

Original Research Article

MiR-155 protects against sepsis-induced cardiomyocyte apoptosis via activation of NO/cGMP signaling pathway by eNOS

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Abstract

Purpose: To examine the impact of miR-155 on sepsis-induced myocardial apoptosis and heart failure, and to explore its molecular mechanism.

Methods: Mice were divided into four groups and septic myocardial dysfunction was induced by intraperitoneal injection of lipopolysaccharide (LPS, 5 mg/kg). The LPS stimulation expression of miR-155 levels was determined by real time-polymerase chain reaction (RT-PCR). In vivo, echocardiography and TUNEL staining were used to investigate the effects of miR-155 in inhibiting cardiac function and myocardial apoptosis. Changes in the expression of eNOS when miR-155 was overexpressed or inhibited were determined by RT-PCR, while double luciferase gene assay assessed the relationship between eNOS and miR-155, eNOS, expression of iNOS, SGC alpha 1, and PKG protein.

Results: MiR-155 was significantly increased after LPS stimulation ($p < 0.01$). In vitro, the inhibition of miR-155 by antagomiR significantly down-regulated the apoptosis of cardiomyocytes ($p < 0.05$), while overexpression of miR-155 by agomiR significantly up-regulated the apoptosis of cardiomyocytes ($p < 0.05$). In vivo, ejection fraction, fractional shortening and heart weight were significantly increased ($p < 0.05$), while apoptosis was significantly decreased ($p < 0.05$). MiR-155 negatively regulated the expression of eNOS ($p < 0.01$), and targeted the expression of eNOS mRNA ($p < 0.001$). In addition, the expression of eNOS, sGC α 1 and PKA were significantly up-regulated ($p < 0.05$), while the expression of iNOS was significantly down-regulated ($p < 0.05$) after the inhibition of miR-155 in LPS mouse model.

Conclusion: MiR-155 regulates sepsis-induced cardiomyocyte apoptosis and heart failure through eNOS/NO/cGMP signaling pathway. Thus, these findings can potentially facilitate the development of an effective strategy for management of heart failure.

Keywords: Apoptosis, Cardiac dysfunction, miRNA-155, Sepsis

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INTRODUCTION

As a common complication of severe sepsis, myocardial dysfunction is a common cause of

death in intensive care units [1]. Infective cardiomyopathy has multiple regulatory mechanisms, including uncontrolled immune and inflammatory responses, disturbances in

myocardial and mitochondrial energy metabolism, and apoptosis [2-5]. Targeted interventions for cardiac dysfunction in sepsis are still lacking, except for antibiotic therapy and symptomatic treatment strategies such as blocking systemic perfusion and blood pressure [6]. MicroRNAs (miRNAs, miR) is the length of about 20 to 25 nucleotides of endogenous non-coding small RNA. They regulate gene expression after transcription. The role of MicroRNAs in tumor, cardiovascular, endocrine metabolism and viral infections has received increasing attention from researchers over the past years [7]. MiRNAs have been reported to play important roles in many cellular processes, including proliferation, apoptosis, differentiation, migration and senescence [8-10]. Several miRNAs, including miR-15a, miR-16, miR-27a, miR-146a, miR-150, miR-223, miR-574-5p and miR-4772-5p, have been shown in human or experimental infective cardiomyopathy disorders, but its mechanism remains unclear.

Abnormal expression of miR-155 plays an important role in atherosclerosis, hypertrophic cardiac remodeling, acute myocardial infarction, myocardial ischemia-reperfusion injury, diabetic cardiomyopathy, and many other cardiovascular diseases [11,12]. In addition, miR-155 is a kind of immune regulatory microRNAs known to all, but lipopolysaccharide (LPS) induces and controls multiple cells and organs of the inflammatory process. However, the role of miR-155 in cardiac dysfunction induced by sepsis is still poorly understood. The aim of this study was to explore the role of miR-155 in septic cardiac dysfunction and related mechanisms in mice.

EXPERIMENTAL

Animals and groupings

Male C57/B6 mice (8 – 10 weeks old) were purchased from the Model Animal Research Centre of Fujian Medical University. This study was approved by the Animal Ethics Committee of Fujian Medical University Animal Center. All procedures were conducted in accordance with the 'Animal Research Reporting: *In vivo* Experiments guidelines 2.0' [13]. Mice were placed in dark autoclaved cages with light for 12 h, and had access to standard chow and water. The animals was divided into 4 groups with 4 in each group: (1) saline + NC antagomiR; (2) saline + miR-155 antagomiR; (3) LPS + NC antagomiR; and (4) LPS + miR-155 antagomiR. Mice were injected intraperitoneally with LPS (5 mg/kg) (Sigma, St. Louis, MO, USA). Twelve hours after LPS injection, cardiac function was measured by two-dimensional echocardiography,

and mice were sacrificed 2 - 3 h later (14 - 15 h after LPS injection). Mouse heart samples were collected and fixed in 4 % paraformaldehyde (PFA) or frozen in nitrous nitrogen, and were further analyzed at -80 °C.

Reagents and kits

Fetal Bovine Serum (FBS), penicillin mixture (100 U/mL), Dulbecco's Modified Eagle Medium (DMEM) (Rockville Gibco, Maryland, USA); pentobarbital (Sigma, St. Louis, MO, USA); TRizol, Lipofectamine 2000, radioimmunoprecipitation assay (RIPA) lysate, bicinchoninic acid (BCA), protein quantification kit (Invitrogen, Carlsbad, CA, USA); reverse transcription and real-time quantitative PCR kit (TaKaRa, Tokyo, Japan); TUNEL kit (Novozymes Biologicals, Bagsvaerd, Denmark); rabbit anti-Rat eNOS, iNOS, sGC α 1, PKG, Bax, Bcl2 and β -actin monoclonal antibodies (Abcam, Cambridge, MA, USA); HRP-labelled goat anti-rabbit IgG (Xi'an Jingcai Biologicals, Xi'an, China); rat miR-155 agomiR, miR-155 antagomiR and their respective random control sequences (agomiR NC/antagomiR NC) (Ribo Biologicals, Guangzhou, China); luciferase assay kit (Promega, Madison, WI, USA); and 4',6-diamidino-2-phenylindole (DAPI) staining solution (Jiangsu Biyuntian Technology Company, Nanjing, China). All other reagents were obtained locally and were of analytical purity.

Cell culture and processing

The isolation and culture of primary neonatal mouse cardiomyocytes were as described previously [14]. Suckling mouse cardiomyocytes was applied with Lipofectamine 2000 for miR-155 antagomiR and miR-155 agomiR. According to the manufacturer's instructions, in the presence or absence of 1 μ g/mL LPS (control), and to detect apoptotic cells, cells were scored by nuclear morphology, using randomly selected *in situ* cell death detection kit. A minimum of 100 cells were examined per sample.

MiR-155 antagomiR-treated mice

A miR-155 antagonist, a 2'OME+5'chol-modified miR-155 inhibitor, was used to modulate the expression level of miR-155 in mice to study the function of miR-155 in LPS-induced cardiac insufficiency effect. The MiR-155 was inhibited by tail vein injection of miR-155 antagomiR or control (80 mg/kg) for three consecutive days prior to LPS treatment. MiR-155 expression levels in the myocardium were measured using real-time quantitative polymerase chain reaction to confirm the effect of antagomiR treatment.

Small animal echocardiography

Echocardiography was performed on mice using a Vevo2100 (visualsonic, Ontario, Canada) 5 h after LPS or saline injection. Left ventricular EF (%) and FS (%) were measured at the level of the parasternal short-axis papillary muscle using m-mode images. Echocardiographic data was recorded and analyzed under different treatment blinding methods.

TUNEL detection

Heart samples were OCT-embedded and cut into tissue slices 10 μ m thick by a frozen slicer. Apoptosis was determined using terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) using an *in situ* cell death detection kit (Novozymes Biologicals, Bagsvaerd, Denmark) according to the kit instructions. Images were obtained using a fluorescent microscope at 200x magnification.

Quantitative real time-polymerase chain reaction (qRT-PCR)

The entire RNA was extracted from the cardiac tissue or the cardiomyocytes using the TRIzol RNA extraction kit, and reverse transcribed to cDNA using the iScript cDNA synthesis kit in line with the manufacturers' instructions. The miR-155 expression levels were measured on an ABI 7900HT fast real-time PCR system using Bulge-Loop miRNA qPCR primers (Ribo-Bio, Guangzhou, China) and SYBR supermix kit (Bio-Rad, Hercules, CA, USA) to detect miR-155 expression levels. A 5S was used as an internal reference for miRNA, and 18S as an internal reference for common genes. Gene expression was performed by 40 cycles of qPCR by means of the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), and the SYBR Green supermix kit. Relative miRNA or gene expression levels were calculated by CT value using the $2^{-\Delta\Delta C_t}$ method and the primer sequences are presented in Table 1.

Protein immunoblotting analysis

After lysis with RIPA lysis buffer, cardiac samples were quantified with the BCA protein assay kit. In 10 % sodium dodecyl sulfate (SDS) -

polyacrylamide gel electrophoresis (PAGE) (the same amount of protein gel separation), and transferred to polyvinylidene fluoride (PVDF) membrane. Membranes were incubated overnight at 4 °C with the following antibodies after blocking in 5 % milk for 1 h: anti-Bax (1:1,000), anti-bcl-2 (1:1,000) and anti-eNOS (1:1,000), anti-iNOS (1:1,000), anti-sGC α 1 (1:1,000), anti-PKG (1:1,000). Actin β -actin (1:1 000) was used as an internal reference. Then, in 5 % milk, the membrane was incubated with appropriate horseradish peroxidase coupled with secondary antibodies. An enhanced chemiluminescence system was adopted to detect protein bands.

Dual luciferase reporter gene assay

Targetscan, an online miRNA target gene prediction website, was adopted to calculate miR-155 target genes and to screen eNOS as its possible target molecule. The day before transfection, HEK 293 cells were seeded in 24-well plates at a rate of 2×10^4 cells/well. Lipofectamine 2000 was used for all transfections following the manufacturer's instructions. Cells were transfected via pGL3 luciferase expression vector that is with the eNOS pRL-TK Renilla luciferase 3'UTR and miR-155 agomiR or a negative control. After 48 h post transfection, a dual-luciferase reporter gene assay system was used to measure luciferase activity and normalize it to renal cell luciferase activity. The predicted miR-155 binding site on the eNOS 3'UTR was mutated so as to observe whether the mutation abolished the reduction of luciferase activity by the miR-155 agomiR.

Statistical analysis

Statistical Package for Social Sciences (SPSS) 19.0 (IBM, Armonk, NY, USA) was used for all analyses. The mean \pm standard error of the mean (SEM) was used to represent the results. Two groups were compared using independent samples t-test. Comparisons between three or more groups were performed via one-way analysis of variance (ANOVA) and Bonferroni's post hoc test. $P < 0.05$ was considered statistically significant.

Table 1: Primer sequences

Gene	Forward	Reverse
eNOS	TACTCCAGGCTCCCGATG	AAGGGCAGCAAACCACTC
mmu-5S	GGTTGTGGTTGGTTGGTTTGT	ATACCACAACCACCCCT
mmu-miR-155-5p	AGCGCTTAATGCTAATTGTGAT	GTTGTGGTTGGTTGGTTTGT

RESULTS

In vivo and *in vitro* miR-155 expressions

Treating cardiomyocytes with (1 µg/mL) LPS, significantly increased the levels of miR-155 expression. The Rt-QPCR results showed that miR-155 expression was significantly up-regulated in mice with infectious cardiac insufficiency induced by intraperitoneal injection of LPS (Figure 1 A). In this study, samples were processed at 6, 12 and 24 h. Through Rt-QPCR detection, miR-155 expression levels increased significantly at 6 h after injection, peaked at 12 h and decreased until 24 h, but were still significantly up-regulated compared with the control group ($p < 0.05$). The Rt-QPCR results showed that LPS-induced miR-155 expression was significantly upregulated at different time points, with the most significant upregulation at 12 h (Figure 1 B). It facilitates further study on the role of miR-155 in LPS-induced septic cardiac insufficiency.

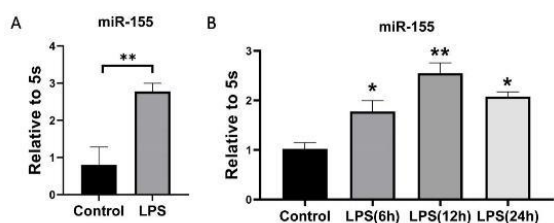


Figure 1: miR-155 expression was up-regulated in LPS-treated cardiomyocytes and mouse models. * $p < 0.05$, ** $p < 0.01$

Effect of miR-155 inhibition on LPS-induced apoptosis

The RT-PCR results showed that the expression of miR-155 was significantly inhibited after transfection of myocytes with miR-155 antagomiR (Figure 2 A). At the cardiomyocyte level, miR-155 antagomiR was first transfected and then treated with LPS (1 µg/mL). TUNEL staining showed that LPS induced apoptosis of the cardiomyocytes, and the transfection of the apoptosis of cardiomyocytes with miR-155 antagomiR was significantly reduced (Figure 2 B).

Effect of overexpression of miR-155 on LPS-induced apoptosis

The results of RT-PCR assay revealed that cardiomyocytes which transfected with miR-155 agomiR could prominently raise the expression of miR-155 (Figure 3 A). The LPS (1 µg/mL) was used to treat cardiomyocytes. TUNEL staining showed that LPS treatment induced apoptosis of

the cardiomyocytes, while transfection of miR-155 agomiR aggravated LPS-induced apoptosis of the cardiomyocytes (Figure 3 B).

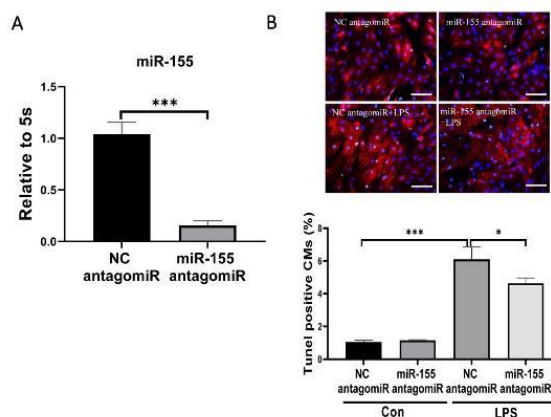


Figure 2: Inhibition of miR-155 reduced myocardial apoptosis. (A) Rt-QPCR demonstrated that miR-155 antagomiR significantly inhibited the expression of miR-155; (B) TUNEL staining demonstrated that miR-155 antagomiR reduced LPS-induced myocardial apoptosis. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$

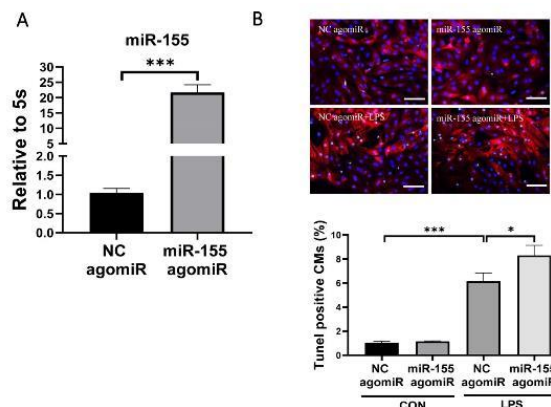


Figure 3: Overexpression of miR-155 worsened cardiomyocyte apoptosis. (A) RT-QPCR showed that miR-155 agomiR significantly overexpressed miR-155. (B) TUNEL staining demonstrated that miR-155 agomiR could increase the effect of LPS induced myocardial apoptosis. * $P < 0.05$, *** $p < 0.001$

Effect of miR-155 inhibition on cardiac function and apoptosis

Injection of miR-155 antagomiR into mice via the tail vein for 3 consecutive days prior to LPS treatment resulted in significant inhibition of miR-155 in the heart. Echocardiographic results showed decreases in EF (%) and FS (%), indicators of cardiac function in mice after LPS treatment, but these were partially reversed by miR-155 antagomiR (Figure 4 A). Heart weight results showed a reduction in heart weight in mice after LPS treatment. However, miR-155 antagonists partially reversed this result (Figure 4

B and C). According to TUNEL staining results, miR-155 inhibition also decreased the ratio of TUNEL-positive nuclei (Figure 5 A), and western blot analysis showed that LPS-induced elevation of bcl-2 /Bax ratio could be partially reversed by miR-155 antagomiR (Figure 5 B). From these results, inhibiting miR-155 improved cardiac function and decreased apoptosis in LPS-treated mice.

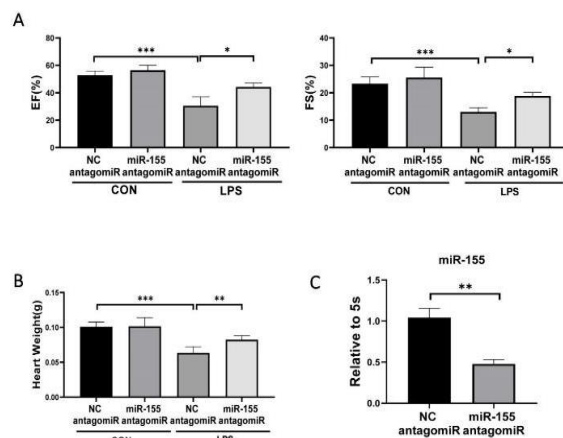


Figure 4: miR-155 antagomiR improved cardiac function in LPS-treated mice. (A) Echocardiography showed that Mir-155 antagomiR significantly improved IPS-induced partial left ventricular shortening (%) and reduced ejection fraction (%). (B) Reduced heart weight in mice (C) reduced relative expression level of Mir-155. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$

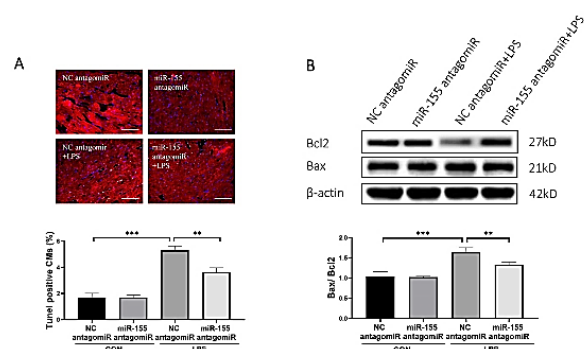


Figure 5: miR-155 antagomiR improved myocardial apoptosis in LPS-treated mice. (A) TUNEL results showing miR-155 antagomiR significantly reduced LPS-induced apoptosis in cardiac tissues. Original magnification: $\times 200$; (B) Western blot results showed that miR-155 antagomiR significantly reduced LPS-induced elevation of Bcl-2/Bax ratio. ** $P < 0.01$, *** $p < 0.001$

MiR-155 protects against myocardial apoptosis

Using RT-PCR, this research found that at the mRNA level, eNOS expression was down-regulated in miR-155 agomiR-treated mice and up-regulated in miR-155 antagomiR-treated mice,

indicating a negative relationship between eNOS and miR-155 expression (Figure 6 A). It was found in the luciferase reporter gene assay that miR-155 agomiR decreased luciferase activity compared to the negative control, and that if the predicted miR-155 binding site on the eNOS 3'UTR is mutated, the binding of miR-155 to the eNOS 3'UTR is blocked, thereby abolishing the decrease in luciferase activity, which indicates that eNOS is a direct target gene of miR-155 (Figure 6 B). This research co-treated cells with miR-155 antagomiR and an eNOS inhibitor (L-NAME HCL), and TUNEL staining showed that the eNOS inhibitor partially reversed the protective effect of miR-155 antagomiR against apoptosis in cardiomyocytes (Figure 7 A). eNOS promotes NO synthesis, and it was detected by immunoblotting the NO/cGMP pathway protein expression of eNOS, iNOS, sGC α 1 and PKG. It was also discovered that miR-155 antagomiR reversed LPS-induced inhibition of the NO/cGMP pathway (Figure 7 B).

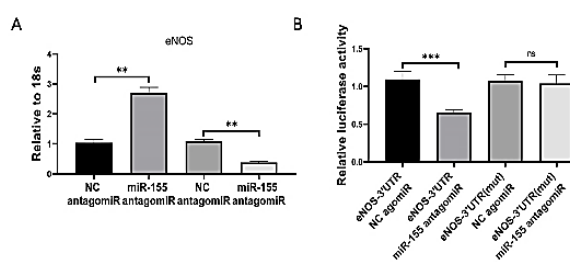


Figure 6: miR-155 targeted regulation of eNOS. (A) QPCR results showed that overexpression of miR-155 inhibited eNOS expression, and inhibition of miR-155 promoted eNOS expression. (B) Wild-type and mutant 3'UTRs were transfected with miR-155 agomiR or negatively controlled HEK293 cells and luciferin activity was determined. ** $P < 0.01$, *** $p < 0.001$.

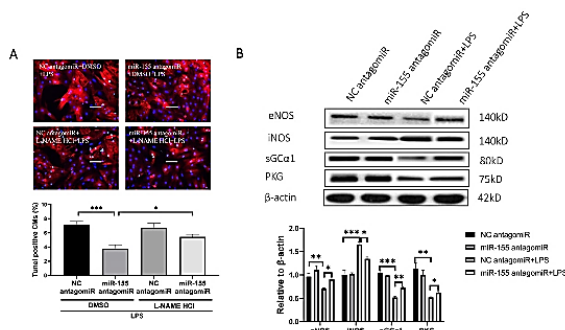


Figure 7: Inhibits miR-155 protects LPS-induced myocardial apoptosis by activating the NO/cGMP signaling pathway through eNOS. (A) TUNEL results demonstrated that eNOS inhibitor (L-NAME HCL) reversed the protective effect of miR-155 antagonist on LPS-induced apoptosis. Original magnification: $\times 200$. (B) Western blot analysis of eNOS, iNOS, sGC α 1, PKG protein expression. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$

DISCUSSION

Sepsis-related myocardial dysfunction is the leading cause of high morbidity and mortality in critically ill patients. There is increasing evidence that various miRNAs, including miR-15a, miR-16, miR-27a, miR-146a, miR-150, miR-223, miR-574-5p, miR-21-3p, and miR-4772-5p, play a role in septic heart disease. They also play a regulatory role in dysfunction and potential clinical value, but the mechanism is yet to be understood. As an important inflammatory regulatory miRNA, miR-155 expression can be induced by LPS *in vitro* and *in vivo*. However, the function of miR-155 in LPS-induced myocardial dysfunction remains unclear. In this study, miR-155 expression level was significantly increased in the heart under LPS stimulation.

In different experimental models, the role of miR-155 in cardiovascular damage is controversial. In animal models of hypertensive cardiac injury and ischemia-reperfusion injury, inhibition of miR-155 protects the heart from pathological hypertrophy and improves cardiac function, according to several studies [13]. In contrast, other studies argue that forced expression of miR-155 attenuates cardiovascular injury under certain pathological conditions e.g., acute viral myocarditis and atherosclerosis [15]. Therefore, it is essential to elucidate the functional role of miR-155 in septic cardiac dysfunction. Based on available data from this study, it was interesting to find that drug inhibition of miR-155 can improve the EF and FS of LPS-stimulated hearts, suggesting that inhibition of miR-155 has a protective effect on septic hearts.

Apoptosis is also an important process in sepsis besides impaired cardiac function [16]. In fact, several drugs such as corticosteroids, cyclosporine A and simvastatin have been shown to attenuate sepsis-related myocardial apoptosis [17]. The present study further revealed that inhibiting miR-155 attenuated LPS-induced myocardial apoptosis through a reduction in TUNEL-positive nuclei and an increase in the Bcl-2/Bax ratio in the myocardium. Furthermore, it is found from available data that inhibiting miR-155 prevents apoptosis in isolated cardiomyocytes, whereas overexpression of miR-155 worsens apoptosis in isolated cardiomyocytes, suggesting that the beneficial effects of inhibiting miR-155 in infective cardiac insufficiency may be primarily related to its anti-apoptotic effects. However, the type of cardiac apoptotic cells in the *in vivo* LPS model is unknown. This should be considered a limitation of this study. The data suggests that miR-155

may be a novel target for reducing apoptosis during sepsis.

Since eNOS was predicted to be a target gene of miR-155, the expression level of eNOS in LPS-stimulated hearts was further examined. As expected, it was negatively regulated *in vitro* by miR-155. eNOS as a direct target of miR-155 was further confirmed by means of luciferase reporter gene analysis. Despite the fact that eNOS has previously been shown to be involved in a variety of biological processes, including cell proliferation, migration, differentiation and apoptosis, little is known about its effect in LPS-induced septic infection in cardiovascular disease. In this present study, eNOS was identified as new miR-155 target gene that may lead to myocardial dysfunction in sepsis by targeting apoptosis. The NO-cGMP signaling pathway play an important role in the treatment of symptoms in patients with obstructive cardiovascular disease [16].

Nitroglycerin was used to relieve angina symptoms as early as the 19th century. Nitric oxide (NO) was later found to be a key contributor to arterial vasodilation [17], but the underlying molecular and cellular mechanisms remain unclear. In the following decades, ornithophosphoryl cyclase was identified as an NO receptor producing cGMP upon activation, and phosphodiesterase (PDE) was activated and degraded by cGMP as a downstream signaling cascade in a negative feedback loop. The cellular functions of cGMP in different cell types are mainly mediated through cGMP-dependent protein kinase phosphorylation proteins [18]. These findings suggest that, in addition to its acute effects on the vascular system, the NO-cGMP pathway regulates the development and/or rupture of coronary plaques [19]. *In vivo*, it was found that the expression of eNOS, sGC α 1, and PKG was downregulated and iNOS was upregulated in response to LPS stimulation, and that inhibition of miR-155 reversed their expression levels.

Limitations of this study

The study has some limitations. The mechanism by which LPS induces miR-155 is not clear. Previous studies have shown that transcription factor Ets2 play a key role in LPS-induced miR-155 induction. In addition, IL-10 inhibited the expression of Ets2, thereby inhibiting LPS-induced miR-155. Recently, it was shown that miR-155 can be delivered *in vivo* and *in vitro* in exocytosis between immune cells in response to LPS treatment. Therefore, further research on the upstream mechanisms of miR-155 induction

and the source of miR-155 upregulation in sepsis-induced cardiac insufficiency would be of great interest. Inhibition of miR-155 is thought to prevent cardiac injury after LPS treatment, but the interaction between miR-155 regulated cardiac insufficiency and sepsis cardiomyocyte apoptosis remains to be demonstrated.

CONCLUSION

This study has demonstrated that miR-155 expression is upregulated in sepsis-induced cardiomyopathy. Pharmacological inhibition of miR-155 improves cardiac functions and reduces cardiac apoptosis in sepsis patients. Therefore, miR-155/eNOS/NO-cGM pathway may be a new target for the treatment of septic myocardial dysfunction. However, more patient clinical plasma samples are needed to confirm the feasibility of miR-155 as a biomarker of sepsis-induced cardiac dysfunction.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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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