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rhTNFR:Fc increases Nrf2 expression via miR-27a mediation to protect myocardium against sepsis injury

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ABSTRACT

Objective: Sepsis is a whole-body inflammation disease and can result in septic shock and multiple organ failure. The previous study demonstrated that miR-27a plays a critical role in inflammation regulation. Here, we investigated that effect and its possible mechanism of rhTNFR:Fc on sepsis treatment.
Methods: LPS induced sepsis mice model was established. 10 mg/kg rhTNFR:Fc was used to treat sepsis mice by intravenous injection.
Results: RhTNFR:Fc improved cardiac function of sepsis mice, and markedly decreased miR-27a but increased Nrf2 expression level of myocardium in LPS treated mice. In H9C2 cells, rhTNFR:Fc also increased Nrf2 expression, elevated cell viability and decreased cell apoptosis. However, the effects were reversed by miR-27a mimic. In addition, miR-27a mimic reduced the activity of Nrf2 3′UTR while miR-27a inhibitor elevated enhanced its level.
Conclusion: rhTNFR:Fc activated Nrf2 pathway to protect myocardium against LPS-induced sepsis injury via miR-27a regulation.

1. Introduction

Sepsis is a potentially fatal whole-body inflammation mainly caused by serious trauma, burning, shock and operation and so on [1]. The development of sepsis can result in septic shock and multiple organ failure [2], which is a most common cause of death among critically ill patients in non-coronary diseases [3]. Because sepsis can continue even after the infection that caused it is gone. Severe sepsis is sepsis complicated by organ dysfunction. Heart is one of most vulnerable organs to be injured [4] and myocardial dysfunction is associated with high mortality of sepsis.

The previous study showed that pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), played a critical part in myocardial dysfunction during sepsis [5,6]. Patients with sepsis with lower left ventricular ejection fraction have increased plasma level of TNF-α and apoptotic cell death of cardiomyocytes [7]. Recombinant human tumor necrosis factor-α receptor II: IgG-Fc (rhTNFR:Fc) fusion protein, which is also known as etanercept, is a soluble TNF receptor fusion protein that binds and inactivates proinflammatory cytokine TNF. It has been reported that rhTNFR:Fc improved the left ventricular contractile function in response to lipopolysaccharide (LPS) in -KO mice [8]. In addition, rhTNFR:Fc was used to the treatment of rheumatoid arthritis [9], ankylosing spondylitis [10] and psoriasis [11], all of above diseases are commonly related to immune dysfunction. It is considered rhTNFR:Fc has a potential role in treatment of sepsis. However, the molecular mechanism underlying it is not fully known.

There is increasing evidence that microRNAs (miRNAs), a new class of endogenous and non-coding RNAs, are implicated in various human diseases via binding to 3′-UTR of target genes [12]. It was also reported dysregulation of miRNAs is common in sepsis [13–15]. Previous study has found that miR-27a was up-regulated in lungs of septic mice, which involved in the regulation of TNF-α and IL-6 via NF-κB subunit phosphorylation [15]. The down-regulation of miR-27a is identified as a negative-regulatory mechanism to prevent inflammatory response [16]. In addition, TargetScan Software predicted that nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is the target gene of miR-27a in our preparatory work. Nrf2 is a member of the cap-N-collar family, which is the principal transcription factor that regulates antioxidant response

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element-mediated expression of detoxifying antioxidant enzymes [17] and plays an important role in protecting organs from injury. In present study, we investigated whether rhTNFR:Fc has a positive effect on sepsis treatment. We hypothesized that miR-27a was involved in the process of protecting myocardium against sepsis injury by activation of Nrf2 pathway. The LPS induced sepsis mice model was established and embryonic rat cardiac myoblasts H9C2 cells were cultured to examine our hypothesis.

2. Materials and methods

2.1. Animal model

The procedures of animal experiments was performed by Guide for the Care and Use of Laboratory Animals and were approved by the Experimental Animal Ethics Committee of People's Hospital of the Ningxia Hui Autonomous Region. Male C57B6/L mice were purchased from Beijing Laboratory Animal Research Center (Beijing, China). Animals were kept in a temperature-controlled room with free access to standard chow and water. Twenty-four mice were randomly divided into four groups. Six mice were administrated with normal saline and defined as control group; sepsis mice model was established by intraperitoneal injection with 4 mg/kg LPS (LPS group, n = 6); the mice in rhTNFR:Fc group (n = 6) were administrated with intravenous injection of 10 mg/kg rhTNFR:Fc; the remaining 6 mice intravenously injected with 10 mg/kg rhTNFR:Fc before intraperitoneal injection with 4 mg/kg LPS (rhTNFR:Fc + LPS group).

2.2. Detection of cardiac function

After 12 h injection of LPS, the mice were anesthetized with intraperitoneal injections of ketamine (80 mg/kg) and xylazine (10 mg/kg) and the cardiac function of mice was detected by Millar pressure-conductance catheter SPR839 (Millar Instruments, USA) were computed as described [18]. Mean artery pressure, stroke work, Left ventricular (LV) pressure at maximal dP/dt, LV end systolic pressure, LV end diastolic pressure, LV + dP/dt, LV-dP/dt and heart rate were computed.

2.3. Cell culture

The embryonic rat cardiac myoblasts H9C2 (ATCC, USA) was cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 5 mg/ml streptomycin. Cells in DMEM co-incubated with 150 ng/ml rhRNFR:Fc for 1 h were considered to be rhTNFR:Fc group; treated with 10 μg/ml LPS for 12 h were considered as LPS group; the cells in LPS + rhTNFR:Fc group were pre-incubated with 150 ng/ml rhTNFR:Fc and then treated with 10 μg/ml LPS for 12 h. All cell experiments were repeated for three times.

2.4. Quantitative real-time PCR

Total RNAs were extracted from myocardial tissue of mice or H9C2 cells using the TRIzol reagent (Invitrogen, USA) and 2 μg of RNA was used to reverse transcription-PCR with ImProm-II™ (Promega, USA) following the manufacturer's instructions. The mRNA levels of Nrf2, NQO1, GST and HO-1 were quantified by using TransStartTM SYBR Green qPCR Supermix (TransGen Biotech, China) and β-actin conducted as internal control gene. MiRNAs of tissue or cells were isolated by MiRNEasy Mini Kit (Qiagen, Germany); miRNAs were reversed to cDNA by TaqMan MicroRNA Reverse Trancription Kit (Applied Biosystems, USA) and then quantified with TaqMan MicroRNA Assay (Applied Biosystems, USA). U6 small nuclear RNA served as an internal normalized reference.

2.5. Western blotting analysis

The myocardial tissues of mice were homogenized with phosphate buffered saline. After centrifugation, the homogenate without supernatant was lysed with lysis buffer including protease inhibitor and then centrifuged at 12,000 g for 20 min to keep supernatants. Cells were washed in ice-cold PBS and lysed in whole cell lysis buffer. After centrifugation, the protein concentrations of myocardial tissues or cells were detected by Bradford's method. Equal amounts of protein were loaded onto sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membrane (Bio-Rad, USA), which was blocked with 5% non-fat dry milk in Tris–HCl buffered saline incubated with the primary antibodies (Cell signaling, USA) according to the manufacturer's recommendations. β-actin was served as a control protein to quantify the expression of target protein.

2.6. Transfection procedures

MiR-27a of cells was over-expressed or knocked down by transfected with miR-27a mimic or miR-27a inhibitor. H9C2 cells were transfected with the final concentration of 100 nM miR-27a or miR-27a inhibitor or negative control miRNA using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions. MiR-27a mimic, miR-27a inhibitor and negative control miRNA were produced by Ribobio Co., Ltd. (RIBOBIO, China).

2.7. Luciferase reporter assay

The PCR-amplified 3′UTR of Nrf2 fragments were cloned into pCDNA3.1/ZEO(+)-luc vector to generate Nrf2 3′UTR reporter constructs, and then vectors with miR-27a binding sites were co-transfected into H9C2 together with pCDNA3 plasmid by Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. After 24 h, relative luciferase activities were measured using the Dual-Luciferase Reporter Assay (Promega, USA).

2.8. MTT assay

H9C2 Cells viability was evaluated by MTT (Sigma, USA) assay. Briefly, 5 mg/mL MTT reagent was added to the medium (the final concentration MTT is 0.5 mg/mL) and incubated with cells for 4 h. After incubation, 150 μL dimethylsulfoxide was used to dissolve the crystal products and then the absorbance was detected at a wavelength of 570 nm by SpectraMax M5 Multimode Reader (Molecular Devices, USA).

2.9. TUNEL assay

The apoptosis of H9C2 cells was analyzed using TUNEL assay (Roche, USA) following the instructions strictly. In brief, cell suspension was attached to the surface of a glass slide with poly-L-Lysine and fixed with 4% paraformaldehyde. Paraffin sections were used and cells were incubated with terminal deoxynucleotidyl transferase using FITC labeled nucleotides. The percentage of TUNEL positive cells was calculated as followed: TUNEL-positive cells/total number of cells × 100%.

2.10. Statistical analysis

All analyses were performed using SPSS 18.0 software. The student *t*-test was performed for comparison between two groups,

and the one-way ANOVA test was used for comparison among multiple groups. All data are shown as mean ± SD, and significance was established at $P < 0.05$.

3. Results

3.1. RhTNFR:Fc improves cardiac function of mice exposed to LPS

C57BL/6 mice were exposed to LPS (4 mg/kg) to induce a sepsis. As shown in Fig. 1A–G, the cardiac function indexes including mean artery pressure, stroke work, left ventricular, pressure at maximal dP/dt, left ventricular end systolic pressure, left ventricular end diastolic pressure and left ventricular (LV) +dP/dt (F) and −dP/dt were significantly decreased by LPS in mice. In addition, injection of rhTNFR:Fc before treatment with LPS, the cardiac function indexes mentioned above were markedly increased. However, the level of heart rate of mice had no difference among four groups as described in Fig. 1H.

3.2. RhTNFR:Fc regulates myocardial miR-27a and Nrf2 expression of mice exposed to LPS

The expression of miR-27a was detected by real-time PCR, the result showed miR-27a was significantly up-regulated in LPS group, and pretreatment of rhTNFR:Fc markedly decreased miR-27a expression of myocardium in LPS treated mice (Fig. 2A). The levels of Nrf2 and its target genes were detected by real-time PCR and western blotting. As shown in Fig. 2B and C, the mRNA and protein expression of Nrf2 and its target genes NQO1, GST and HO-1 was down-regulated by LPS, the down-regulation of which was reversed by rhTNFR:Fc.

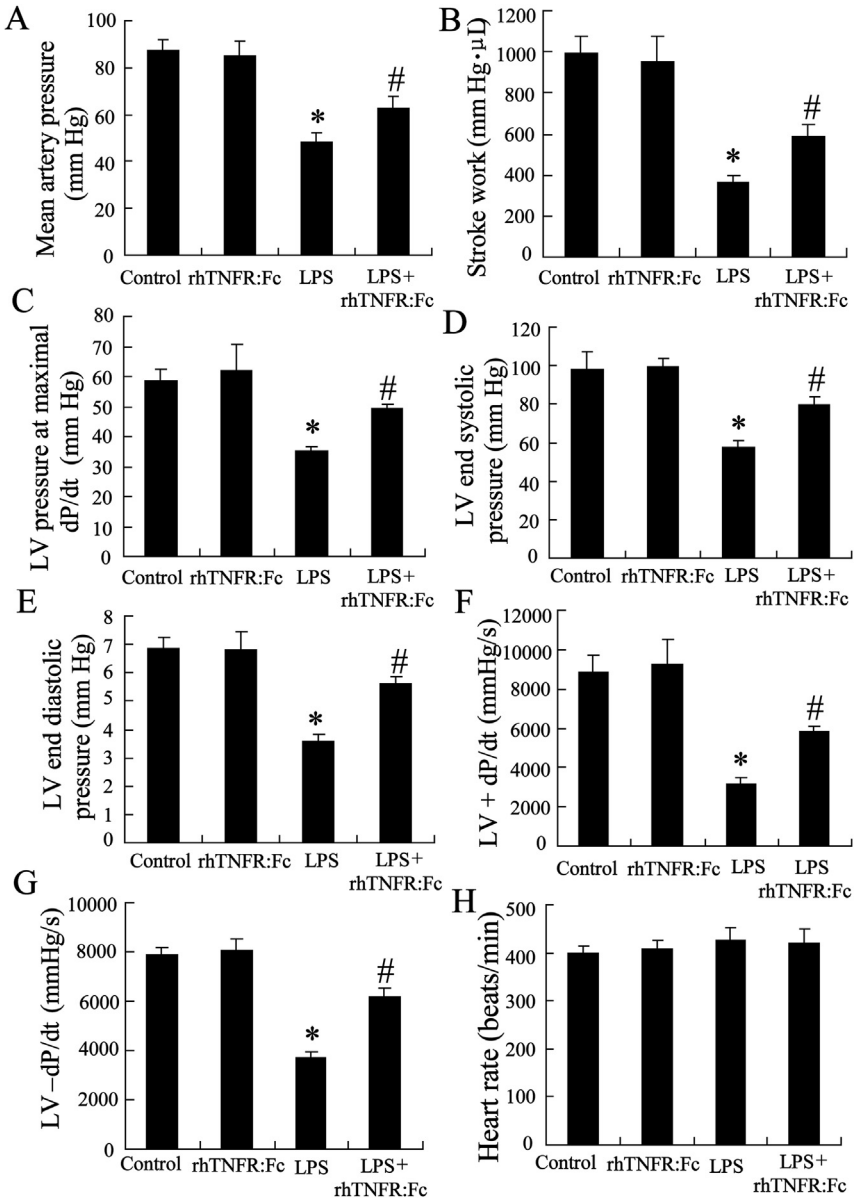

Fig. 1. RhTNFR:Fc improves cardiac function of mice exposed to LPS. A) Mean artery pressure; B) Stroke work; C) Left ventricular (LV) pressure at maximal dP/dt; D) Left ventricular end systolic pressure; E) Left ventricular end diastolic pressure; F) left ventricular (LV) +dP/dt; G: LV −dP/dt. n = 6 mice per group. * VS control group, $P < 0.05$; # VS LPS group, $P < 0.05$.

3.3. RhTNFR:Fc regulates the expression of miR-27a and Nrf2 in H9C2 cells exposed to LPS

Treated H9C2 cells with 10 μg/ml LPS for 12 h, miR-27a expression was significantly decreased (Fig. 2D), and the levels of Nrf2 and its target genes were significantly increased (Fig. 2E and F). When pre-treated cells with 150 ng/ml rhTNFR:Fc for 1 h before exposed to LPS, H9C2 cells had a lower level of miR-27a, and higher levels of Nrf2, NQO1, GST and HO-1 compared with LPS groups.

3.4. MiR-27a regulates Nrf2 expression in H9C2 cells

To investigate the effect of miR-27a on Nrf2 expression, H9C2 cells were transfected with miR-27a mimic or inhibitor to over-express or down-regulate the expression of miR-27a. As shown in Fig. 3A and D, the quantity of miR-27a was effectively regulated by mimic and inhibitor. Over-expressed miR-27a reduced the binding of miR-27a and 3′UTR of Nrf2 (Fig. 3B), and the level of Nrf2 was also down-regulated by miR-27a mimic (Fig. 3C). In addition,

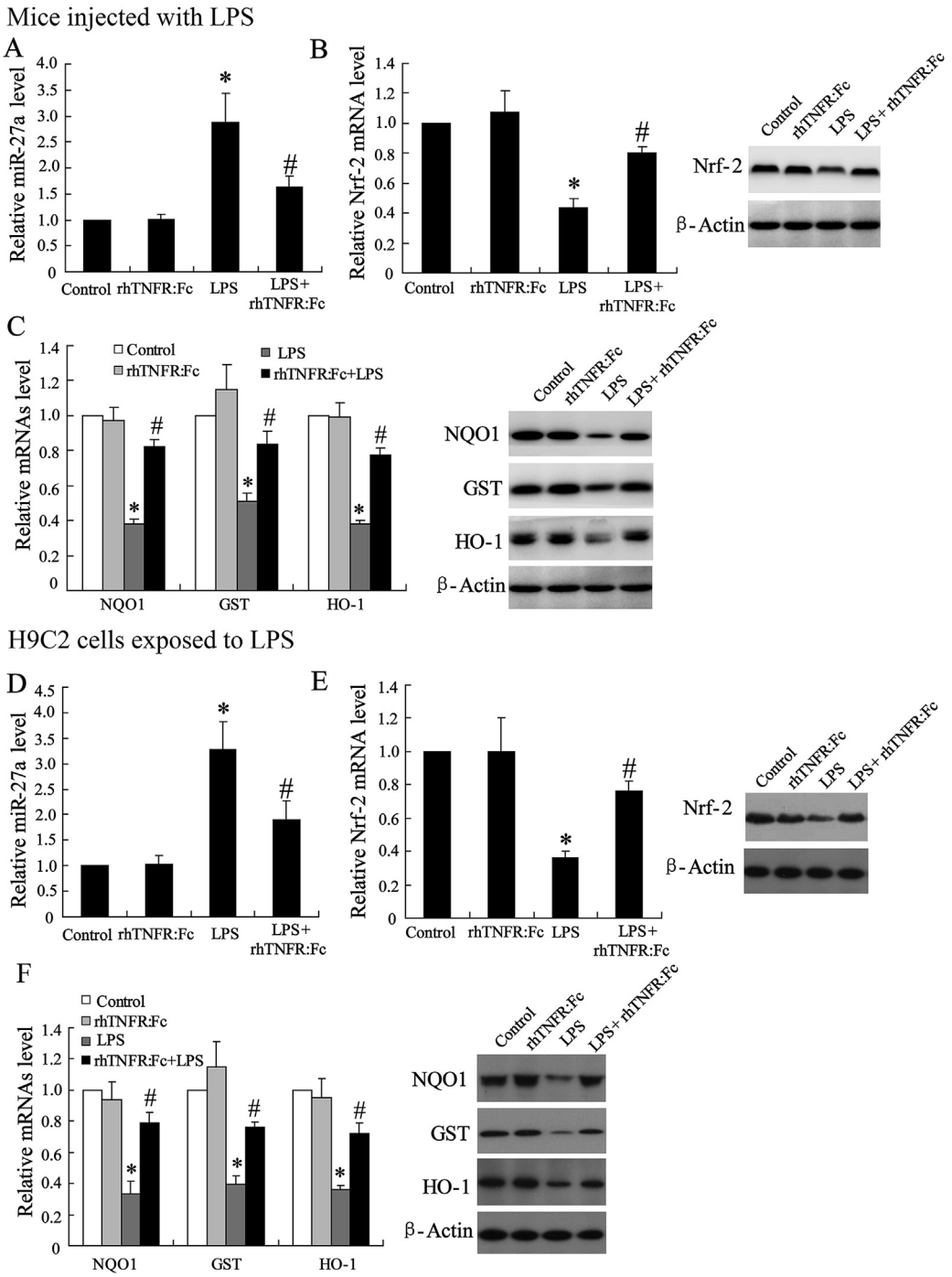

Fig. 2. RhTNFR:Fc regulates myocardial miR-27a and Nrf2 expression in vivo and in vitro *In vivo* experiments, the expression levels of miR-27a (A), Nrf2 (B) and Nrf2 target genes (C) were determined. n = 6 mice per group. *In vitro* experiments, the expression levels of miR-27a (D), Nrf2 (E) and Nrf2 target genes (F) were also detected. All results were repeated for three times. * VS control group, $P < 0.05$; # VS LPS group, $P < 0.05$.

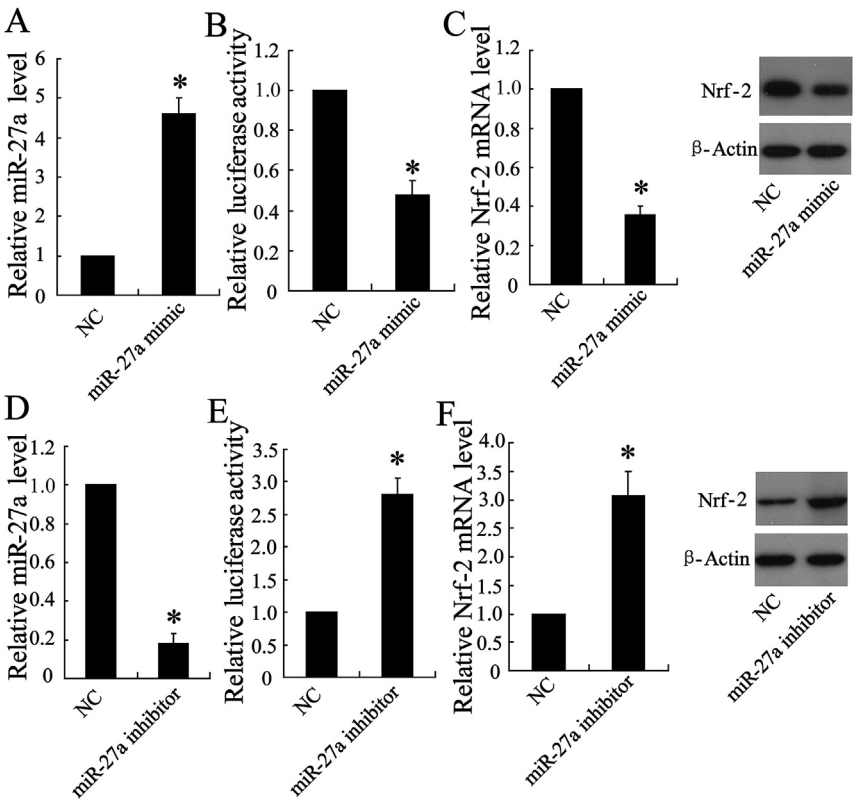

Fig. 3. MiR-27a regulates Nrf2 expression in H9C2 cells H9C2 cells were transfected with miR-27a mimic or miR-27a inhibitor by Lipofectamine 2000. The cells in control group were transfected with negative control miRNA and defined as NC group. All results were repeated for three times.* VS NC group, $P < 0.05$.

down-regulation of miR-27a enhanced the binding of miR-27a to 3′UTR of Nrf2 (Fig. 3E) and increased the level of Nrf2 (Fig. 3F).

3.5. Overexpression of miR-27a reversed the effects of rhTNFR:Fc on Nrf2 pathway

As indicated previously, LPS significantly down-regulated the expression of Nrf2 and its target genes, pretreatment with rhTNFR:Fc up-regulated their levels in H9C2 cells. As shown in Fig. 4A, we also observed miR-27a mimic reversed the effects of rhTNFR:Fc on Nrf2 pathway in H9C2 cells exposed to LPS.

3.6. miR-27a involves in the regulation of rhTNFR:Fc on H9C2 survival and apoptosis

As shown in Fig. 4B and C, LPS significantly decreased cell viability and elevated the level of cell apoptosis, rhTNFR:Fc increased cell viability and reduced apoptosis of H92C cells exposed to LPS. The effects of rhTNFR:Fc on preventing cells against LPS-induced injury was attenuated by transfecting cells with miR-27a mimic.

4. Discussion

Sepsis-induced organ failure is the major cause of death, and is characterized by a massive dys-regulated inflammatory response. In present study, the effects of rhTNFR:Fc on LPS-induced sepsis mice were investigated and the underlying mechanisms were also examined in both in vivo and in vitro experiments. The major findings was rhTNFR:Fc improved the myocardial function in sepsis mice induced by LPS via activating Nrf2 pathway by miR-27a.

rhTNFR:Fc is a soluble TNF receptor fusion protein that binds and inactivates proinflammatory cytokine TNF. The inflammasome is necessary to the innate immune response to infection in sepsis [19]. In present study, we found rhTNFR:Fc markedly increased the mean artery pressure, stroke work, left ventricular, pressure at maximal dP/dt, left ventricular end systolic pressure, left ventricular end diastolic pressure and left ventricular (LV) +dP/dt (F) and −dP/dt of sepsis mice induced by LPS. LPS, a complex glycoprotein that resides in the outer membranes of gram-negative bacteria, has been implicated as a causative agent in apoptosis in cardiomyocytes and endothelial cells, a pivotal event that can lead to septic shock and associated syndromes. Jobe et al. [20] also demonstrated that rhTNFR:Fc attenuated the progression to heart failure, which was confirmed by in vivo experiments of this study. In addition, we also found that treatment with rhTNFR:Fc elevated cell viability and reduced cell apoptosis rate in embryonic rat cardiac myoblasts H9C2 cells.

To investigate the molecular mechanism of rhTNFR:Fc on myocardial protection of sepsis mice, the expression of miRNA was detected by real-time PCR. Wang et al. demonstrated that miR-27a relieved pulmonary inflammation and promotes survival of septic mice via up-regulating PPARγ and down-regulating TNF-α expression [15]. We hypothesized miR-27a may be involved in the regulation of rhTNFR:Fc on sepsis. In vivo experiment demonstrated that rhTNFR:Fc significantly reduced the miR-27a expression of heart in mice administrated with LPS. In cardiac myoblasts H9C2 cells, down-regulation of miR-27a was also observed in rhTNFR:Fc group. These results indirectly confirmed that TNF-α pathway is involved in the pathophysiology of sepsis, which was described previously [21].

In addition, there is increasing evidence that this innate response can be dramatically influenced by the cellular redox state.

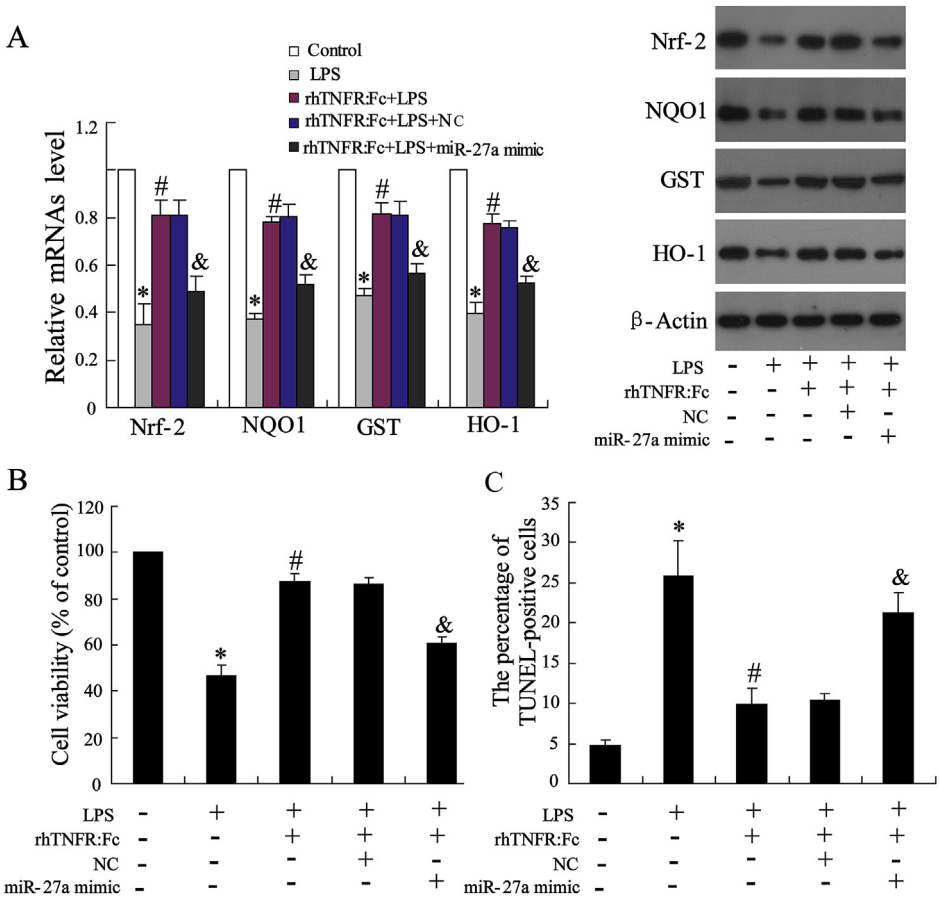

Fig. 4. miR-27a is involves in the effects of rhTNFR:Fc on Nrf2 pathway, cell survival and cell apoptosis in H9C2 cells. A) The expression levels of Nrf2 and its target genes in cells was detected; B) The cell viability was detected by MTT; B) cell apoptosis was detected by TUNEL assay. All results were repeated for three times. * VS control group, $P < 0.05$; # VS LPS group, $P < 0.05$; & VS rhTNFR:Fc + LPS + NC group.

Oxidase-dependent reactive oxygen species (ROS) play significant roles in the pathophysiology of sepsis [22]. Nrf2, a master regulator of antioxidant defenses, caused a dysregulation of innate immune response that resulted in greater mortality in a polymicrobial sepsis and LPS shock model [22]. Previous study revealed that cellular glutathione and other antioxidants activated by Nrf2 were critical for inflammatory signaling pathways in response to LPS and TNF-α [23]. Glutathion transfer enzyme (GST), quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO1) are antioxidant-associated defenses enzymes regulated by Nrf2 [24]. In present study, we found that rhTNFR:Fc significantly increased the levels of Nrf2, GST, NQO1 and HO-1 in LPS administrated mice. In H9C2 cells exposed to LPS, the same results were also observed. These findings are agreed with the conclusion indicated previously [25]. In addition, in vitro experiments we also found over-expression or silence miR-27a effectively regulated the expression of Nrf2 and its target genes. Luciferase reporter assay confirmed miR-27a reduced the binding of miR-27a to 3′-URT of Nrf2. These results suggest that regulation of Nrf2 pathway via miR-27a is involved in the LPS induced myocardial function.

In conclusion, our study demonstrated that rhTNFR:Fc protects myocardium against LPS-induced sepsis injury via increasing Nrf2 expression by miR-27a regulation. The present findings offer a theoretical basis for better application of rhTNFR:Fc to organ protection in sepsis.

Conflict of interest

All authors have no conflict of interest.

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Transparency document

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