

Original Article

miR-222-3p reduces myocardial injury in sepsis by regulating NFATc3

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Abstract: Objectives: To investigate the function and basic molecular pathways of miR-222-3p in sepsis-induced myocardial injury (SIMI), focusing on its post-transcriptional regulation of the NFATc3 signalling network, and to explore its use as a therapeutic target for SIMI. Methods: Septicemia was induced in rodents by cecal ligation and puncture (CLP). The animals were divided into the following seven groups: Sham, Sepsis, Sepsis + miR-222-3p, Sepsis + anti-222-3p, Sepsis + ad-GFP, Sepsis + miR-222-3p + NFAT inhibitor, and Sepsis + anti-222-3p + NFAT inhibitor. A NFAT inhibitor (100 µg/d) was administered intraperitoneally for three days before CLP surgery, and 24 hours prior to CLP, miR-222-3p, anti-222-3p, or ad-GFP (100 µL each) were infused intravenously through the tail vein. The levels of miR-222-3p in myocardial tissue were determined by qRT-PCR, and NFATc3 protein level 24 hours after surgery was determined by western blot. H&E staining of the myocardial cells and TUNEL assays were used to detect apoptosis in myocytes. Echocardiogram was performed to assess the heart, and the plasma levels of CK-MB, cTnI, IL-6, IL-10 and TNF-α were measured by ELISA. Results: Myocardial miR-222-3p expression was significantly upregulated in septic mice compared to sham-operated controls. NFATc3 levels decreased in the miR-222-3p injection group and increased in the anti-miR-222-3p group. miR-222-3p reduced myocardial cell apoptosis, improved left ventricular ejection fraction, lowered plasma concentrations of CK-MB, cTnI, IL-6 and TNF-α, and promoted IL-10. Suppressing miR-222-3p produced the inverse outcome. The activity of NFAT was also reduced somewhat. Conclusions: MiR-222-3p inhibits NFATc3 to reduce myocardial damage in septic mice, suggesting that miR-222-3p may represent a therapeutic target for sepsis-induced heart failure.

Keywords: miR-222-3p, NFATc3, sepsis, myocardial injury, cardiomyocyte apoptosis, inflammation

Introduction

Sepsis, a life-threatening organ dysfunction caused by a dysregulated host response to infection, remains a leading cause of mortality in intensive care units worldwide [1]. Among the various organ dysfunctions associated with sepsis, cardiac impairment, known as septic cardiomyopathy, is a critical complication that significantly contributes to the high mortality rate [2]. Patients with septic cardiomyopathy exhibit reduced left ventricular ejection fraction, ventricular dilatation, and impaired contractility, which are associated with poor prognosis [3-5]. Despite extensive research, the molecular mechanisms underlying sepsis-induced myocardial injury remain incompletely

understood, and effective therapeutic strategies are limited.

MicroRNAs (miRNAs) are short, non-coding RNA molecules that regulate gene expression through post-transcriptional mechanisms by binding to the 3'-untranslated regions (3'-UTR) of target messenger RNAs (mRNAs) and promoting mRNA degradation or inhibiting protein synthesis [6, 7]. Based on these observations, a variety of microRNAs (miRNAs) have been implicated in the development of various cardiac diseases, such as myocardial infarction, heart failure, and septic cardiomyopathy [8-11]. Among these, miR-222-3p has been linked to various cell functions, such as proliferation and apoptosis, as well as inflammatory responses

[12, 13]. Several microRNAs that are related to the development of heart disease and damage to the heart after myocardial ischemia-reperfusion have been identified in previous studies [14-16]. The functions and specific modes of action of miR-222-3p in the injury of the heart caused by sepsis are not yet known.

The nuclear factor of activated T-cell (NFAT) family comprises four calcium-dependent isoforms: NFATc1, NFATc2, NFATc3, and NFATc4. In the heart, NFAT signaling is canonically activated by calcineurin-mediated dephosphorylation in response to elevated intracellular calcium, leading to nuclear translocation and transcriptional reprogramming [17]. Among these isoforms, NFATc3 is predominantly expressed in cardiomyocytes and serves as a critical pathologic mediator driving cardiac hypertrophy, maladaptive remodeling, apoptosis, and pro-inflammatory cytokine production under stress conditions [18, 19]. Bioinformatic analysis has predicted NFATc3 as a potential target for miR-222-3p; however, no experimental evidence has yet been provided to support such a regulatory relationship in septic cardiomyopathy. This study provides the first direct evidence for this particular targeting connection. However, this regulatory relationship has not been experimentally validated in sepsis-induced cardiac injury.

To determine whether miR-222-3p promotes cardiac injury in sepsis and whether this specific miRNA is indeed targeting NFATc3, a rodent sepsis model was constructed utilizing cecal ligation and puncture (CLP). The effects of exogenous miR-222-3p mimic delivery and silencing concerning cardiac inflammation, damage, and systemic indices were investigated. To investigate whether the effects of miR-222-3p are mediated by NFATc3, an NFAT inhibitor was administered in conjunction with miR-222-3p modulation. According to these findings, novel perspectives regarding the cellular pathways of sepsis-related cardiac damage were suggested, and thus miR-222-3p might serve as an effective therapeutic candidate for this condition.

Materials and methods

Animals and experimental design

Four-week-old male Kunming mice (20-25 g) were sourced from the Animal Center of the

Third Hospital of Hebei Medical University. All the experimental protocols involving these animals were authorized by the Animal Care and Use Committee of the Third Hospital of Hebei Medical University and performed following the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. During the initial week, the mice were maintained in a standard environment (12-hour light-dark cycle, $22 \pm 2^\circ\text{C}$, $50 \pm 10\%$ relative humidity) and had unrestricted access to food and water.

The following groups of mice were used (n=5 per group): 1) Sham group: underwent laparotomy but not CLP; 2) Sepsis group: performed CLP procedure; 3) Sepsis + ago-222-3p group: received a tail vein infusion of ago-222-3p (100 μL) 24 hours before CLP; 4) Sepsis + antago-222-3p group: received a tail vein infusion of antago-222-3p (100 μL) 24 hours before CLP; 5) Sepsis + ad-GFP group: received a tail vein infusion of ad-GFP (100 μL) 24 hours before CLP; 6) Sepsis + ago-222-3p + NFAT inhibitor group: received both treatments; 7) Sepsis + antago-222-3p + NFAT inhibitor group: received both treatments. NFAT inhibitors [TargetMol] (100 $\mu\text{g/d}$) were given intraperitoneally over three days before the CLP experiment. Twenty-four hours before the operation, tail-vein infusions of ago-222-3p, antago-222-3p, or ad-GFP [Genecfcs] (100 μL per dose) were administered to the mice. At the end of the experiment, all mice were humanely euthanized through intraperitoneal injection of sodium pentobarbital (150 mg/kg), a dosage exceeding three times the standard sedative dose. Death was confirmed by the absence of observable respiration or cardiac activity.

Sepsis model construction

Sepsis was induced by CLP surgery as described previously. Pentobarbital sodium (50 mg/kg) was used as the anaesthetic agent and given by intraperitoneal injection. An abdominal median incision was made and then the cecum was exposed. Approximately 1 cm above the cecum's apex, the region was ligated and subsequently accessed via dual 21-gauge punctures. A small amount of feces was extruded to ensure patency. The cecum was then returned to the abdominal cavity, and the incision was closed in layers. Sham-control animals underwent the same surgery but did not have the ligation and puncture steps. All of the participants received a single 1 ml subcutaneous

injection of saline for post-operative fluid resuscitation.

Sample collection

At 24 hours post-operation, the mice were anaesthetized, and blood samples were collected from inferior vena cava puncture. The serum was isolated by spinning at 3000 rpm for 15 minutes at 4°C, followed by storage at -80°C prior to conducting every subsequent experiment. After blood collection, collection, the mice were euthanized by cervical dislocation, and the hearts were quickly dissected. The four cardiac ventricles were isolated, and four separate fractions were obtained for different purposes: one for fixation in 4% paraformaldehyde to obtain histologic sections, one for fast freezing in liquid nitrogen for further protein extraction, and another for RNA storage to prepare RNA samples.

Quantitative real-time PCR (qRT-PCR)

To obtain total cardiac cell RNA, TRIzol reagent (Invitrogen) was used according to the above procedures. RNA quantity and quality were evaluated through spectrophotometric techniques. Then cDNA was synthesized utilizing a reverse transcription kit [GeneCopoeia] for subsequent quantitative real-time PCR analysis with SYBR Green PCR Master Mix [GeneCopoeia]. The specific primers are listed below: miR-222-3p forward: 5'-AGCTACATCTGGCTACTGGGT-3', reverse: 5'-GTGCAGGGTCCGAGGT-3'; U6 forward: 5'-CTCGCTTCGGCAGCA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'. The relative expression levels of miR-222-3p were calculated using the $2^{-(\Delta\Delta Ct)}$ formula, with U6 acting as the internal control.

Western blot analysis

Tissue samples were collected, and RIPA lysis buffer with protease inhibitors were added to lyse cells for protein extraction. Protein concentration was determined using a BCA assay. Protein samples (30 µg) were separated on 10% SDS-PAGE gel and then transferred onto PVDF membranes. The membranes were blocked with 5% skim milk for 60 minutes at room temperature, and then incubated overnight at 4°C with primary antibodies against NFATc3 [Proteintech] (1:1000) and GAPDH [Proteintech] (1:5000). The blots were washed, and then, using HRP-conjugated secondary

antibodies [Proteintech] (1:5000), incubated for 60 minutes at ambient temperature to produce the signal. A chemiluminescence substrate was added, and we used ImageJ software to determine the relative amounts of protein expression. GAPDH served as a reference housekeeping gene.

Histology analysis

Cardiac tissues were preserved in 4% paraformaldehyde prior to embedding in paraffin for 5 µm sections. Histologic characteristics were assessed by doing Hematoxylin and Eosin (H&E) staining on these slides. A pathologist blinded to the group allocation evaluated the architectural changes with optical microscopy. Myocardial injury was scored based on the extent of cardiomyocyte necrosis, edema, and leukocyte infiltration.

TUNEL assay

Apoptosis was detected using a TUNEL kit. Paraffin-embedded cardiac tissues were first deparaffinized and then rehydrated; subsequently, a 15-minute incubation with proteinase K at room temperature was carried out. Then, the above slides were placed in TUNEL reagent for 60 minutes in the dark at 37°C. Samples were washed and then stained with DAPI for nuclear visualization. Necrotic cells appear in green fluorescence, and all nuclei are shown under blue fluorescence by a fluorescence microscope. The ratio of apoptotic cells represents the count of TUNEL-stained cells relative to the overall cell population.

Echocardiography

A Vevo 2100 Imaging System for transthoracic echocardiography was employed to assess the heart. We used 1.5% isoflurane for sedation of the animals, and then removed their chest hair. A 2D and M-mode image were acquired in the parasternal short-axis window next to the papillary muscle. LVEF and FS indices of the left ventricle were extracted from M-mode traces. A skilled sonographer who did not know the allocation of the experimental group conducted all the examinations.

Enzyme-linked immunosorbent assay (ELISA)

Using the producer's protocol and proprietary ELISA reagents [Jiyinmei], we measured blood

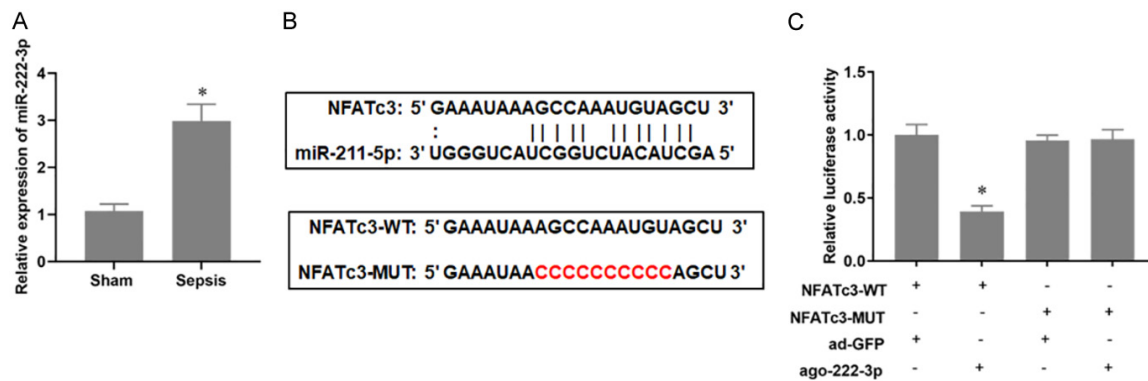


Figure 1. miR-222-3p expression is upregulated in the myocardial tissue of septic mice. A. The relative expression level of miR-222-3p was determined by qRT-PCR. B. Binding sites between miR-222-3p and NFATc3, and the wild-type/mutant sequences of NFATc3. C. Relative luciferase activity. Data are presented as mean $\pm$ SD. N=5. *P < 0.001 vs. Sham group.

concentrations of cardiac damage indicators (CK-MB and cTnI) as well as pro-inflammatory molecules (IL-6, IL-10, and TNF- α). The plasma specimens and controls were transferred to the pre-coated wells, followed by a 2-hour room-temperature binding period. After the above washes, we added biotin-labelled detection reagents and incubated for 1 hour. There was one more rinse, followed by the streptavidin-HRP solution, and incubation for 30 minutes. Finally, TMB substrate was added and a stop reagent used to stop the reaction. We used the generated standard curve to calculate the concentration of the above-mentioned markers.

Luciferase reporter gene assay

Bioinformatic analysis of the StarBase website (<http://starbase.sysu.edu.cn/>) indicated that NFATc3 was a potential target of miR-222-3p. Dual-luciferase reporter systems containing wild-type and altered NFATc3 fragments were constructed to confirm this association. Then, these constructs were co-transfected with miR-222-3p mimics or ad-GFP into laboratory cells in 24-well microplates. We measured the relative luminescence signals of the cells after 48 hours in the dark incubation period.

Statistical analysis

All results are shown as the mean $\pm$ standard deviation (SD). SPSS 29.0 was used for statistical analysis. One-way ANOVA was used, and then Tukey's HSD *post-hoc* tests were performed on all groups. A P value < 0.05 was considered statistically significant.

Results

miR-222-3p was elevated in the myocardium of septic mice

To determine whether miR-222-3p promotes myocardial injury in sepsis at the level of cardiac tissue, qRT-PCR was used to measure its expression. As shown in the above data, the level of miR-222-3p was significantly higher in the sepsis group than in the sham group (**Figure 1A**). Therefore, it was concluded that miR-222-3p is involved in the pathogenesis of cardiac dysfunction in sepsis. To identify potential target genes of miR-222-3p, StarBase 3.0 (<http://starbase.sysu.edu.cn/>) was used; miR-222-3p putatively binds to the 3'UTR of NFATc3 (**Figure 1B**, upper panel). Next, a mutant sequence of NFATc3 was created (**Figure 1B**, lower part), and then a luciferase reporter assay was performed. miR-222-3p significantly reduced the luciferase activity of the wild-type NFATc3 vector; however, no such decrease was observed for the mutant version (**Figure 1C**).

miR-222-3p regulates NFATc3 expression in the myocardial tissue of septic mice

Next, we investigated the association between miR-222-3p, NFATc3 and myocardial injury in sepsis. Western blot analysis revealed that NFATc3 protein levels were lower in the sepsis group than in the sham group. miR-222-3p mimic significantly suppressed NFATc3 levels in the sepsis group compared to the untreated sepsis group, while silencing miR-222-3p (antago-222-3p group) increased NFATc3 expres-

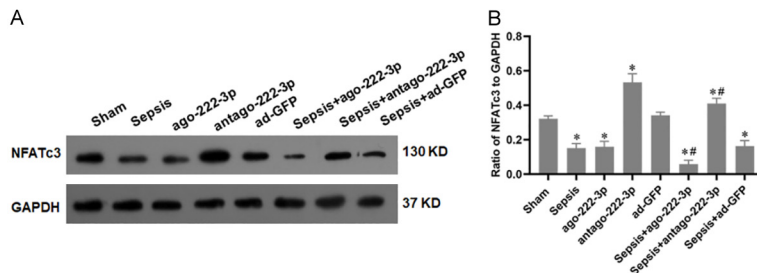


Figure 2. miR-222-3p negatively regulates NFATc3 protein expression. A. Representative western blot bands for NFATc3 and GAPDH. B. Densitometric analysis of NFATc3 protein levels normalized to GAPDH. Data are presented as mean $\pm$ SD. N=5. *P < 0.001 vs. Sham group. #P < 0.001 vs. Sepsis group.

sion, The amount of NFATc3 protein in the sepsis+ago-222-3p group (miR-222-3p mimic before sepsis) remained relatively low compared to the sepsis and antago-222-3p groups, whereas NFATc3 levels in the sepsis+antago-222-3p group (miR-222-3p inhibition before sepsis) were higher than in the sepsis group (**Figure 2A** and **2B**). These observations suggest that miR-222-3p negatively regulates NFATc3 NFATc3 protein expression in the myocardium of septic mice.

miR-222-3p reduces myocardial histopathologic injury in septic mice

H&E staining of heart tissue showed the following histologic alterations. The sham group exhibited normal cardiac histology without significant pathologic alterations. On the other hand, the sepsis group also showed serious damage to the heart, such as myofibrillar disorganization, cytoplasmic expansion, apoptosis, and infiltration by inflammatory cells. Treatment with ago-222-3p effectively reduced the histologic changes, and inhibition of miR-222-3p (antago-222-3p group) exacerbated them. However, the protective effect of exogenous miR-222-3p mimic was counteracted by the joint treatment of ago-222-3p and an NFAT inhibitor (ago-222-3p + NFAT inhibitor group). Similarly, the harmful effects of miR-222-3p knockdown were reduced by NFAT inhibition (antago-222-3p + NFAT inhibitor group) (**Figure 3A, 3B**).

miR-222-3p inhibits cardiomyocyte apoptosis in septic mice

TUNEL assays were performed on the seven experimental groups to detect apoptosis in cardiomyocytes. According to quantitative evalua-

tion, the percentage of TUNEL-positive cardiomyocytes was significantly increased in the sepsis cohort compared to the sham reference group, and this increase was not affected by ad-GFP administration. Upregulation of ago-222-3p effectively inhibited sepsis-induced necrosis, whereas inhibition of 222-3p exacerbated cardiomyocyte apoptosis. Critically, treatment with an NFATc3 inhibitor effectively

rescued the aggravated apoptosis induced by antago-222-3p, restoring apoptotic levels comparable to the Sepsis group. Furthermore, co-treatment with ago-222-3p and the NFATc3 inhibitor did not produce additional anti-apoptotic effects compared with ago-222-3p treatment alone. These findings confirm that NFATc3 serves as the primary downstream mediator of miR-222-3p in regulating cardiomyocyte apoptosis during sepsis. Representative TUNEL images have been rigorously verified to correspond accurately with the quantitative data presented in **Figure 4A** and **4B**.

miR-222-3p promotes cardiac function in septic mice

Evaluation of Myocardial Function by Echocardiography: Based on the aforementioned observations, the LVEF levels within the sepsis group were significantly lower than those in the sham control, suggesting a decline in cardiac function. Administration of ago-222-3p markedly improved the left ventricular ejection fraction in the sepsis group compared to the baseline, while silencing miR-222-3p further reduced LVEF. However, such aforementioned results were inhibited by NFAT blockade (**Figure 5A-C**).

miRNA-222-3p modulates serum levels of cardiac injury markers and inflammatory cytokines in septic mice

ELISA was used to measure the levels of myocardial injury (CK-MB and cTnI) and pro-inflammatory factors (IL-6, IL-10 and TNF- α) in the serum of septic mice. The above assays provided quantitative data on the blood concentration of cardiac and immunologic factors. As depicted in **Figure 6**, the results corresponded

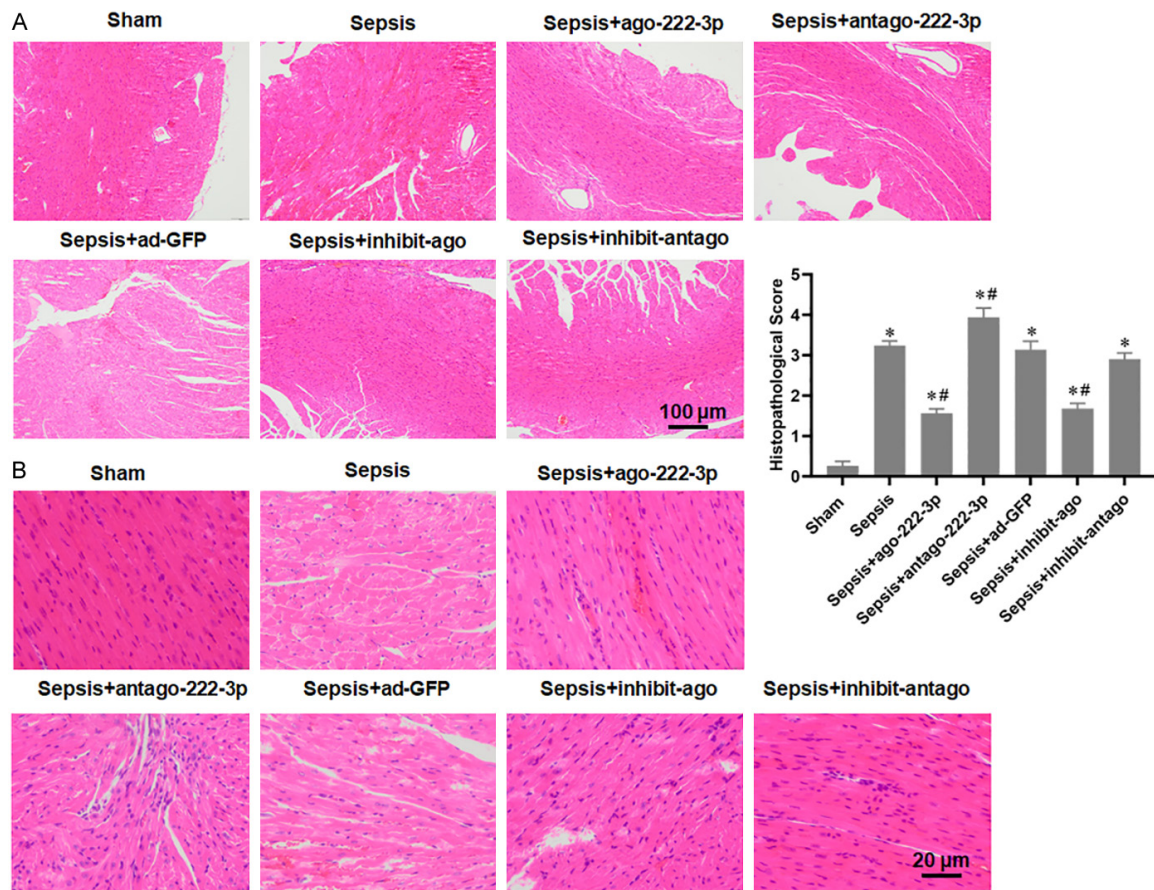


Figure 3. MiR-222-3p alleviates myocardial histopathologic damage. A. Representative photomicrographs of H&E-stained myocardial sections. B. Quantitative histopathologic scoring based on inflammatory infiltration, myofiber disarray, interstitial edema, and cardiomyocyte necrosis (0-4 scale). Scale bar = 100 μ m and 20 μ m. N=5. *P < 0.001 vs. Sham group. #P < 0.001 vs. Sepsis group.

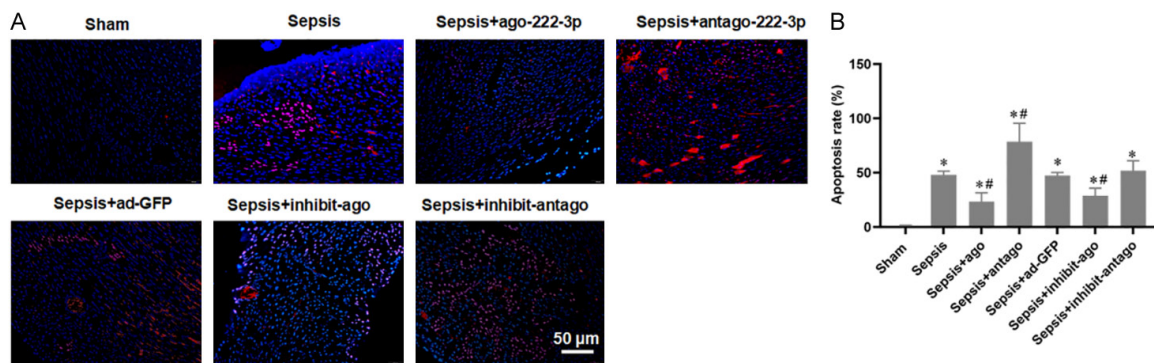


Figure 4. MiR-222-3p inhibits cardiomyocyte apoptosis in septic mice. A. Representative fluorescence micrographs of TUNEL-stained myocardial sections from all seven experimental groups (red: apoptotic cells; blue: DAPI-stained nuclei). B. Quantitative analysis of cardiomyocyte apoptosis. Data are presented as mean $\pm$ SD. N=5. *P < 0.001 vs. Sham group. #P < 0.001 vs. Sepsis group. Scale bar = 50 μ m.

uniformly. Among the sepsis group, plasma concentrations of CK-MB, cTnI, IL-6, and TNF- α were all higher than in the sham-surgery control

group, and IL-10 was lower. Administration of ago-miR-222-3p led to a substantial reduction in CK-MB levels (**Figure 6A**), and a decrease in

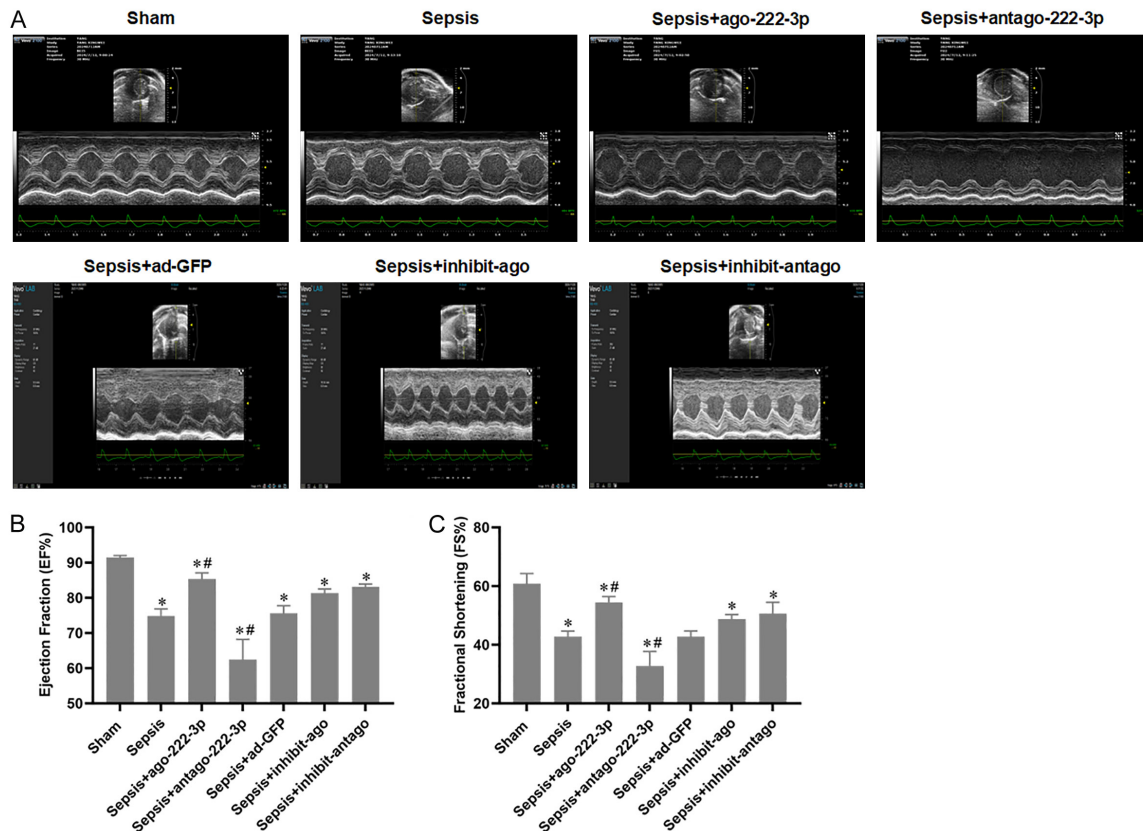


Figure 5. MiR-222-3p improves cardiac function. Left ventricular ejection fraction (LVEF) and fractional shortening (FS) was measured by echocardiography. A. Representative echocardiography from all seven experimental groups. B, C. Quantitative analysis of LVEF and FS. Data are presented as mean $\pm$ SD. N=5. *P < 0.001 vs. Sham group. #P < 0.001 vs. Sepsis group.

cTnI (Figure 6B), IL-6 (Figure 6C), and TNF- α (Figure 6E) was also observed. Concurrently, the blood levels of IL-10 similarly rose following this treatment (Figure 6D). NFAT inhibitors were used to show that the modifying effects of both ago-miR-222-3p and antago-miR-222-3p were abolished. The amounts of these molecules in the i+ago+CLP group matched those in the sepsis group. Based on the above data, it can be concluded that modulation of miR-222-3p by the NFAT pathway is indeed effective.

Discussion

Myocardial damage in sepsis is responsible for much of the death in septic patients. Although there have been some developments in intensive care, practical strategies for treating septic cardiomyopathy in clinical practice are still lacking. This study aimed to explore the role of miR-222-3p in sepsis-induced cardiac injury and to

elucidate the underlying molecular mechanisms. Our results demonstrated that exogenous overexpression of miR-222-3p reduced myocardial injury and cell death, improved heart function, and inhibited inflammation by suppressing NFATc3-related genes.

Recently, numerous microRNAs regulated genes have been identified in cardiovascular diseases, and miR-222-3p is one such microRNA that has displayed various functions in the cardiac system and diseases. Studies indicate that miR-222-3p modulates cardiac failure and myocardial ischemia/reperfusion damage [20-22]. Nevertheless, the role of miR-222-3p in myocardial damage triggered by sepsis remained to be identified. Based on the above analysis, we found that miR-222-3p showed an upward trend in the cardiac tissue of septic mice and may promote the pathology of sepsis-induced heart damage.

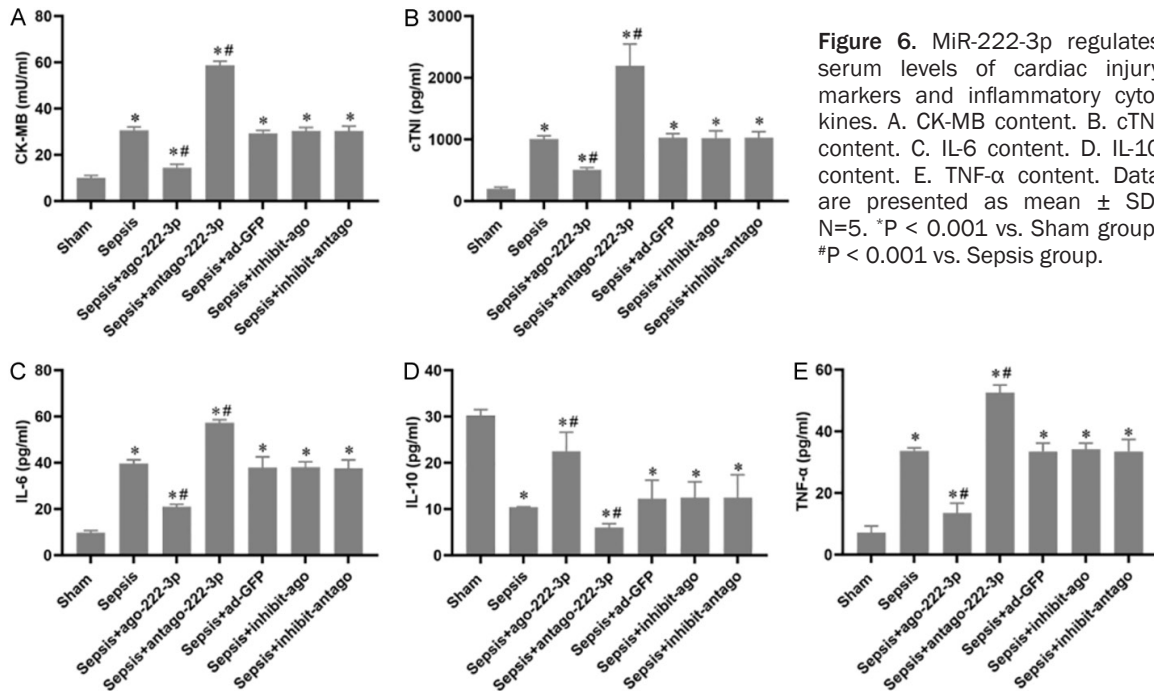


Figure 6. MiR-222-3p regulates serum levels of cardiac injury markers and inflammatory cytokines. A. CK-MB content. B. cTNI content. C. IL-6 content. D. IL-10 content. E. TNF- α content. Data are presented as mean $\pm$ SD. N=5. *P < 0.001 vs. Sham group; #P < 0.001 vs. Sepsis group.

NFATc3 is a member of the NFAT family of transcription factors, and dysregulation of NFATc3 has been implicated in cardiac hypertrophy, heart failure, and ischemia-reperfusion injury [23-26]. Previous studies have identified NFATc3 as a plausible target for miR-222-3p; however, the role of miR-222-3p in myocardial injury triggered by sepsis via this specific pathway remains largely uninvestigated. Myocardial NFATc3 levels were elevated in septic mice. Conversely, miR-222-3p overexpression suppressed NFATc3 levels, while miR-222-3p silencing led to their further increase. Based on these results, miR-222-3p appears to be suppressing NFATc3 expression within the myocardium of septic patients.

To investigate the role of miR-222-3p in sepsis-induced cardiac injury, miR-222-3p was overexpressed or knocked down *in vivo* to observe changes in cardiac structure, cardiomyocyte death, heart function, and inflammation. The above results show that ago-222-3p can reduce myocardial cell damage, inhibit programmed cell death of cardiomyocytes, improve heart function, decrease plasma concentrations of myocardial injury markers (CK-MB and cTNI) and pro-inflammatory molecules (IL-6 and TNF- α), and increase the level of an anti-inflammatory factor (IL-10). miR-222-3p deficiency increased myocardial damage, promoted apop-

toxis of cardiomyocytes, impaired heart function, raised plasma levels of myocardial injury markers and pro-inflammatory cytokines, and reduced concentrations of anti-inflammatory mediators. These findings suggest that miR-222-3p attenuates sepsis-induced cardiac injury.

To determine whether the protective effect of miR-222-3p is mediated by NFATc3, an NFAT inhibitor was employed in the experiment, and then the results were observed. NFAT inhibition abrogated the protective effect of miR-222-3p and attenuated the detrimental effects of miR-222-3p knockdown. Based on the above results, miR-222-3p may protect cardiac cells from damage caused by sepsis via NFATc3.

The precise mechanism by which miR-222-3p exerts its cardioprotective effect in sepsis remains unclear. NFATc3 is a known regulator of many genes involved in cell death, inflammation and cardiac remodelling [27-30]. NFATc3 may regulate downstream signaling pathways involved in apoptosis and inflammation and thus reduce myocardial damage; other targets of miR-222-3p that are regulated by this stress have not been identified yet.

Our study has several restrictions. First, miR-222-3p expression was examined only at the

24-hour time point after CLP. The long-term effects of miR-222-3p modulation on cardiac remodeling and function have not been investigated. A pharmaceutical agent was used to inhibit the NFAT pathway; thus, there was a risk of non-specific off-target effects. Conditional knockout mice in genetic studies could be used to study whether NFATc3 regulates the effect of miR-222-3p more precisely. Finally, the particular molecular pathways by which NFATc3 promotes apoptosis, inflammation, and cardiac failure in sepsis are still mostly unknown.

Conclusion

miR-222-3p expression is upregulated in the myocardium of septic mice, mice, and its over-expression attenuates cardiac injury by inhibiting cardiomyocyte death and improve cardiac function and regulate inflammation through the NFATc3 pathway. Therefore, miR-222-3p may serve as a novel therapeutic target for sepsis-induced myocardial injury. Later research will focus on revealing the specific molecular mechanisms and clinical applications of miR-222-3p-targeting therapy for sepsis.

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Disclosure of conflict of interest

None.

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