**MiR-210-3p Enhances Cardiomyocyte Apoptosis and Mitochondrial Dysfunction by Targeting the NDUFA4 Gene in Sepsis-Induced Myocardial Dysfunction**

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**Summary**

Sepsis-induced myocardial dysfunction (SIMD) is a common complication with high incidence rates in sepsis patients. This study aimed to investigate the roles of miR-210-3p in regulating cardiomyocyte apoptosis and mitochondrial dysfunction associated with SIMD pathogenesis.

A rat sepsis model was established by cecal ligation and puncture. Serum inflammatory factors, myocardial tissue apoptosis, and expression of miR-210-3p were evaluated. In vitro, miR-210-3p expression in H9C2 cells was altered by transfection with its mimics or inhibitors. H9C2 viability was assessed via CCK-8 assay, and reactive oxygen species (ROS) production and apoptosis were detected through flow cytometry. The targeting regulatory relations between miR-210-3p and NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4 (NDUFA4) were validated by dual luciferase reporter assay.

The rat sepsis model showed increased serum TNF-α and IL-6 levels, significant myocardial tissue injuries and apoptosis with decreased Bcl-2 and increased Caspase-1 protein levels. In vitro, septic rat serum suppressed viability, promoted ROS production and apoptosis, impaired COX IV activities and increased cytochrome release in H9C2 cells. The expression of miR-210-3p was greatly increased in myocardial tissues of septic rats and septic serum-treated H9C2 cells. miR-210-3p directly binds to the 3’ UTR of the NDUFA4 gene. Septic rat serum suppressed NDUFA4 and Iron-Sulfur Cluster Assembly Protein U gene expressions in H9C2 cells. The above cellular and molecular alterations in H9C2 cells induced by septic serum were enhanced by miR-210-3p mimics and abrogated by miR-210-3p inhibitors.

miR-210-3p promoted SIMD pathogenesis by targeting NDUFA4 to enhance cardiomyocyte apoptosis and impair mitochondrial function.

**Key words:** Reactive oxygen species, Inflammation, Myocardium, Mitochondrion, MicroRNA
model and to regulate the development of sepsis-associated cardiac dysfunction by targeting the SH3 domain-containing protein 2 gene.15 The specific suppression of miR-199a repressed the progression of acute lung injury induced by sepsis through targeting of the expression of sirtuin 1 in alveolar macrophages.16 In addition, miR-210 was differentially expressed in sepsis-induced acute kidney injury.17 However, its association with SIMD remains unexplored.

NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4 (NDUFA4), alternatively known as COX subunit FA4, has been characterized as a new subunit of the mammalian mitochondrial cytochrome-c oxidase (COX) complex, associated with HIV infection and the electron transport chain.14-16 Mutations in the NDUFA4 gene and the resultant mitochondrial dysfunctions have been closely implicated in the pathogenesis of multiple human diseases such as diabetes mellitus and neurological disorders.13,18 A recent report showed that the expression of NDUFA4 could be directly targeted and suppressed by miR-147b in colorectal cancer (CRC) cells, leading to decrease of CRC cell proliferation, cell cycle progression, and increase of apoptosis.19 Moreover, miR-7 was also demonstrated to regulate the growth and metastasis of lung cancer cells by directly associating with NDUFA4, a process mediated by the Akt (protein kinase c) and ERK (extracellular signal-regulated kinase) signaling pathways.20 More importantly, the expression of NDUFA4 as well as that of Iron-Sulfur Cluster Assembly Protein U (ISCU) associated with mitochondrial functions, were also shown to be targeted by miR-210, which was critically involved in mitochondrial energy metabolism regulation during clear cell renal cell carcinoma development and mitochondrial dysfunction in preeclampsia and first trimester placental dysfunction.21,22 However, little is known about the targeting of ISCU and NDUFA4 by miR-210 in SIMD.

In this study, we aimed to investigate the effects of serum collected from a rat sepsis model, established using the cecal ligation and puncture method, on the cellular functions of and differential expressions of miRNAs in the rat myocardial cell line H9C2, followed by further analysis of the roles of miR-210-3p and its targeting of NDUFA4 in the above processes.

Methods

Rat sepsis model and serum collection: Male specific pathogen free-grade Sprague-Dawley (SD) rats bought from the Experimental Animal Center of the Central South University (Changsha, China) were used for establishment of a sepsis model as previously described.23 Briefly, a total of 16 SD rats were randomly divided into the Sham group (n = 8) and the Sepsis group (n = 8). After being anesthetized by intraperitoneal injection of 10% chloral hydrate, the abdominal cavity of the rats in the Sepsis group was opened with a median abdominal incision, and the cecum was then separated, ligated with a No. 4 silk, punctured twice at the distal end, and placed back in the abdominal cavity, followed by closing the abdominal incision. For the Sham group, the same surgical operations were performed as in the Sepsis group, except that no ligature and puncturing were made. Twenty-four hours after the operation, all rats were sacrificed after anesthesia and sera were prepared from the collected blood samples for further assays. The study was approved by the Animal Care and Use Committee of the Hainan General Hospital in advance.

ELISA: The relative levels of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) in the rat serum samples were detected by enzyme-linked immunosorbent assay (ELISA) using the Rat TNF-α ELISA KIT (#SEKR-0009) and the Rat IL-6 ELISA KIT (#SEKR-0005) purchased from the Solarbio Life Science Company (Beijing, China) following the producer's instructions. At least three biological replicates were done for statistical analysis of TNF-α and IL-6 levels.

Histological evaluations: Histological alterations and cell apoptosis in the rat myocardial tissues were evaluated by the hematoxylin and eosin (H&E) staining and the terminal deoxynucleotidyl transferase mediated dUTP nick end-labeling (TUNEL) assay, respectively. Briefly, rat myocardial tissues were fixed with 4% paraformaldehyde and embedded in routine paraffin. After being de-waxed using dimethyl benzene and hydrated with ethanol, the rat myocardial tissue slides were subjected to hematoxylin staining for 5 mins and eosin staining for 3 mins. Finally the slides were de-waxed again and neutral balata fixed. Further, TUNEL assay was performed by using the TdT Frag DNA Fragmentation Imaging Kit (Sigma-Aldrich) following the manufacturer’s instructions. The nucleus of apoptotic myocardial cells containing internucleosomal DNA cleavage were stained green during the TUNEL assay.

Immunohistochemistry: The expression of B-cell lymphoma 2 (Bcl-2) and caspase-1 in the rat myocardial tissues were analyzed by immunohistochemistry. Briefly, rat myocardial tissue slides prepared as described above were dehydrated and washed with TBST twice, and then blocked at room temperature with 5% BSA solution for 15 minutes. The slides were then washed with PBS and incubated with diluted primary antibodies targeting Bcl-2 (#ab59348; Abcam; 1: 500) or caspase-1 (#ab74279; Abcam; 1:500) overnight at 4°C. After washing with PBS for 5 minutes, the rat myocardial tissue slides were incubated with horseradish peroxidase-conjugated secondary antibodies (1:500) for 1.5 hours at room temperature, developed with DAB, stained with hematoxylin, dehydrated, cleared with xylene, and finally observed under a fluorescence microscope.

Cell culture, treatment, and transfection: The rat cardiomyocyte H9C2 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Thermo Fisher Scientific) containing 10% fetal bovine serum (Gibco) and 1% penicillin and streptomycin (Sigma-Aldrich) at 37°C in a humidified atmosphere with supply of 5% CO₂. For analysis of the impacts of sepsis on myocardial dysfunction, H9C2 cells were cultured in DMEM supplemented with 10% rat serum collected from the Sepsis or Sham group. For modulation of miR-210 expression, H9C2 cells were transfected with miR-210 mimics (CTG TGC GTG TGA CAG CCG CTG...
tion reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. 

Cell viability and apoptosis: The viabilities of cultured H9C2 cells were measured by the Cell counting kit-8 (CCK-8; Dojindo, Japan) assay according to the protocols provided by the manufacturer. Briefly, H9C2 cells at the logarithmic growth phase (10^5 cells) were precipitated by centrifugation at 700 × g for 6 minutes, rinsed with PBS, resuspended in annexin binding buffer, incubated in the dark for 15-18 minutes at room temperature, and finally analyzed by flow cytometry. Three biological replicates were performed.

Cell apoptosis and reactive oxygen species (ROS) contents: The percentages of apoptotic H9C2 cells were measured by labeling with the Dead Cell Apoptosis Kit (#AB-113851; Abcam) according to the manufacturer’s instructions. After incubation with primary and secondary antibodies (horseradish peroxidase-conjugated), the protein levels were finally determined by developing using an ECL Substrate Kit (#ab133406; Abcam). GAPDH was used as an internal control. The primary antibodies used in this study were the following: anti-p65 (#ab16502; Abcam), anti-IL-1β (#ab234437; Abcam), and anti-ASC (#ab47092; Abcam), and anti-GAPDH (#ab8245; Abcam).

Quantitative RT-PCR (qRT-PCR): The mRNA and miRNA levels in H9C2 cells were analyzed by the qRT-PCR method. Briefly, total RNA samples were extracted from H9C2 cells using the Invitrogen TRIzol Reagent (#15596018; Thermo Fisher Scientific) following the manufacturer’s instructions. Then, cDNA samples were prepared from 2.5 μg total RNA samples by reverse transcription using the QuantiTect Reverse Transcription Kit (#205311; QIAGEN) according to the manufacturer’s instructions. The expression of GAPDH and U6 were detected as the internal standards of protein in each group were separately boiled at 100°C for 5 minutes in protein loading buffer. Subsequently, the proteins were separated into SDS-PAGE gels (10%-12%), transferred onto PVDF membranes (Millipore), and blocked with 5% BSA solution for 1-2 hours at room temperature. After incubation with primary and secondary antibodies (horseradish peroxidase-conjugated), the protein levels were finally determined by developing using an ECL Substrate Kit (#ab133406; Abcam). GAPDH was used as an internal control. The primary antibodies used in this study were the following: anti-p65 (#ab16502; Abcam), anti-IL-1β (#ab234437; Abcam), and anti-GAPDH (#ab8245; Abcam).

Western blotting: Total protein samples from cultured H9C2 cells were prepared using the Total Protein Extraction Kit (#AMJ-KT0007; AmyJet Scientific, Wuhan, China) following the producer’s instructions. Protein concentrations were measured using the BCA method and 25-35 μg of protein in each group were separately boiled at 100°C for 5 minutes in protein loading buffer. Subsequently, the proteins were separated into SDS-PAGE gels (10%-12%), transferred onto PVDF membranes (Millipore), and blocked with 5% BSA solution for 1-2 hours at room temperature. After incubation with primary and secondary antibodies (horseradish peroxidase-conjugated), the protein levels were finally determined by developing using an ECL Substrate Kit (#ab133406; Abcam). GAPDH was used as an internal control. The primary antibodies used in this study were the following: anti-p65 (#ab16502; Abcam), anti-IL-1β (#ab234437; Abcam), and anti-GAPDH (#ab8245; Abcam).

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Dual luciferase reporter assay: The binding of miR-210-3p to the 3’ UTR region of NDUF4 was validated by the dual luciferase reporter assay using the psiCHECK-2 plasmids and related kit (#E1910; Promega), according to the manufacturer’s instructions. Briefly, the sequence of the

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**Table.** Primer Sequences Used for qRT-PCR

| Name    | Primer sequences (5’–3’) |
|---------|------------------------|
| GAPDH F | CCTGCTCTCATAGACAAAGATGGT |
| GAPDH R | GGGTAGCTAATGGAACACATG |
| ISCU F  | CAGGGCTTACCCACAAAGAGGT |
| ISCU R  | TCATGACGTCACCACATATG |
| NDUF4 A F | CAAGGCCAGAACACATCCACG |
| NDUF4 A R | ATGCCAAAGGCCATCACATA |
| U6 F | CTGGCTTGGCAAGACA |
| U6 R | AACGCTTACGAATTTTCG |
| miR-29a-5p RT | CTCAACTGTGTCGTCGAGGCATAAGTCGTAAGCAC |
| miR-29a-3p F | ACACTCCAGCTGGGACTTTTGTGGTT |
| miR-37a-3p RT | CTCAACTGTGTCGTCGAGGCATAAGTCGTAAGCAC |
| miR-18a-5p RT | CTCAACTGTGTCGTCGAGGCATAAGTCGTAAGCAC |
| miR-18a-5p F | ACACTCCAGCTGGGACTTTTGTGGTT |
| miR-210-3p RT | CTCAACTGTGTCGTCGAGGCATAAGTCGTAAGCAC |
| miR-210-3p F | ACACTCCAGCTGGGACTTTTGTGGTT |
| miR-322-5p RT | CTCAACTGTGTCGTCGAGGCATAAGTCGTAAGCAC |
| miR-322-5p F | ACACTCCAGCTGGGACTTTTGTGGTT |
| miR-320-3p RT | CTCAACTGTGTCGTCGAGGCATAAGTCGTAAGCAC |
| miR-320-3p F | ACACTCCAGCTGGGACTTTTGTGGTT |
| All miRNA R | CTCAACTGTGTCGTCGTAAGCAC |

F indicates forward primer; R, reverse primer; and RT, Stem-loop reverse transcription primer.
wild-type NDUFA4 3’ UTR region (NDUFA4 F: CCC TCG AGG TGA ACG TGG ACT ACA GCA AAC; NDUFA4 R: ATT TGC GCC CGC CAC ACC CTT ATT AAA GCA GCA TA) and the mutant NDUFA4 3’ UTR (NDUFA4-MUT-F: AAG GTC TTT CAG AAG CCA TCG GTG TTT TTT CAC ACT TAA GCA GGA AC; NDUFA4-MUT-R: GTT CCT GCT TAA GTG GAA AAA ACA CAG GAT GGC TTC TGG AAC ACC TT) amplified by RT-PCR were ligated to psiCHECK-2 plasmids, which were then transfected into H9C2 cells using the Lipofectamine™ 3000 transfection reagent (Thermo Fisher Scientific) following the manufacturer’s instructions, together with miR-210-3p mimics or a negative control as specified. The luciferase activities of the cell lysates were finally measured using a GloMax-20/20 luminometer.

**Immunofluorescence:** H9C2 cell slides were fixed with 4% formaldehyde solution for 12 minutes at room temperature, washed with PBS solution three times, blocked with 5% BSA solution for 2 hours at room temperature, and incubated with antibodies targeting NDUFA4 (#ab 129752; Abcam; 1: 500) overnight at 4°C. The slides were then washed with PBS, incubated in the dark with fluorescent-conjugated secondary antibodies for 1-2 hours at room temperature, and mounted with the Prolong Gold Antifade Reagent (#9071; CST). The fluorescence intensities were finally evaluated by observation under a fluorescence microscope.

**COX IV activity:** The activities of COX IV were detected using the COX Activity Kit (#KA0323; Abnova Corporation) according to the manufacturer’s instructions. Briefly, H9C2 cells grown in 96-well plates were incubated with Tris-phenol buffer, hematin solution, and COX-I or COX-II preparations for 5 minutes at room temperature and incubated with 25 μL NSAID inhibitor for 50 minutes at room temperature. Subsequently, the H9C2 cells were incubated with 50 μL cold COX Chemiluminescent Substrate and cold arachidonic acid solution for 5 seconds, and immediately measured using luminometer in the Relative Light Units.

**Statistical analysis:** Quantitative data produced from at least biological repeats were analyzed for statistical significance with the SPSS 20.0 software. Student’s t-test and ANOVA were performed to evaluate differences between two or more groups, respectively. A P-value of < 0.05 was set as the threshold for significant differences.

**Results**

**Establishment of the rat sepsis model with myocardial injuries:** In order to explore the mechanisms underlying myocardial dysfunction induced by sepsis, we first established a rat sepsis model using the cecal ligation and puncture method. An ELISA assay showed that the levels of two inflammatory factors, IL-6 and TNF-α, in the serum of sepsis model rats were much higher than those in the control group (Figure 1A). In addition, H&E staining showed significant tissue injuries in the myocardial tissues of the sepsis model group compared with the control group (Figure 1B). Moreover, the TUNEL method showed significantly enhanced cell apoptosis in the myocardial tissues of the sepsis model group, in contrast to the control group (Figure 1C, D). Consistently, the protein levels of Bcl-2 in the rat myocardial tissues of the sepsis group were remarkably lower compared with the control group (Figure 1E, F). On the other hand, the caspase-1 protein levels in the myocardial tissues of the sepsis model group were significantly higher than in the control group (Figure 1G, H). The elevated inflammatory factors, enhanced myocardial tissue damage, and cardiomyocyte apoptosis indicated the successful induction of sepsis in the rats.

**Septic rat serum enhanced apoptosis and elevated miR-210-3p expression in H9C2 cells:** To investigate the cellular alterations of myocardial cells during sepsis, we treated cultured H9C2 cells with serum collected from septic rats. We found that the viabilities of H9C2 cells were greatly suppressed by the septic rat serum compared with that from the control group (Figure 2A). Additionally, the ROS levels in H9C2 cells treated with septic serum were significantly higher than those treated with serum from the control group (Figure 2B). By flow cytometry, we showed that the apoptosis of H9C2 cells was greatly enhanced by treatment with septic rat serum compared with the control group (Figure 2C). Consistently, we found by western blot that the abundances of the nuclear factor-kappa B (NF-κB) p65 subunit, NLR family, pyrin domain-containing 3 (NLRP3), cleaved caspase-1, apoptosis-associated speck-like protein (ASC), and IL-1β proteins in H9C2 cells were all remarkably elevated in comparison with the control group (Figure 2D-G). Contrarily, the Bcl-2 protein levels in H9C2 cells treated with septic serum were significantly lower than in the control group (Figure 2F, G). Importantly, we demonstrated here that the expressions of miR-378a, miR-181a, and miR-210-3p were substantially increased in H9C2 cells treated with septic rat serum, in contrast to the control group (Figure 2H). Moreover, the significant increase of miR-210-3p expression was validated in the myocardial tissues of septic rats compared with the control group (Figure 2I). The significant alterations in miRNAs levels suggested their roles in the myocardial dysfunctions induced by sepsis pathogenesis.

**miR-210-3p directly targets NDUFA4 gene expression in H9C2 cells with sepsis-induced mitochondrial dysfunction:** For better understanding of the molecular mechanisms underlying SIMD, we analyzed the downstream genes that could be potentially targeted by miR-210-3p in H9C2 cells. Through bioinformatics analysis, we predicted that miR-210-3p mimics induced a sharp decrease in the luciferase activity in H9C2 cells expressing the wild-type NDUFA4 sequence but not in those expressing a mutant version of NDUFA4, supporting the direct association between miR-210-3p and NDUFA4 (Figure 3B). We further found that the expressions of both the NDUFA4 and ISCU, implicated in mitochondrial function, were significantly reduced in H9C2 cells treated with septic rat serum, compared with the control group (Figure 3C-E). The sharp decrease in NDUFA4 expression in H9C2 cells cultured with septic rat serum was also confirmed by im-
Figure 1. Induction of sepsis and myocardial tissues injuries in rat model. A: Relative IL-6 and TNF-α levels in the serum of rats of the control and sepsis groups. IL-6 and TNF-α levels were detected by the ELISA method. B: Myocardial tissue injuries in rats of the sepsis group. Rat myocardial tissue injuries were analyzed by H&E staining. C, D: Enhancement of cardiomyocyte apoptosis in the myocardial tissues of the sepsis group. Cell apoptosis in rat myocardial tissues was assessed by the TUNEL method. E-H: Alterations in Bcl-2 and caspase-1 expression in the myocardial tissues of the sepsis group. Immunohistochemistry was used to detected Bcl-2 and caspase-1 expression. ***P < 0.001.

miR-210-3p mediated the septic serum-induced apoptosis and mitochondrial dysfunctions in H9C2 cells: To clarify the roles of miR-210-3p in the above processes, we overexpressed or repressed the expression of miR-210-3p in H9C2 cells under septic rat serum treatment. As shown in Figure 4A, miR-210-3p was highly expressed in H9C2 cells treated with septic rat serum compared with the control cells. Meanwhile, compared with control cells, miR-210-3p level was overexpressed in cells transfected with miR-210-3p mimics and significantly down-regulated in cells transfected with miR-210-3p inhibitor. First, we showed that the inhibition of H9C2 cell viability by septic rat serum was greatly aggravated by miR-210-3p mimics, but significantly rescued by transfection with miR-210-3p

munofluorescence (Figure 3F). In agreement with these results, we further showed that the COX IV activities of H 9C2 cells were greatly impaired by treatment with septic rat serum (Figure 3G). The cytochrome C content in H9C 2 cells also significantly increased upon treatment with serum from septic rats (Figure 3H, I). These results indicated that miR-210-3p could repress NDUFA4 expression by directly binding its 3' UTR region in H9C2 cells, which showed mitochondrial dysfunction associated with sepsis development.
Role of Mir-210 in sepsis-induced cardiac injury

Figure 2. Septic rat serum promoted apoptosis and changed miRNA expression in H9C2 cells. A: Repressed viabilities of H9C2 cells caused by serum from septic rats. The CCK-8 assay was performed to detect H9C2 cell viability. B: Elevated ROS contents in H9C2 cells following treatment with septic rat serum. ROS contents in H9C2 cells were measured by flow cytometry. C: Promotion of H9C2 cell apoptosis by serum collected from septic rat group. Cell apoptosis was evaluated through flow cytometry. D-G: Alterations in NF-κB p65, NLRP3, Bcl-2, ASC, and IL-1β and caspase-1 cleavage in H9C2 cells induced by septic rat serum. Protein levels in H9C2 cells were detected by western blot. HDAC-1 and GAPDH were used as internal controls. H: Differential expression of representative miRNAs in H9C2 cells treated with serum from septic rats. I: Great increase in miR-210-3p expression in myocardial tissues in septic rats. The relative expression of miRNAs was analyzed by qRT-PCR. ***P < 0.001.

Inhibitors (Figure 4B). Moreover, miR-210-3p mimics further promoted the production of ROS in H9C2 cells treated with serum from septic rats, whereas miR-210-3p inhibitors suppressed ROS production (Figure 4C). Flow cytometry demonstrated that miR-210-3p mimics further promoted the apoptosis of H9C2 cells under septic serum treatment, while miR-210-3p inhibitors suppressed the septic serum-induced H9C2 cell apoptosis (Figure 4D). Moreover, the alterations in the NLRP3, Bcl-2, cleaved caspase 1, ASC, IL-1β, ISCU, and NAUFA4 protein levels in H9C2 cells induced by septic rat serum were all enhanced by miR-210-3p mimics and suppressed by miR-210-3p inhibitors (Figure 4E, F). Furthermore, qRT-PCR, western blotting, and immunofluorescence assays confirmed that miR-210-3p mimics decreased and miR-210-3p inhibitors promoted NAUFA4 and ISCU expression in H9C2 cells treated with septic rat serum (Figure 5A-D). Consistently, the COX IV activities in H9C2 cells treated with septic rat serum were repressed by miR-210-3p mimics and enhanced by miR-210-3p inhibitors (Figure 5E). In addition, the cytochrome C content in H9C2 cells under septic serum treatment was increased by miR-210-3p mimics and reduced by miR-210-3p inhibitors (Figure 5F, G). These results showed that sepsis-induced myocardial cell apoptosis and mitochondrial dysfunctions were mediated by miR-210-3p.
The SIMD, featured by myocardial systolic and diastolic dysfunctions, is a severe complication commonly presented by septic patients with high incidence. Previous research demonstrated that the pathogenesis of myocardial dysfunction caused by sepsis was mediated by multiple miRNAs and resultant alteration of functional gene expression. miR-210-3p was recently implicated in the development of sepsis-induced acute kidney injury; however, little is known about its expression and functions in SIMD. In the present study, we first established a rat sepsis model by cecal ligation and puncture and serum samples from these septic rats were used to treat rat cardiomyocyte H9C2 cells. We showed here that septic rat serum significantly suppressed the viability and promoted ROS production and apoptosis in H9C2 cells, which was accompanied by great expressional increases of multiple miRNAs such as miR-210-3p. Furthermore, we validated a bioinformatic prediction that miR-210-3p repressed NDUFA4 expression in H9C2 cells via directly targeting its 3′ UTR region, thus resulting in repressed ISCU expression and impaired COX IV activities. Finally, we confirmed the roles of miR-210-3p expressional increase in septic rat serum-induced changes in H9C2 viability, ROS production, apoptosis, and mitochondrial dysfunction by transfecting these cells with miR-210-3p mimics or inhibitors. This study established a new miR-210-3p/NDUFA4/ISCU axis underlying the pathogenesis of myocardial dysfunctions during sepsis development.
Figure 4. Roles of miR-210-3p in septic serum-induced H9C2 cell apoptosis. A: Relative expression of miR-210-3p in septic serum-treated H9C2 cells transfected with/without miR-210-3p mimics or inhibitors. B: Viabilities of H9C2 cells transfected with miR-210-3p mimics or inhibitors under septic serum treatment. Cell viabilities were quantified with the CCK-8 assay. C: Effects of miR-210-3p mimics or inhibitors on ROS production in H9C2 cells treated with septic rat serum. D: Effect of miR-210-3p mimics or inhibitors on ROS production in H9C2 cells treated with septic rat serum. H9C2 cell apoptosis was measured by flow cytometry. E, F: Alterations of apoptosis and mitochondrial function-related proteins in H9C2 cells treated with septic rat serum after miR-210-3p mimics or inhibitor transfection. Protein levels were relatively compared by western blot using GAPDH as the internal standard. ***P < 0.001, versus Control. ###P < 0.001, versus Sepsis.
miR-210-3p regulates the mitochondrial respiratory chain function by inhibiting NDUF4. A: Relative mRNA levels of ISCU and NDUF4 in H9C2 cells transfected with miR-210-3p mimics or inhibitors under septic rat serum treatment. B, C: Relative protein levels of ISCU and NDUF4 in H9C2 cells transfected with miR-210-3p mimics or inhibitors under septic rat serum treatment. D: NDUF4 protein levels in H9C2 cells transfected with miR-210-3p mimics or inhibitors under septic rat serum treatment showed by Immunofluorescence. E: Impact of miR-210-3p mimics or inhibitors on COX IV activities in H9C2 cells treated with septic rat serum. F, G: Alterations in cytochrome C levels in H9C2 cells transfected with miR-210-3p mimics or inhibitors and treated with septic rat serum. ***P < 0.001, versus Control. **P < 0.01, ***P < 0.001, versus Sepsis.

Previous reports showed that the promoted myocardial cell apoptosis critically contributes to myocardial dysfunctions induced by sepsis.24-26 In this study, the anti-apoptotic protein Bcl-2 showed great decrease in the myocardial tissues of septic rats, while caspase-1 protein abundance was greatly increased in myocardial tissues of sep-
tic rats, which confirmed the successful model establishment and induction of myocardial dysfunction in model rats, combined with inflammatory factor expression and TUNEL assay. Moreover, the septic rat serum substantially suppressed the viability and enhanced the apoptosis of H9C2 cells as well as induced elevated expression of NF-κB p65, NLRP3, cleaved caspase-1, and IL-1β protein levels, reduced Bcl-2 levels, and promoted cytochrome C release. In addition, the oxidative stress mediated by ROS production also contributed to the apoptosis of cardiomyocyte associated with sepsis-induced organ damages such as myocardial dysfunction.12,13) We also observed here that the ROS production in H9C2 cells was significantly enhanced by septic rat serum. Furthermore, the abnormal mitochondrial signaling and disrupted mitochondrial functions were also shown to be involved in the development of SIMDs.28,29) Significant inhibition of the mitochondrial COX IV activities were observed in H9C2 cells treated with septic rat serum in this study. All these cellular and molecular alterations in septic serum-treated H9C2 cells fully validated the induction of cardiomyocyte dysfunctions by serum progression, which was used as the cellular model for the following assays.

As described above, miRNAs were critically implicated in damage in multiple organs induced by sepsis, such as acute kidney and lung damage, mainly due to their great potency of suppressing target gene expression.12,13) However, the pathogenic roles of miRNAs in myocardial dysfunctions associated with sepsis progression still remain largely unknown. In this study, we found that the expression of miR-378, miR-181a, and miR-210-3p were all greatly elevated in H9C2 cells treated with septic rat serum. Among them, miR-210-3p expression exhibited the most significant increase in H9C2 cells after septic rat serum treatment. Therefore, we further investigated the roles of miR-210-3p in the regulation of H9C2 functions by serum from septic rats. Importantly, we found that the modulations of H9C2 cell viability, ROS production, apoptosis, and COX IV activities were effectively abrogated by miR-210-3p inhibitors, but significantly enhanced by miR-210-3p mimics. These observations indicate a pathogenic role of miR-210-3p in myocardial dysfunctions caused by sepsis pathogenesis. Abnormally increased miR-210-3p might be used as diagnostic biomarker for predicting myocardial dysfunction development during sepsis progression. Our results also demonstrated that the inhibition of miR-210-3p expression might be further explored as a potent strategy to prevent myocardial dysfunctions in septic patients. It should be noted that miR-378 and miR-181a also play essential roles in several pathogenic conditions associated with sepsis; their functions in sepsis-induced myocardial dysfunctions deserve further exploration considering their great expressional increases in H9C2 cells treated with septic rat serum.

Previous reports have established NDUFA4 as an important component of COX, which is involved in the COX IV assembly and electron transportation in mitochondria.15,16) NDUFA4 was predicted to be a target gene of miR-210-3p by bioinformatics analysis in this study, which is consistent with the impaired COX IV activities in H9C2 cells caused by rat septic serum. Subsequently, we verified the direct binding of miR-210-3p to the 3’ UTR region of NDUFA4 by dual luciferase assay in H9C2 cells. The targeting of NDUFA4 expression by miR-210-3p was further supported by the significant reduction of NDUFA4 expression in septic serum-treated H9C2 cells. Moreover importantly, miR-210-3p mimics induced further inhibition of NDUFA4 expression in H9C2 cells treated with septic rat serum, while the opposite occurred upon treatment of H9C2 cells with miR-210-3p inhibitors. ISCU is another functional protein regulating mitochondrial functions whose expression could also be targeted by miR-210-3p.21,22) The regulation of myocardial energy metabolism shift during ischemic heart disease development by miRNA-210 was mediated by its targeting of ISCU in cardiomyocytes.30) The major weakness of this study is that we did not evaluate phenotypic consequence of the experimental animal models including cardiac function and survival, in response to sepsis and the effect of miR-210-3p suppression. A more scientific design of in vivo experiments should be application of miR-210-3p-knockout mouse model. We disclosed in this study that the ISCU expression in H9C2 cells was greatly suppressed by septic rat serum, which was enhanced by miR-210-3p mimics but mitigated by miR-210-3p inhibitors. These investigations further broadened our understanding of the downstream molecular events in miR-210-3p-regulated myocardial dysfunctions associated with sepsis.

**Conclusion**

In summary, we unveiled in this study that miR-210-3p, whose expression was greatly increased expressed in H9C2 cells treated with septic rat serum, promoted the apoptosis and mitochondrial damages in cardiomyocytes during sepsis progression through targeting NDUFA4 expression. These observations provided novel insights into the molecular pathogenic mechanisms underlying sepsis-induced myocardial dysfunctions, which might be further explored for myocardial dysfunction prevention and clinical treatments.

**Disclosure**

**Conflicts of interest:** None.

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