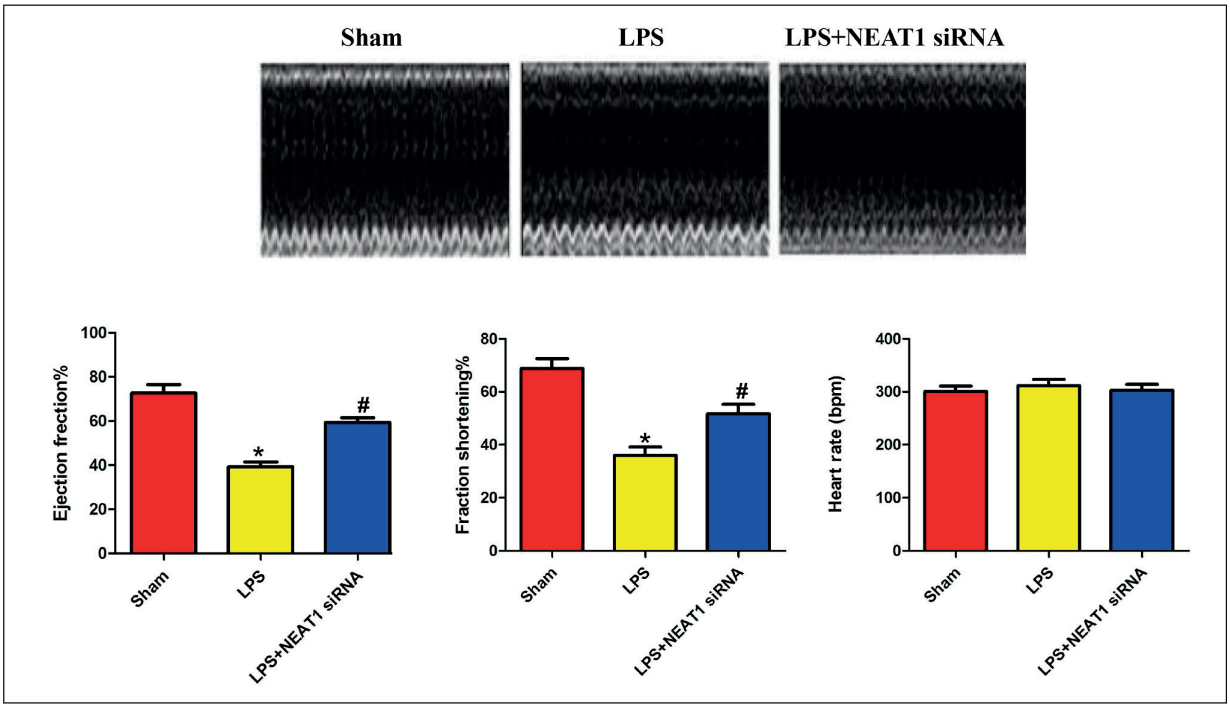


Figure 2. Cardiac function of the three groups of mice. Sham group: control group, LPS group: sepsis-induced myocardial injury group, and LPS + NEAT1 siRNA group: NEAT1 knockdown group. *$p<0.05$ *vs.* Sham group, and $^{\#}p<0.05$ *vs.* LPS group.

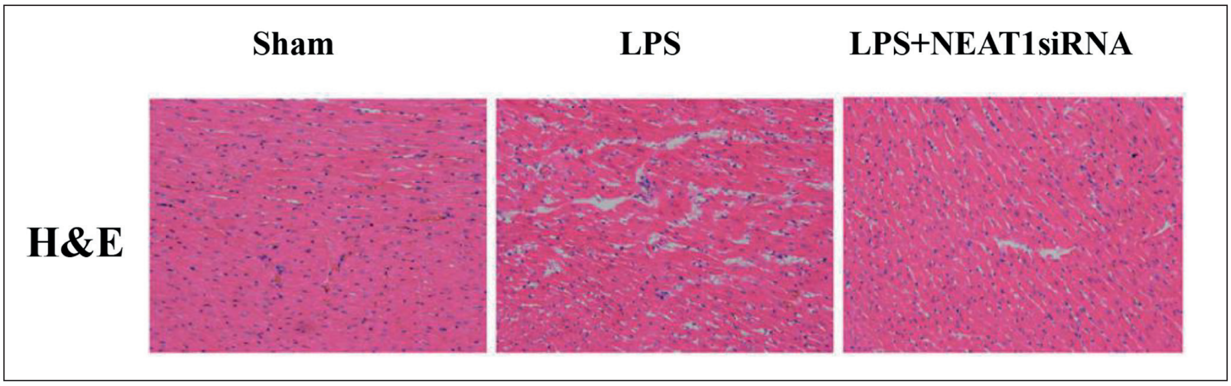


Figure 3. H&E staining results of myocardial tissues of mice in the three groups. Sham group: control group, LPS group: sepsis-induced myocardial injury group, and LPS + NEAT1 siRNA group: NEAT1 knockdown group. $^*p<0.05$ *vs.* Sham group, and $^\#p<0.05$ *vs.* LPS group.

significant edema, disordered myofilament arrangement, degradation and necrosis to varying degrees. However, after knockdown of NEAT1, the edema of myocardial tissues in mice was markedly reduced, and the abnormality of myofilament was significantly improved (Figure 3). The above results suggested that NEAT1 siRNAs could alleviate LPS-induced myocardial injury.

Knockdown of NEAT1 Inhibited LPS-Induced Myocardial Apoptosis in Mice

Through the TUNEL staining, the apoptotic level of myocardial cells in the three groups of mice was detected. The results revealed that after LPS injection for 12 h, the number of apoptotic myocardial cells was evidently elevated ($p<0.05$), with about (33.48±2.43) times as much as that in the Sham group. Nevertheless, NEAT1 knockdown decreased the number of apoptotic myocardial cells to (3.02±1.63) times as much as that in the Sham group ($p<0.05$; Figure 4). The above results demonstrated that NEAT1 knockdown can markedly inhibit the apoptosis of mouse myocardial cells.

Knockdown of NEAT1 Reduced Oxidative Stress in LPS-Induced Mouse Myocardial Tissues

Considering that the oxidative stress is an important pathological change in sepsis-induced myocardial injury, immunohistochemistry was adopted to detect the expression of antioxidant enzyme 4-HNE in mouse heart. According to the results, the expression of 4-HNE in mouse heart of the LPS group was markedly higher than that in the Sham group ($p<0.05$), which was significantly inhibited after injection of NEAT1 siRNAs ($p<0.05$; Figure 5). The above results indicate that NEAT1 siRNAs can alleviate the oxidative stress level in the myocardial tissues of mice with sepsis-induced myocardial injury.

Knockdown of NEAT1 Alleviated Inflammation in LPS-Induced Mice

The expression levels of inflammatory cell markers, CD45 and CD68 in the myocardial tissues of mice in each group were further detected *via* Western blotting. It was found that after NEAT1 knockdown, the number of inflammatory cells in myocardial tissues of septic mice decreased significantly. Therefore, NEAT1 inhibition could be considered to reduce myocardial inflammation in septic mice (Figure 6).

Furthermore, the mRNA levels of inflammatory cytokines in mouse myocardial tissues were determined using RT-PCR. The results showed that the levels of inflammatory markers (IL-1, IL-6, MCP-1 and TNF-α) in mouse myocardial tissues of the LPS group were evidently higher than those in the Sham group ($p<0.05$), which were significantly lower in NEAT1 siRNA group than those in the LPS group ($p<0.05$; Figure 7).

Expression of the Toll-Like Receptor (TLR)/Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NF-κB) Signaling Pathway in Mouse Myocardial Tissues

To further explore the molecular mechanism of NEAT1 in the development of sepsis-induced myocardial injury, the activation of the TLR2/NF-κB signaling pathway was examined. As shown in Figure 8, the expression levels of TLR2 and p-p65 in mouse myocardial tissues of the LPS group were up-regulated ($p<0.05$), while the expression levels of these two proteins were re-

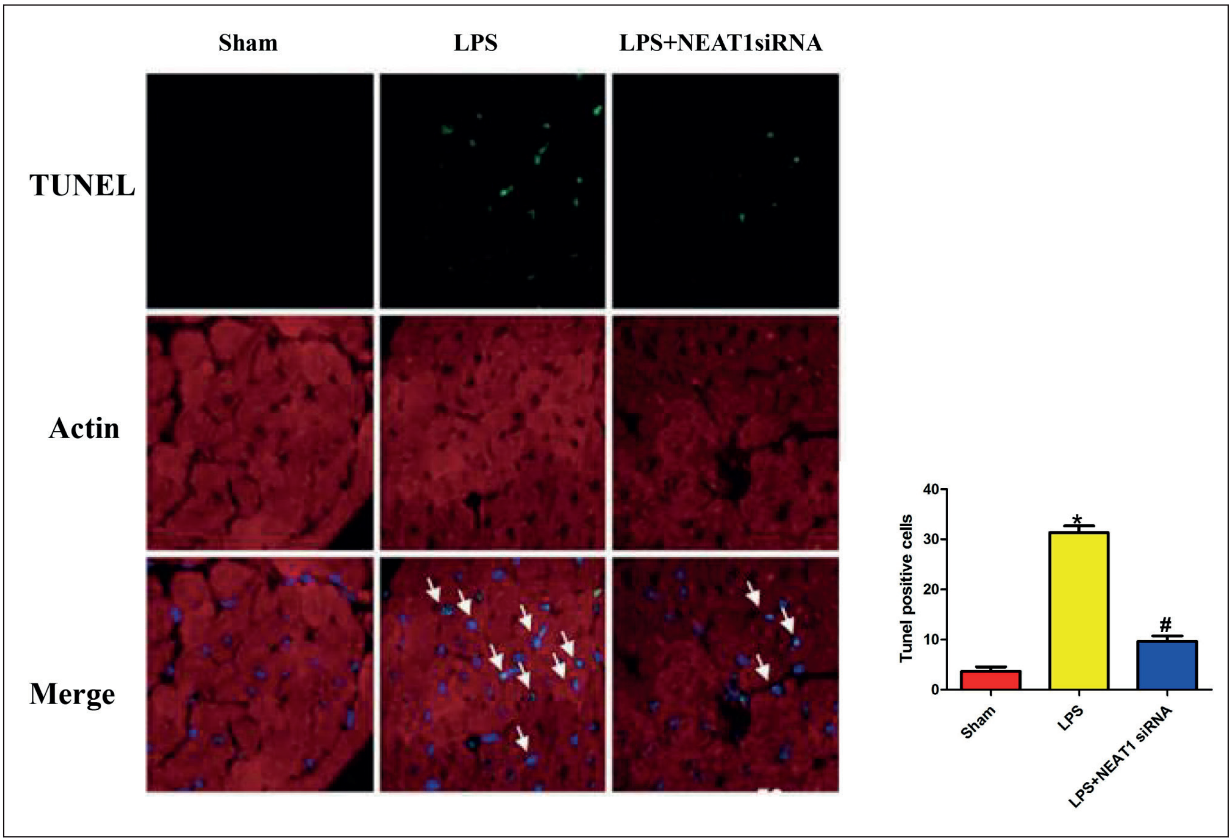


Figure 4. TUNEL staining results of myocardial tissues of mice in the three groups. Sham group: control group, LPS group: sepsis-induced myocardial injury group, and LPS + NEAT1 siRNA group: NEAT1 knockdown group. *$p<0.05$ *vs.* Sham group, and $^{\#}p<0.05$ *vs.* LPS group.

markably inhibited after the injection of NEAT1 siRNAs ($p<0.005$). Therefore, it was speculated that lncRNA NEAT1 might cause sepsis-induced myocardial injury by activating the TLR2/NF-κB signaling pathway.

Discussion

Sepsis is a highly fatal disease, and effective treatment at present is still lacking[12]. The heart is one of the organs most vulnerable to sepsis[13].

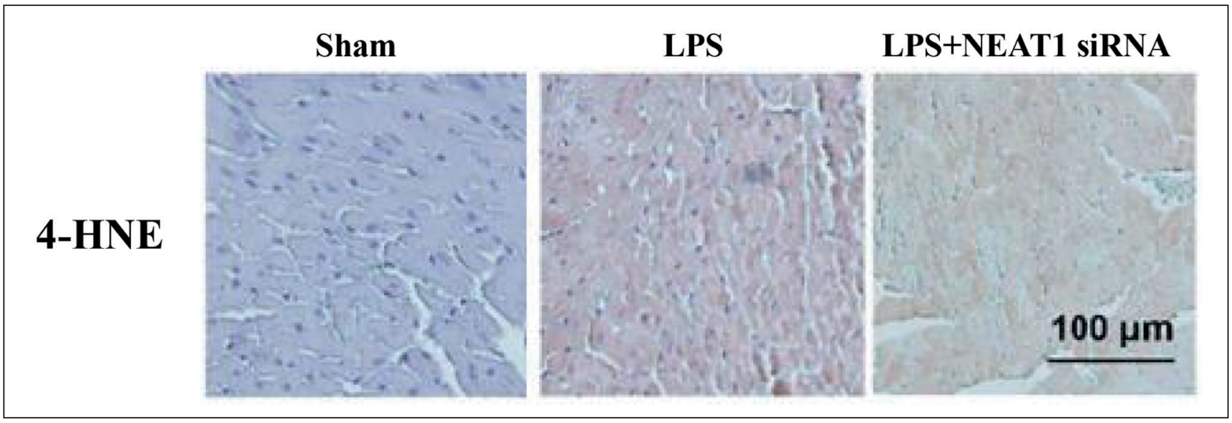
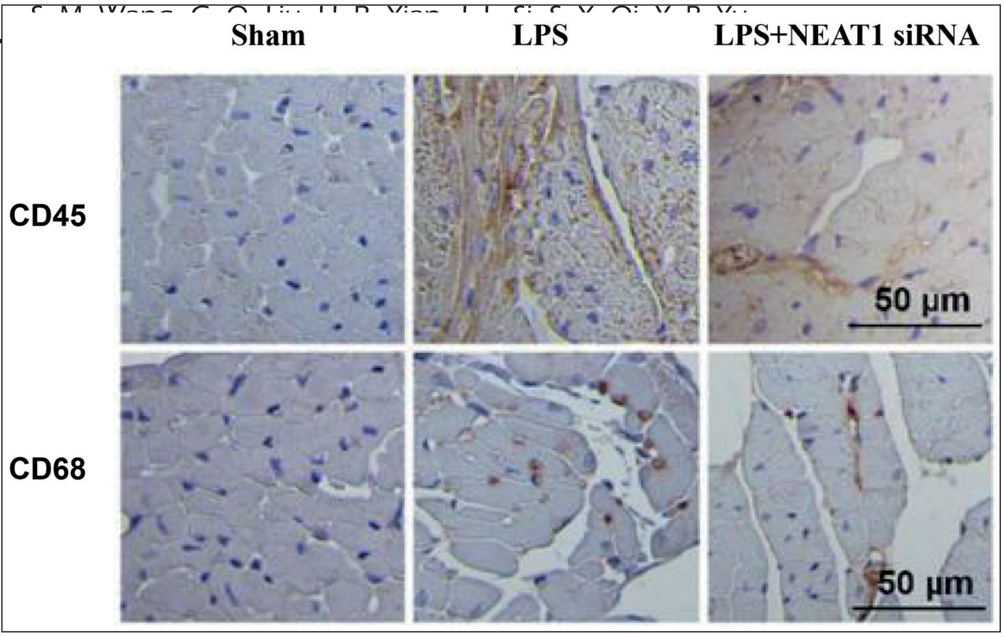


Figure 5. Immunohistochemical staining results of 4-HNE in myocardial tissues of mice in the three groups. Sham group: control group, LPS group: sepsis-induced myocardial injury group, and LPS + NEAT1 siRNA group: NEAT1 knockdown group. *$p<0.05$ *vs.* Sham group, and $^{\#}p<0.05$ *vs.* LPS group.

Figure 6. Western blotting results of CD45 and CD68 in the myocardial tissues of mice in the three groups. Sham group: control group, LPS group: sepsis-induced myocardial injury group, and LPS + NEAT1 siRNA group: NEAT1 knockdown group. $*p<0.05$ *vs.* Sham group, and $^{\#}p<0.05$ *vs.* LPS group.



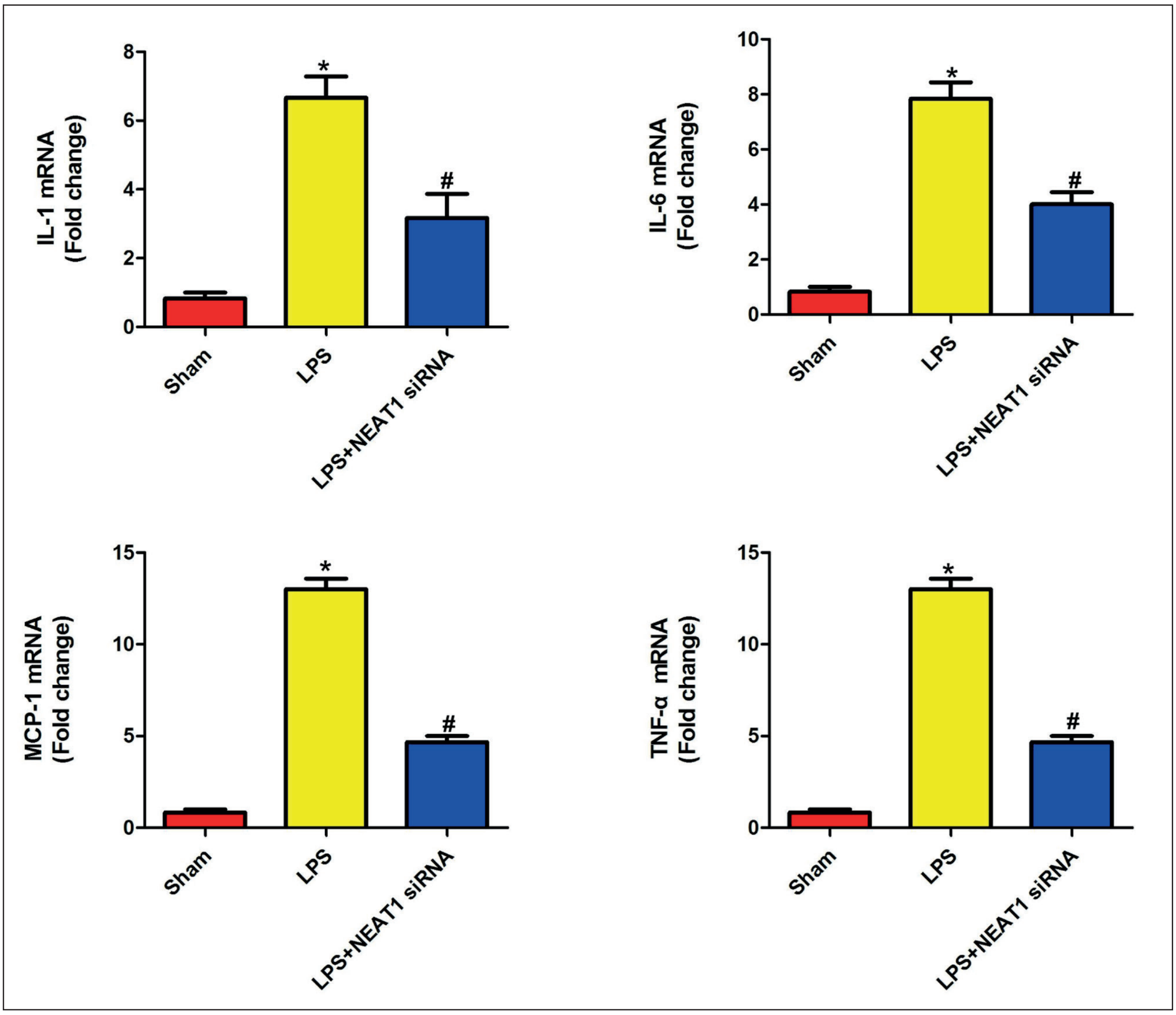


Figure 7. MRNA expressions of inflammatory factors in myocardial tissues of mice in the three groups. Sham group: control group, LPS group: sepsis-induced myocardial injury group, and LPS + NEAT1 siRNA group: NEAT1 knockdown group. $*p<0.05$ *vs.* Sham group, and $^{\#}p<0.05$ *vs.* LPS group.

Previous work[14] have shown that about 50% of patients with severe sepsis suffer from cardiac dysfunction, and the recovery of cardiac function is considered to be a key factor for the survival of patients. Current studies[15-17] have revealed that the pathogenesis of sepsis-induced myocardial injury mainly includes: 1) microcirculation and microvascular abnormalities, 2) autonomic nervous disorder, 3) metabolic abnormality, 4) mitochondrial dysfunction, 5) cell death, 6) activation of inflammatory cells and inflammatory factors, and 7) cardiac systolic and diastolic dysfunction.

Researches have shown that endotoxin can mediate the development of sepsis-induced myocardial injury in human and animal models during the activation of inflammatory signaling pathways. The induction of endotoxin to sepsis-induced myocardial injury mainly depends on the TLR family and CD14[18]. In sepsis, IL-1 and TNF-α can directly bind to TLRs, thus initiating downstream cascade reactions[19]. Among the various members of the TLR family, TLR2 binds to the cell wall components of Gram-positive bacteria, thus inducing an inflammatory response. On the one hand, inactivated *Staphylococcus aureus* can cause massive production of TNF-α, IL-1 and NO in the heart, leading to cardiac dysfunction and even heart failure[20,21]. On the other hand, *Staphylococcus aureus* can induce TLR2 upregulation, and a relevant study[22] has shown that TLR2$^{-/-}$ mice are resistant to myocardial dysfunction and cytokine production caused by *Staphylococcus aureus*. Therefore, in sepsis-induced myocardial injury, targeting TLR2 can effectively inhibit LPS-induced myocardial cell death. In addition, oxidative stress and cardiac cell apoptosis are important pathological changes in sepsis-induced myocardial injury. MiR-30A-3P overexpression can inhibit myocardial cell apoptosis by activating the PTEN/PI3K/Akt signaling pathway[23]. It has also been revealed that the activation of PPAR-γ can inhibit cardiac dysfunction and myocardial cell necrosis and apoptosis caused by sepsis-induced myocardial injury[24].

In this work, NEAT1 knockdown could evidently improve the cardiac function of LPS mice, manifesting as increased EF and FS of mice. In addition, H&E staining results also demonstrated that NEAT1 knockdown significantly alleviated LPS-induced heart tissue injury in mice. Furthermore, the apoptotic level in mouse heart was evaluated by TUNEL staining. The total number of apoptotic cells in mouse heart markedly decreased after NEAT1 knockdown in LPS mice. Besides,

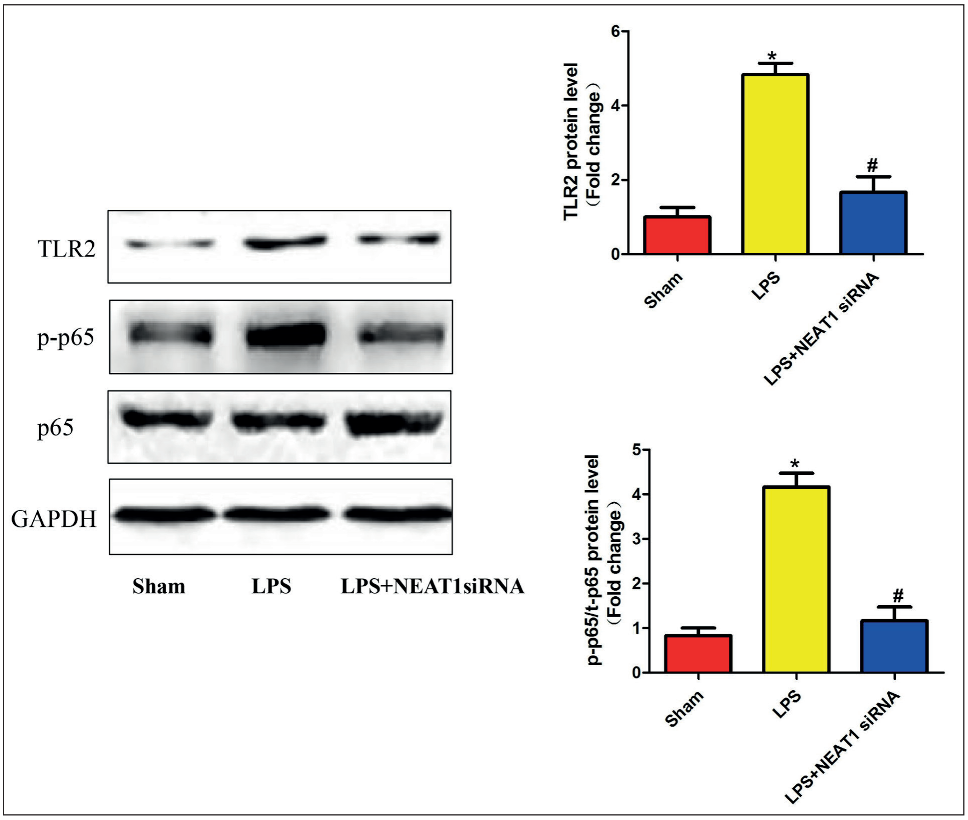


Figure 8. Expressions of TLR2 and p65 in myocardial tissues of mice in the three groups. Sham group: control group, LPS group: sepsis-induced myocardial injury group, and LPS + NEAT1 siRNA group: NEAT1 knockdown group. *$p<0.05$ *vs.* Sham group, and $^{\#}p<0.05$ *vs.* LPS group.

NEAT1 siRNAs could also oxidize the level of the stress-related indicator (4-HNE). At the same time, the levels of inflammatory cells and pro-inflammatory factors in myocardial tissues of mice were further detected by immunohistochemistry and RT-PCR. The results validated that NEAT1 siRNAs could remarkably reverse the activation of myocardial inflammation induced by LPS. Finally, the mechanism of NEAT1 siRNAs affecting sepsis-induced myocardial injury was investigated. The results showed that the expression of TLR2 and the abnormal increase in p65 phosphorylation in NEAT1 siRNA group were reversed. However, there are still some limitations in this study: 1) We did not design *in vitro* experiments to verify. 2) The serum level of lncRNA NEAT1 in the Sham group and LPS group was not detected. 3) No direct target for lncRNA NEAT1 was found.

Conclusions

We revealed, for the first time, the role of lncRNA NEAT1 in sepsis-induced myocardial injury triggered by LPS in mice. LncRNA NEAT1 is expected to become an important target for the prevention and treatment of sepsis-induced myocardial injury in the future.

Conflict of Interests

The authors declared no conflict of interest.

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