Original Article

MicroRNA-214-3p Ameliorates LPS-Induced Cardiomyocyte Injury by Inhibiting Cathepsin B

(miR-214-3p / cathepsin B / oxidative stress / cardiomyocyte injury / lipopolysaccharide)

W. YAN, Y. FENG, Z. LEI, W. KUANG, C. LONG

The First Affiliated Hospital, Department of Cardiovascular Surgery, Hengyang Medical School, University of South China, Hengyang, Hunan, 421001, China

Abstract. Myocardial injury is a common complication of sepsis. MicroRNA (miRNA) miR-214-3p is protective against myocardial injury caused by sepsis, but its mechanism in lipopolysaccharide (LPS)-induced cardiomyocyte injury is still unclear. An AC16 cell injury model was induced by LPS treatment. Cell Counting Kit-8 and flow cytometry assay showed decreased cell viability and increased apoptosis in LPS-treated AC16 cells. The levels of caspase-3, Bax, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), myosin 6 (Myh6), myosin 7 (Myh7), reactive oxygen species (ROS), and malondialdehyde (MDA) were increased in LPS-treated AC16 cells, but the levels of Bcl-2 and superoxide dismutase (SOD) were decreased. MiR-214-3p was down-regulated and cathepsin B (CTSB) was up-regulated in LPS-treated AC16 cells. At the same time, miR-214-3p could target CTSB and reduce its expression. We also found that a miR-214-3p mimic or CTSB silencing could significantly reduce LPS-induced apoptosis, decrease ROS, MDA, caspase-3, and Bax and increase SOD and Bcl-2. CTSB silencing could significantly reduce ANP, BNP, Myh6, and Myh7 in LPS-treated AC16 cells. The effects of CTSB silencing were reversed by a miR-214-3p inhibitor. In summary, miR-214-3p could inhibit LPS-induced myocardial injury by targeting CTSB, which provides a new idea for myocardial damage caused by sepsis.

Introduction

Sepsis is a common disease in critical care medicine and the leading cause of death for severely infected people worldwide. It is characterized by systemic inflammation, multiple organ dysfunction and high mortality (Lelubre and Vincent, 2018). Sepsis-evoked myocardial injury is the most common severe sepsis and septic shock complication, related to poor prognosis and high mortality (Landesberg et al., 2012; L'Heureux et al., 2020). However, the specific pathogenesis of cardiomyopathy caused by sepsis is still unknown.

Research studies have revealed that lipopolysaccharide (LPS) is an important factor in myocardial injury caused by sepsis (Pfalzgraff and Weindl, 2019; Xin and Lu, 2020). LPS-induced cardiomyocyte injury is mainly achieved by excessive reactive oxygen species (ROS) (Gölz et al., 2014). Excessive ROS production will lead to increased oxidative stress, causing cell death and tissue injury (Su et al., 2019; Tan et al., 2019). At the molecular level, ROS-induced oxidative stress of cardiomyocytes promotes protein oxidation and lipid peroxidation (Haileselassie et al., 2019), which can lead to contractile dysfunction of cardiomyocytes, cardiac pump failure and hypoperfusion in distant organs or tissues (Chang et al., 2019). Consequently, it was necessary to investigate the specific mechanism of LPS-induced oxidative stress in cardiomyocytes.

MicroRNAs (miRNAs) are highly conserved endogenous small RNAs that do not encode proteins and can play their biological role by inhibiting genes at the post-transcriptional level (Liu et al., 2008b; Ho et al., 2016). Prior studies have shown that miR-214 regulates various cardiovascular diseases, comprising myocardial infarction, myocardial hypertrophy, pulmonary hypertension, coronary artery diseases and cardiomyopathy caused by sepsis (Zhao et al., 2017; Ge et al., 2018). Existing evi-

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Corresponding author: Chaozhong Long, The First Affiliated Hospital, Department of Cardiovascular Surgery, Hengyang Medical School, University of South China, No. 69, Chuanshan Avenue, Hengyang, Hunan, 421001, China. E-mail: chaozhonglong@163.com

Abbreviations: ANP – atrial natriuretic peptide, BNP – brain natriuretic peptide, CTSB – cathepsin B, LPS – lipopolysaccharide, miRNA, miR – microRNA, MDA – malondialdehyde, MUT – mutant, Myh6 – myosin 6, Myh7 – myosin 7, NC – negative control, ROS – reactive oxygen species, SOD – superoxide dismutase, WT – wildtype.

dence showed that miR-214 had protective effects on hypoxia/reoxygenation-induced cell damage and myocardial ischemia/reperfusion injury (Wang et al., 2016). Nevertheless, further exploring the underlying mechanism of miR-214-3p in cardiomyocyte injury caused by sepsis is necessary.

Cathepsin B (CTSB) is a typical cysteine lysosomal protease correlated with apoptosis, autophagy, oxidative stress and inflammation (Liu et al., 2020). CTSB can aggravate cardiomyocyte apoptosis and oxidative stress in doxorubicin-induced cardiomyocyte inujury (Bai et al., 2018; Liow and Chow, 2018). Whether CTSB participates in regulating LPS-mediated cardiomyocyte damage has not been studied. This study aimed to verify whether miR-214-3p affected LPS-induced cardiomyocyte injury by regulating CTSB.

Material and Methods

Establishment of a cardiomyocyte injury model and transfection

AC16 cells (human cardiomyocyte cell line, iCell Bioscience Inc., Shanghai, China) were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Burlington, MA) at a temperature of 37 °C in a 5 % CO_2 atmosphere. The LPS cardiomyocyte cell injury model was established by culturing AC16 cells with 0, 1, 2, and 3 μg/ml of LPS for 12, 24 and 36 hours. The 3 μg/ml LPS concentration induced the most expressed changes in AC16 cells. Thus, 3 μg/ml of LPS concentration was chosen for subsequent studies and referred to as the control group when not further manipulated.

A miR-214-3p mimic (5′-ACAGCAGGCACAGACA GGCAGU-3′), miR-214-3p inhibitor (5′-ACUGAAUG UCUGUGCCUGCUGU-3′), small interfering RNA specifically targeting CTSB (si-CTSB, 5′-TTGGAACTT CTGGACAAGAAA-3′), and corresponding controls (negative control (NC) mimic 5′-CGAUCGCAUCAG CAUCGAUUGC-3′, NC inhibitor 5′-CUAACGCAUG CACAGUCGUACG-3′, si-NC 5′-UUCUCCGAACGU GUCACGUTT-3′) were obtained from Changsha Abiowell Biotechnology Co. (Changsha, China). According to the manufacturer's protocol, AC16 cells exposed to 3μg/ml of LPS were transfected by the molecular constructs with the Lipofectamine 2000 reagent (Invitrogen, Waltham, MA) for 48 h.

Cell Counting Kit-8 (CCK-8) assay

AC16 cells were inoculated into 96-well plates at a density of 1×10^4 cells/well (100 μl/well). The cells were cultured with various concentrations of LPS (0, 1, 2, 3 μg/ml) for 12, 24 and 36 hours. Then, we removed the medicated medium and added 100 μl DMEM containing 10 % CCK-8 solution to each well. After incubation at 37 °C and 5 % CO_2 for 4 hours, a Bio-Tek microplate analyser (MB-530, Shenzhen Huisong Technology Development, Shenzhen, China) was applied to analyse the absorbance (OD) value at 450 nm.

Flow cytometry assay

Apoptosis of AC16 cells was determined by an apoptosis-detecting kit (KGA1030, KeyGEN BioTECH, Nanjing, China). We collected about 2×10^5 cells and added 500 μl of the buffer solution. Next, we added annexin V-FITC and propidium iodide to the culture maintained in the dark for 10 min. The cell apoptosis was detected in a flow cytometer (A00-1-1102, Beckman Coulter, Indianapolis, IN).

Western blot

The expression of apoptosis-related proteins and CTSB was studied by Western blot in AC16 cells. The cells were homogenized in precooled RIPA buffer (AWB0136, Changsha Abiowell Biotechnology) to obtain total protein. The total protein was resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. Primary antibodies Bax (1 : 4000, 50599-2-Ig, Proteintech, Rosemont, IL), Bcl-2 (1 : 600, 26593-1-AP, Proteintech), caspase-3 (1 : 1000, #9661, CST), CTSB (1 : 500, 12216-1-AP, Proteintech), and GAPDH (1 : 5000, 10494-1-AP, Proteintech) were incubated with the membrane overnight at 4 °C. Then, the secondary antibody HRP goat anti-rabbit IgG (1 : 7000, SA00001-2, Proteintech) was incubated with the membrane for 1.5 hours at room temperature. SuperECL Plus (AWB0005, Changsha Abiowell Biotechnology) was used to visualize the target band. Chemiluminescence Imaging System (ChemiScope6100, Clinx Science Instruments, Shanghai, China) was used for protein band imaging analysis.

Quantitative real-time PCR (qRT-PCR)

Total RNA of AC16 cells was extracted by Trizol (15596026, ThermoFisher, Waltham, MA). A reverse transcription kit (CW2569 and CW2141, Beijing Cowin BiotechCo., Beijing, China) was applied to reverse transcribe RNA to cDNA. qRT-PCR was performed in the following conditions using UltraSYBR Mixture (CW2601, Beijing Cowin Biotech), cDNA and primers: 95 °C for 10 min, 95 °C for 15 s, 60 °C for 30 s, with 40 cycles. *GAPDH* was an internal control of genes. *U6* served for internal control of miR-214-3p. The calculation formula was $2^{-\Delta\Delta Ct}$. The primers used in this study are given in Table 1.

Biochemical detection

AC16 cells (1×10^6) were placed in PBS (500 μl) and homogenized with an ultrasonic cell disruptor. The supernatant was produced from the cell homogenate by centrifugation (1500 × g, 10 min). The ROS, malondialdehyde (MDA) and superoxide dismutase (SOD) levels were measured with biochemical test kits (E004-1-1, A003-1, and A001-3, Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Table 1. Primer sequences

Gene	Sequences (5'-3')
ANP	forward: CTGCTCACTGCCCCTCGGA
	reverse: AGCCTCTTGCAGTCTGTCCCTA
BNP	forward: CAGCCTCCGCAGTCCCT
	reverse: TGCAGCTCCGACAGTTTGCC
Myh7	forward: GCCCACTTCTCCCTGATCCAC
	reverse: ACTTCTGATACAAGCCCACGAC
Myh6	forward: GAGCCAAGAGCCGTGACATT
	reverse: GGCAAGAGTGAGGTTCCCG
CTSB	forward: GCCCTCTTTCCATCCCCTGTCG
	reverse: CTTCAAGTAGCTCATGTCCACGTT
miR-214-3p	forward: ACAGCAGGCACAGACAGGCAGT
	reverse: GCTGTCAACGATACGCTACGTAA
GAPDH	forward: ACAGCCTCAAGATCATCAGC
	reverse: GGTCATGAGTCCTTCCACGAT
U6	forward: CTCGCTTCGGCAGCACA
	reverse: AACGCTTCACGAATTTGCGT

Dual-luciferase reporter assay

According to the manufacturer's instructions, CTSB-3'UTR WT (wildtype) or CTSB-3'UTR MUT (mutant) and miR-214-3p mimic or NC mimic were co-transfected into 293A cells (Changsha Abiowell Biotechnology) with Lipofectamine 2000 reagent (Invitrogen) for 48 hours. The luciferase activity was detected by a dual-luciferase assay kit (E1910, Promega, Madison, WI).

Data statistics and analysis

All experiments were carried out three times independently. All results were expressed as mean ± standard deviation. The statistical data analysis was performed by the GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA). An unpaired *t*-test determined the statistical significance between two groups, and one-way ANOVA determined three or more groups. Then, the Tukey's multiple comparison test was used for pairwise comparisons after the ANOVA. $P < 0.05$ was regarded as a statistically significant difference.

Results

Establishment of a cardiomyocyte injury model

We established a cardiomyocyte injury model induced by LPS (0, 1, 2, 3 μg/ml). The results of the CCK-8 assay reflected a cell viability decrease at increasing LPS concentration and treatment time (Fig. 1A). Flow cytometry assay results demonstrated that the apoptosis rate increased with increasing LPS concentration (Fig. 1B). Then, we measured the levels of apoptosis-related proteins (caspase-3, Bax and Bcl-2). The data showed that with increasing LPS concentration, the caspase-3

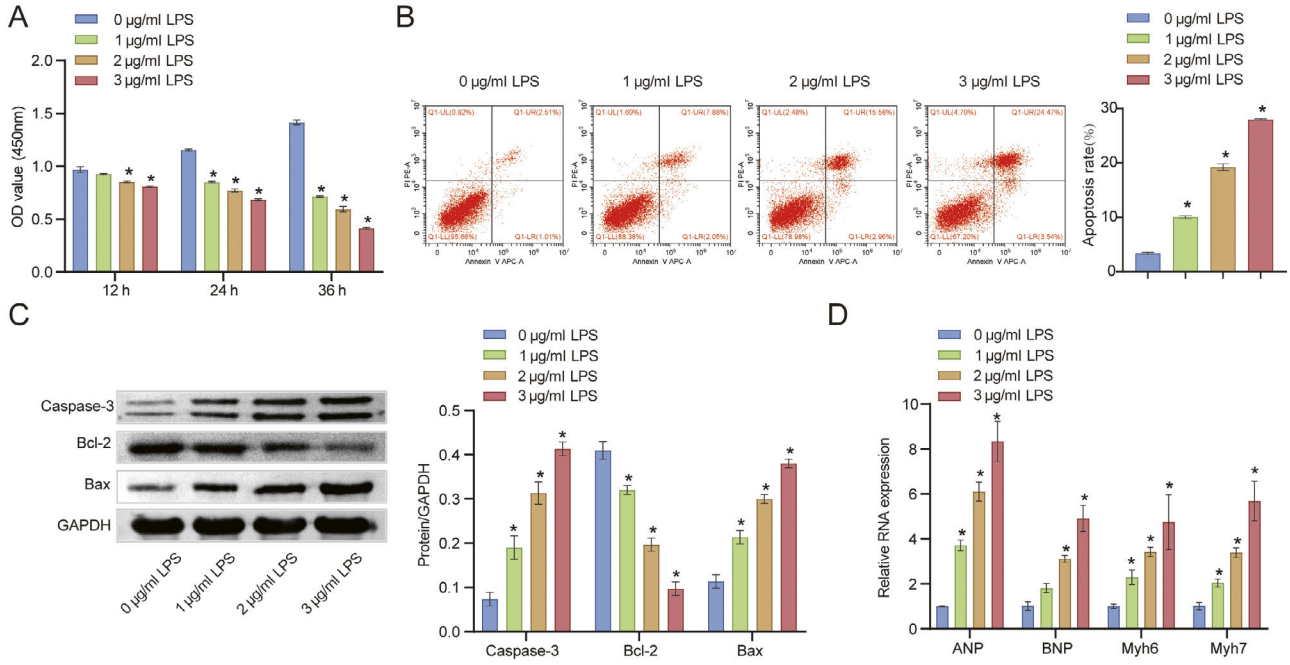

Fig. 1. Establishment of cardiomyocyte injury model. **A**. CCK-8 assay in AC16 cells measured cell viability at various concentrations of LPS (0, 1, 2, 3 μg/ml) for 6, 12 and 24 hours. **B**. Flow cytometry assay detected the apoptosis rate of AC16 cells after LPS acting at various concentrations (0, 1, 2, 3 μg/ml) for 24 hours. **C**. Caspase-3, Bax and Bcl-2 in AC16 cells after the treatment with various LPS concentrations (0, 1, 2, 3 μg/ml) for 24 hours. **D**. Gene expression of *ANP*, *BNP*, *Myh6* and *Myh7* in AC16 cells after the treatment with various concentrations of LPS (0, 1, 2, 3 μg/ml) for 24 hours. *P < 0.05 vs 0 μg/ml LPS.

and Bax levels were increased, while Bcl-2 was decreased (Fig. 1C). Detection of marker genes related to heart failure by qRT-PCR showed that ANP, BNP, Myh6 and Myh7 levels were also increased with growing LPS concentration (Fig. 1D). These results implicated that LPS could induce a cardiomyocyte injury model. Among the tested concentrations, 3 μg/ml LPS treatment for 24 hours had the most significant effect. Therefore, 3 μg/ml LPS was used as intervention conditions for subsequent experiments.

MiR-214-3p improved cardiomyocyte injury

MiR-214-3p has some protective effects against myocardial injury caused by sepsis (Ge et al., 2018). Our qRT-PCR results showed that miR-214-3p in LPS-treated AC16 cells was down-regulated compared with the untreated group (Fig. 2A). To study the association of miR-214-3p with cardiomyocyte injury, we up-regulated miR-214-3p levels in LPS-treated AC16 cells by transfection with a miR-214-3p mimic. The levels of ROS and MDA in cardiomyocytes over-expressing miR-214-3p were decreased, while SOD levels were increased compared with the control group (Fig. 2B–D). Flow cytometry assay results also showed that the apoptosis rate decreased after miR-214-3p over-expression (Fig. 2E). We discovered that after the treatment with the miR-214-3p mimic, caspase-3 and Bax levels decreased, while the expression of Bcl-2 increased. However, after the miR-214-3p inhibitor treatment, these results were opposite to the miR-214-3p mimic group (Fig. 2F). These data indicated that miR-214-3p could reduce LPS-induced oxidative stress and apoptosis, thus alleviating cardiomyocyte injury.

MiR-214-3p targeted CTSB

To verify the molecular relationships of miR-214-3p, we predicted the regulatory relationship between miR-214-3p and mRNAs using the TargetScanHuman database. The prediction results revealed a target site between miR-214-3p and CTSB, as shown in Fig. 3A. Next, we used a dual-luciferase reporter assay to verify whether miR-214-3p directly targeted mRNA. The relative luciferase activity of CTSB-WT cells transfected with the miR-214-3p mimic was lower than that of the cells transfected with the NC mimic. However, there was no difference in relative luciferase activity among different treatments in CTSB-MUT cells (Fig. 3B). The relative expression of CTSB in LPS-induced cardiomyocytes was higher than that in the untreated group. In addition, after over-expression of miR-214-3p, CTSB levels were reduced compared with the NC mimic group. After inhibition of miR-214-3p, CTSB levels were increased compared with the NC inhibitor group (Fig. 3C). The Western blot results correlated with the qRT-PCR results. The above data demonstrated that miR-214-3p could target and regulate the expression of CTSB.

MiR-214-3p improved cardiomyocyte injury by inhibiting CTSB

To verify whether CTSB participates in the protection of miR-214-3p against LPS-induced cardiomyocyte injury, we transfected si-NC and si-CTSB into AC16 cells. The results showed that CTSB expression was reduced in cardiomyocytes transfected with si-CTSB compared to that in the si-NC group (Fig. 4A and B). Under LPS exposure, the si-CTSB treatment reduced the expression of ROS and MDA but facilitated the expression of SOD. These results showed that silencing of CTSB could reduce LPS-induced oxidative stress in cardiomyocytes (Fig. 4C–E). Compared with the si-NC group, the apoptosis rate of the si-CTSB group cells decreased and the levels of caspase-3 and Bax also declined, while Bcl-2 increased (Fig. 4F and G). In addition, the heart failure indicator levels were detected by qRT-PCR. The results revealed that ANP, BNP, Myh6, and Myh7 in the si-CTSB group were significantly reduced compared with those in the control group (Fig. 4H and I). After transfection with the miR-214-3p inhibitor, the levels of these indicators were reversed. The above data showed that miR-214-3p might protect cardiomyocyte injury induced by LPS by partially regulating the expression of CTSB.

Discussion

Our work found that miR-214-3p in cardiomyocytes declined but CTSB levels increased after LPS incubation. Over-expression of miR-214-3p or silencing of CTSB could reduce LPS-induced oxidative stress and apoptosis, thus alleviating cardiomyocyte injury. However, these effects were reversed by miR-214-3p inhibition. Also, miR-214-3p could regulate the levels of CTSB in a targeted way. Therefore, we speculated that miR-214-3p over-expression could reduce LPS-induced cardiomyocyte injury by inhibiting the expression of CTSB.

Oxidative stress and apoptosis were considered important events of LPS-induced myocardial injury (Wang et al., 2013; Zhang et al., 2016). LPS combined with serum endotoxin-binding protein produced many inflammatory cytokines, leading to oxidative stress and excessive ROS production, eventually leading to cardiomyocyte dysfunction (Bao et al., 2018; Zhang et al., 2018; Qiu et al., 2019). Two μg/ml LPS treatment promoted apoptosis of cardiomyocytes (Xue et al., 2021). Our study showed that after LPS treatment of cardiomyocytes, the apoptosis increased significantly, apoptotic proteins caspase-3 and Bax were augmented, and Bcl-2 was reduced. Among the tested concentrations, 3 μg/ml LPS treatment for 24 hours had the most significant effect. In addition, ANP, BNP, Myh6 and Myh7 expression also increased with LPS concentration, which was consistent with previous studies (Liu et al., 2008a; Hinkelbein et al., 2017). These data indicated that LPS

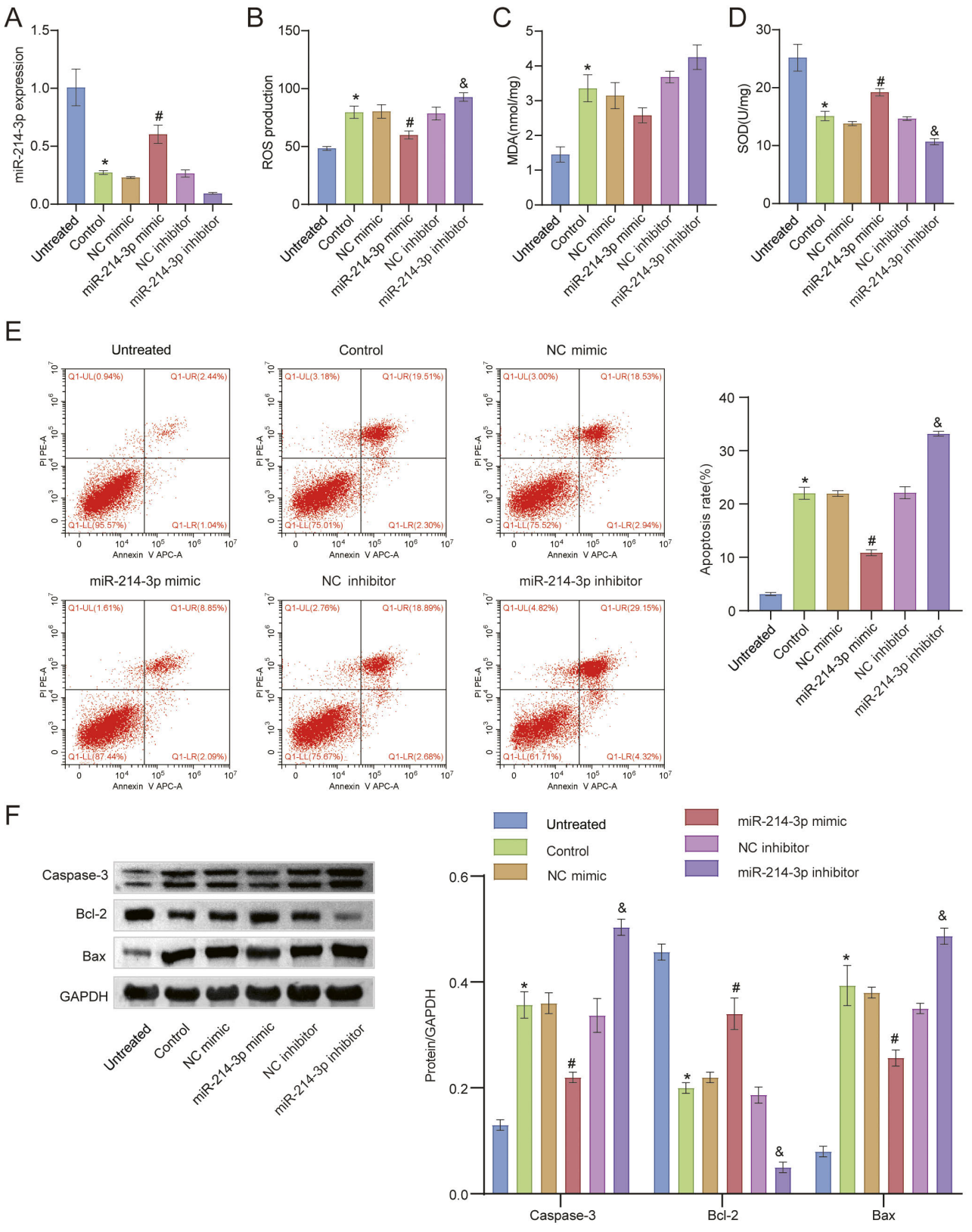

Fig. 2. MiR-214-3p improved cardiomyocyte injury. Transfection of C16 cells with the miR-214-3p mimic, miR-214-3p inhibitor and corresponding controls (NC mimic, NC inhibitor) for 48 h and treatment with LPS (3 μg/ml) for 24 hours. **A**. The levels of miR-214-3p were determined by qRT-PCR. **B**, **C**, and **D**. The ROS level, MDA content and SOD activity were measured by biochemical kits. **E**. Flow cytometry assay was applied to determine the apoptosis rate in each group. **F**. Caspase-3, Bax and Bcl-2 were detected by Western blot. *P < 0.05 vs untreated group. #P < 0.05 vs NC mimic group. &P < 0.05 vs NC inhibitor group.

might induce oxidative stress and apoptosis, leading to cardiomyocyte injury and dysfunction.

MiR-214 was considered an important regulator in the mechanism of cardiomyocyte injury (Liu et al., 2014; Park et al., 2016), which was involved in the occurrence and development of many heart diseases (Yang et al., 2019; Shirazi-Tehrani et al., 2020). MiR-214 expression is low in sepsis-induced cardiomyopathy (Li et al., 2022). This work revealed that the level of miR-214-3p in LPS-treated cardiomyocytes was down-regulated. MiR-214-3p was down-regulated in hypoxia-induced cardiomyocytes and promoted apoptosis (Gong et al., 2019). In addition, miR-214-3p could target CaMKII to inhibit oxidative stress damage to cardiac stem cells (Wang et al., 2018b). These data demonstrated that over-expression of miR-214-3p could inhibit LPS-induced cardiomyocyte injury, including inhibition of apoptosis, regulation of apoptosis-related genes and inhibition of heart failure gene expression. In addition, over-expression of miR-214-3p inhibited oxidative stress in LPS-induced cells. Based on these data, we speculated that miR-214-3p might be an important target in LPS-treated cardiomyocytes.

Recent studies have revealed that miR-124-3p could inhibit LPS-induced inflammatory injury by down-regulating expression of E2F3 in H9C2 cells (Chen et al., 2021). Our research revealed that miR-214-3p could target CTSB through the predicted site and confirmed their targeting relationship by the dual-luciferase reporter assay. A previous study has shown that CTSB aggravated viral myocarditis induced by coxsackievirus B3 by activating inflammation (Wang et al., 2018a). Knock-out of CTSB alleviated angiotensin II-induced cardiomyocyte hypertrophy and apoptosis (Wu et al., 2015). Our study has found that transfection of si-CTSB could inhibit LPS-induced cardiomyocyte injury. The inhibition of miR-214-3p could reverse these effects.

Generally speaking, our research indicated that miR-214-3p might inhibit cell apoptosis and reduce oxidative stress through targeted reduction of CTSB expression, thus reducing LPS-induced cardiomyocyte injury. These data have shown that the miR-214-3p/CTSB axis might be a valuable target in LPS-injured cardiomyocytes, providing a new idea for treating sepsis-induced cardiomyopathy.

Competing interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

Author contributions

Study conception and design: C.L.; data collection: W.Y., Y.F., Z.L., W.K.; analysis and interpretation of results: W.Y., Y.F., Z.L., W.K.; draft manuscript preparation: W.Y. All authors approved the final manuscript version.

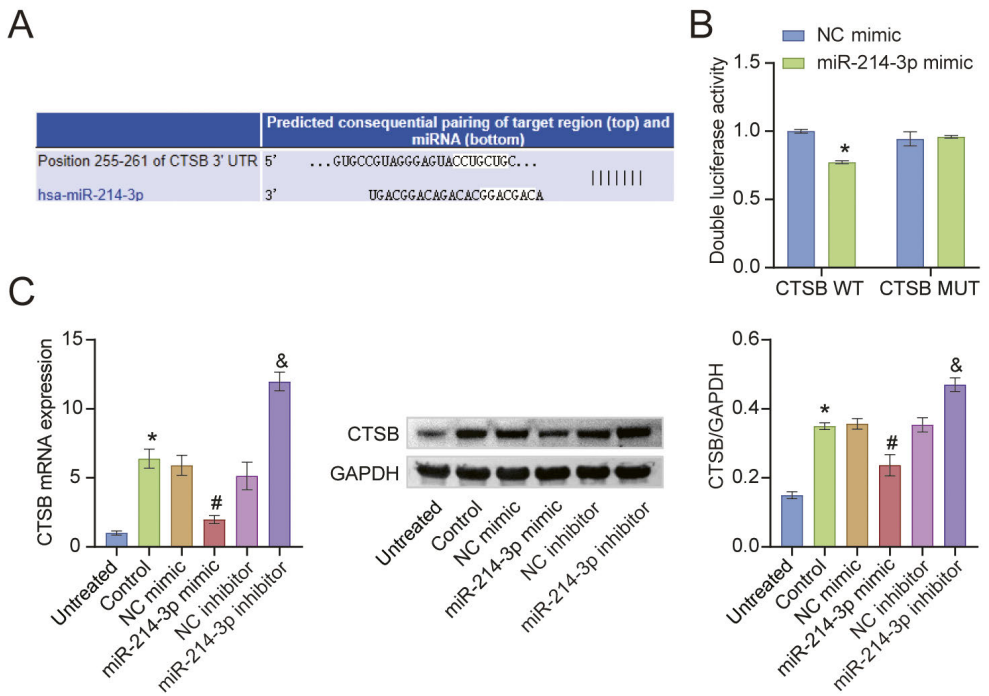

Fig. 3. MiR-214-3p targeted CTSB. **A.** The target site of miR-214-3p and CTSB. **B.** Dual-luciferase reporter assay using 293A cells confirmed that miR-214-3p targets mRNA. **C.** Transfection of AC16 cells with the miR-214-3p mimic, miR-214-3p inhibitor and corresponding controls (NC mimic, NC inhibitor) for 48 h and treatment with LPS (3 μg/ml) for 24 hours. The expression of CTSB in AC16 cells was evaluated by qRT-PCR and Western blot. *$P < 0.05$ vs untreated group. $^{\#}P < 0.05$ vs NC mimic group, $^{\&}P < 0.05$ vs NC inhibitor group.

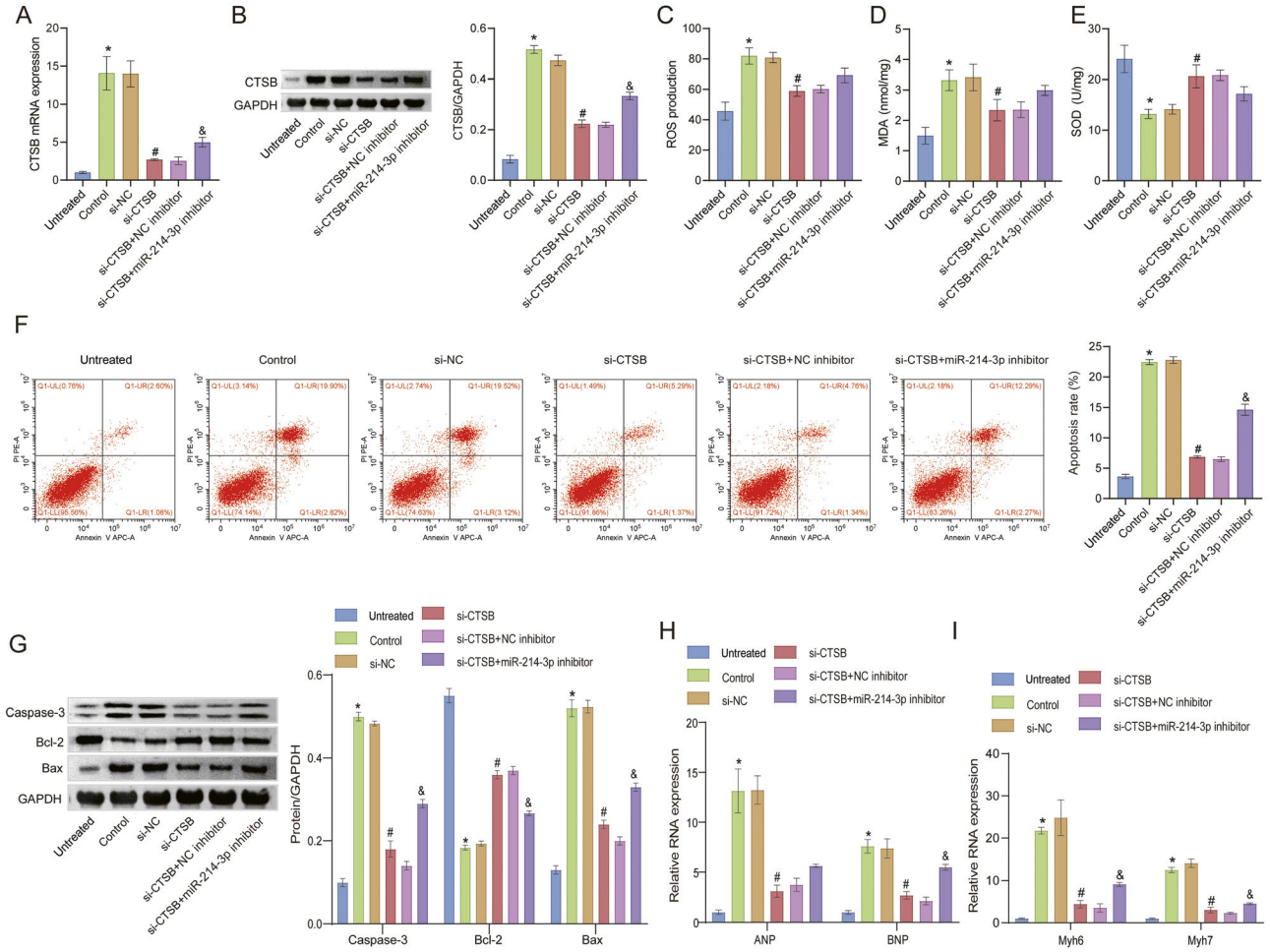

Fig. 4. MiR-214-3p improved cardiomyocyte injury by inhibiting CTSB. Transfection of AC16 cells with si-CTSB, miR-214-3p inhibitor and corresponding controls (si-NC, NC inhibitor) for 48 h and treatment with LPS (3 μg/ml) for 24 hours. **A** and **B**. The expression of CTSB was evaluated by qRT-PCR and Western blot in each group. **C**, **D** and **E**. Biochemical kits were used to determine ROS, MDA and SOD levels. **F.** The apoptosis rate was evaluated by flow cytometry assay in each group. **G**. Caspase-3, Bax and Bcl-2 were determined by Western blot. **H** and **I**. The gene expression of *ANP*, *BNP*, *Myh6* and *Myh7* was detected by qRT-PCR. *P < 0.05 vs untreated group. #P < 0.05 vs si-NC group. &P < 0.05 vs si-CTSB + NC inhibitor group.

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