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Effects of MiR-214-3p Regulation of SERCA2a Expression on Contractility of Cardiomyocytes in Heart Failure Model

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ABSTRACT

This study aimed to explore the targeted regulation of microRNA-214-3p (miR-214-3p) on sarcoplasmic/endoplasmic reticulum Ca($^{2+}$)-ATPase 2a (SERCA2a) and its mechanism on heart failure (HF). In this study, a rat model of HF was established by injecting isoproterenol to detect the changes in heart function. Then the primary rat cardiomyocytes were extracted and cultured. The cells were divided into the normal group, HF model group, miR-214-3p mimic group, and inhibitor group according to treatment methods. The expression differences of SERCA2a in each group were detected. The binding sites of miR-214-3p and SERCA2a were predicted, wild-type or mutant SERCA2a was prepared and co-transfected into cardiomyocytes with mimic, and the targeting effect was detected by the dual-luciferase reporter gene. Finally, the systolic function of each group was detected by a single-cell systolic dynamic edge detection system. The results showed that cardiac output and left ventricular ejection fraction of HF rats were significantly lower than those of normal rats (P<0.05). The results of the cell test showed that messenger ribonucleic acid (mRNA) and protein expression levels of SERCA2a in the model group and the mimic group were significantly lower than those in the mimic group (P<0.05), but there were no differences between normal group and inhibitor group (P>0.05). Target prediction revealed that miR-214-3p had a complementary pairing of 6 bases with the SERCA2a 3'non-coding region. After co-transfection with miR-214-3p mimic and wild-type SERCA2a expression vector, the dual-luciferase activity was significantly decreased (P<0.05). The percentage of maximal contraction amplitude, peak contraction time, and 50% diastolic time of cells in the model group and mimic group decreased significantly. The mimic group was significantly smaller (P<0.05), but there were no differences between the normal group and the inhibitor group (P>0.05). These results indicated that SERCA2a expression was significantly reduced in HF cells, and miR-214-3p could inhibit SERCA2a expression by targeting the SERCA2a 3'UTR region. Inhibition of miR-214-3p could promote the expression of SERCA2a, which in turn promoted the contractile function of HF rat cardiomyocytes.

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Introduction

With the deepening of the aging degree of society, the prevalence of heart failure (HF) shows an increasing trend year by year. Chronic HF, the end-stage manifestation of many cardiovascular diseases, has become a serious health hazard to human beings (1). Statistics show that even if the treatment of HF is optimized, the mortality rate of patients within 5 years is still higher than 50% (2). Therefore, finding a more effective treatment is of great significance for improving the prognosis and survival rate of patients with HF. Studies have shown that Ca^{2+} homeostasis imbalance in cardiomyocytes can lead to a series of Ca^{2+} dependent signaling pathway abnormalities. Moreover, it can directly affect the contractile or diastolic ability of cardiomyocytes, leading to changes in the structure and function of cardiomyocytes, and then play an important role in the occurrence and development of HF (3,4). Sarcoplasmic/endoplasmic reticulum Ca($^{2+}$)-ATPase 2a (SERCA2a) can reabsorb Ca^{2+} into the sarcoplasmic reticulum during cardiac diastole, thus maintaining Ca^{2+} homeostasis in cells and regulating the coupling effect of myocardial excitation or contraction states (5). Previous studies have confirmed that SERCA2a gene transfer therapy can significantly improve the cardiac function of patients with HF (6). However, experimental results of HF cell models in vitro show that overexpression of SERCA2a can significantly improve the contractility of cardiomyocytes (7).

MicroRNAs are a class of small non-coding RANs, typically 22 nucleotides long. MicroRNA can

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complement and pair with the 3'non-coding region (3'UTR) of target messenger ribonucleic acid (mRNA) to inhibit the translation of target genes, thus playing a regulatory role in biological processes (8). With the gradual deepening of research, microRNA has been found to play a regulatory role in heart development and other processes (9). Other studies have shown that microRNA plays an important role in cardiac hypertrophy, arrhythmia and HF (10-12). MicroRNA-214 can be involved in various diseases, such as cancer and cardiovascular diseases, especially ischemic heart disease, myocardial hypertrophy, and HF (13). Studies have shown that microRNA-214 shows a tendency of high expression in the serum of patients with HF (14). However, whether microRNA-214 can regulate the HF process by affecting SERCA2a expression hasn't been verified yet.

To verify the mechanism of microRNA-214 regulating the expression of SERCA2a and affecting the systolic function of cardiac cells in HF models. In this study, a rat model of HF was constructed, and the effect of microRNA-214 transfection on SERCA2a expression level was detected after the extraction of cardiac myocytes. Subsequently, the targeting effect of MicroRNA-214 and SERCA2a and its effect on the contractility of myocardial cells were predicted and verified. This study provided experimental data to understand the regulatory effect of microRNA-214 on SERCA2a and the mechanism of action on the HF process.

Materials and methods

Test materials

Male Sprague-Dawley (SD) rats were purchased from Nanjing Junke Biological Engineering Co., LTD. Isoproterenol was purchased from Med Chem Express. Pentobarbital sodium was purchased from Merck Sigma-Aldrich. Type II collagenase, hyaluronidase, 0.25% trypsin, fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM), 1% penicillin-streptomycin, and opti-(modified eagle medium) MEM were all purchased from Gibco. Lipo2000 was purchased from Merck Sigma-Aldrich. miRNA mimic and inhibitor were designed and synthesized by Thermo Fisher Scientific. Trizol reagent, enterochromaffin-like (ECL) chemiluminescence assay kit, restriction enzyme, and (dermatitis herpetiformis) DH5 α competent cells were purchased from Thermo Fisher Scientific. Complementary DNA (cDNA) reverse transcription kit and reverse transcription-real-time polymerase chain reaction (RT-qPCR) detection kit was purchased from Takara. A polymerase chain reaction (PCR) detection kit was purchased from Shanghai Sangon Bioengineering Co., LTD. The bicinchoninic acid (BCA) protein quantitative kit, SERCA2a protein primary antibody, β-actin protein primary antibody, horseradish peroxidase-labeled IgG secondary antibody, and double luciferase reporter gene detection kit were purchased from Abcam. Thyroxine (T4) carrier and pcDNA3.1 (+) carrier were purchased from Youbao Bio.

Preparation of rat model of HF

Male clean grade SD rats weighing 180 ~ 250g were selected as research subjects. The rats were kept in cages in the animal laboratory and were given a free diet and alternating light for 12 hours. The room temperature was kept at about 25°C and the relative humidity was kept at about 50%. After 7 days of adaptive feeding, isoproterenol (dose: 300mg·kg-1) was injected for 2 consecutive days. The cardiac function of the rat model was measured 2 months later, and the cardiomyocytes were cultured after grouping.

Detection of cardiac function in rats

Normal rats were used as a control group, and the average body weight of the rats was recorded. After modeling, the rats were anesthetized by intraperitoneal injection of pentobarbital (6.0mg·100g-1 body weight (BW)). After removing chest hair, the rats were fixed on the operating table in the supine position. The parameters were measured by cardiac ultrasound. Cardiac output (CO) and left ventricular ejection fraction (LVEF) were then calculated.

Isolation and culture of myocardial cells

The rats were anesthetized by intraperitoneal injection of pentobarbital (6.0mg·100g-1 BW) and then sacrificed. The heart was quickly removed and perfusion was performed into the aortic cannula. Krebs solution was used for 5min, and the low-calcium solution was used for 3min. The digestive solution containing type II collagenase (dose:

1mg·mL-1) and hyaluronidase (dose: 0.8mg·mL-1) was replaced for digestion for 10min. After the heart was cut, other tissues except the left ventricle were removed, and the left ventricle was cut into pieces and digested with 0.25% trypsin for 10min. After filtration, primary cardiomyocytes were obtained by centrifugation at 2500rpm for 2min. Finally, the cells were suspended in a DMEM cell containing 10% FBS. The cells were inoculated on a 12-well cell culture plate and placed in a 5% carbon dioxide 37°C constant temperature incubator for 2h, after which Brdo was added to remove fibroblasts. Then the culture was continued for 48h and the solution was changed.

Transfection of miRNA mimic or inhibitor

The primary rat cardiomyocytes were removed one day before infection and replaced with a culture medium without 1% penicillin-streptomycin. The cells were seeded into 24-well plates at a density of 1.0 × 104 cells per well. Serum-free reduced-serum media was used for the dilution of transfection reagent lipo2000 and miRNA mimic or inhibitor (concentration: 100 nmol·L-1). According to the transfection method, the cells were divided into normal rat cardiomyocytes group (normal), HF rat cardiomyocytes group (model), microRNA-214-3p (miR-214-3p) mimic to HF cells group (mimic), and miR-214-3p infection to HF cell group (inhibitor). Incubation of miRNA mimic or inhibitor with lipo2000 reagent at room temperature. After incubation for 20min, it was added to the myocardial cell culture plate, mixed and infected for 8h. Then it was replaced with a DMEM medium containing serum, and the infection continued for 48h. Real-time polymerase chain reaction (RT-qPCR) and western blot were used to detect the changes of SERCA2a mRNA and protein expression levels in the infected cells. The detection procedures can be illustrated in Sections 2.6 and 2.7

Reverse transcription-polymerase chain reaction test

Total RNA was extracted from rat myocardium by Trizol method, and cDNA reverse transcription was carried out using a cDNA reverse transcription kit. The quantitative primers of SERCA2a and internal reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed and synthesized by Shanghai Sangon Biological Co., LTD. The primer information was shown in Table 1. cDNA was used as a template, and SERCA2a 3'UTR was amplified according to the instructions of the PCR detection kit. SERCA2a expression level was detected using RT-qPCR kit instructions, and the amplified products were detected using 2% agarose gel electrophoresis.

Table 1. Primers detected by PCR.

Names of the gene	Primer sequences (5'→3')	Gene (bp)
WT SERCA2a	F: CGAGCTCCTGACAGCGCTACATGCAG R: CCCAACGTTACTGAGGTAGCAGGACTCTG	—
SERCA2a	F: CTCTGAAGAACTCGGAAATCG R: GATGAGGTAGAGGATGAACTG	167
GAPDH	F: TGGATTTGGACGCATTGGTC R: TTTGCACTGGTACGTGTTGAT	211

Western blot

After transfection with miRNA mimic/inhibitor for 48h, myocardial cells in each group were collected and added to the protease inhibitor mixture. The protein concentration was quantified according to the BCA method and the standard curve was drawn after the cells were broken by ultrasound. The severity of dependence scale-page gel electrophoresis was performed with 10% separated gel and 4% concentrated gel, and the loading volume of protein was 40μg. After protein isolation, the protein bands were transferred to a polyvinylidene fluoride membrane and then placed in a blocking solution for 3h at room temperature. Diluted SERCA2a (1:1000) and β-actin (1:1000) primary antibodies were added and incubated overnight at 4°C. After washing the membrane, a horseradish peroxidase-labeled IgG secondary antibody (1:10,000) was added and incubated at room temperature for 2h. After washing the film with the tailored breast screening trial, an ECL chemiluminescence detection kit was used to develop the target protein bands. Then the relative expression level of the target gene protein was detected by Image J software in a gel imager.

Double luciferase reporter gene assay

PCR products of the 3'UTR fragment of wild-type and mutant SERCA2a gene obtained by amplification were connected to the T4 vector. Then, the cells were transformed into DH5α competent cells, coated on cell culture plates, cultured upside down, and single

colonies were selected for propagation. The plasmid was extracted according to the instruction of the plasmid extraction kit, and the restriction endonucleases Hind III and Sac I were used for double-enzyme digestion verification of the extracted plasmid and the positive plasmid were sent to the company for sequencing verification. Finally, PCR products that met the requirements were connected to the pcDNA3.1 (+) vector to prepare the SERCA2a expression vector. Then, according to the infection method, pcDNA3.1 (+)-SERCA2A (nitidine chloride (NC)), pcDNA3.1 (+)-WT-SERCA2A + miR-214-3p mimic, PcDNA3.1 (+)-MUT-SERCA2a + miR-214-3p mimic was co-infected according to lipo2000. The cells were lysed 48h after infection, and the luciferase activity was determined according to the instruction of the double luciferase reporter gene test kit.

Determination of systolic function of myocardial cells

The star cell length-time curve was recorded according to the single-cell dynamic edge detection cells. Krebs solution was continuously irrigated with a peristaltic pump after 5min of settlement of cardiomyocytes in cell perfusion trough, and continuous electric field stimulation was given at 0.5Hz 40V. IonWIizard software was used to measure the percentage of the systolic amplitude of cardiomyocytes [(initial length of cardiomyocytes-length of cardiomyocytes after contraction) or initial length of cardiomyocytes × 100%], time to peak (TTP) and 50% diastolic time (R50).

Statistical treatments

All experimental data were expressed by mean ± standard deviation (SD), and SPSS 19.0 software was used for statistical processing and analysis of experimental data. Differences between groups were compared by independent sample *t*-test or single-factor analysis of the variance process. The differences between groups were considered statistically significant ($P<0.05$).

Results and discussion

Detection of cardiac function index in HF rats

The differences in cardiac function indicators CO and LVEF between the control group and HF model group were detected, and the results are shown in Figure 1. Compared with control group, CO index (63.49 ± 9.44 mL·min-1 vs 40.26 ± 5.28 mL·min-1) and LVEF index (83.59 ± 7.58% vs 56.17 ± 8.94 %) in model group were significantly decreased ($P<0.01$).

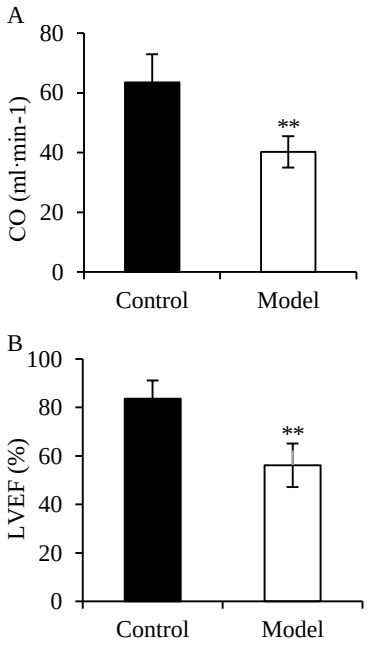

Figure 1. Cardiac function test results of rats. Notes: Compared with the control group, (**$P<0.01$), A: the difference in cardiac CO index in rats. B: the difference in cardiac LVEF index in rats.

Effect of miR-214-3p on SERCA2a expression in HF rat cardiomyocytes

RT-qPCR and western blot were used to detect the effects of infection with miR-214-3p mimic and inhibitor on SERCA2a mRNA and protein expression levels in cells, as shown in Figure 2. Compared with the normal group, SERCA2a mRNA and protein expression levels in the model group and the mimic group were significantly decreased ($P<0.01$). There were no significant differences in SERCA2a mRNA and protein expression between the normal group and inhibitor group ($P>0.05$).

Results of dual-luciferase reporter assay for miR-214-3p targeting SERCA2a

The dual-luciferase reporter gene was used to detect the target effect of miR-214-3p on SERCA2a, and the results were shown in Figure 3. TargetScan and miRbase databases were used to predict the targeted binding sites of miR-214-3p and SERCA2a. There were a total of 6 base complementary pairs.

Subsequently, miR-214-3p mimic and wild-type or mutant SERCA2a expression vector were co-transfected into cardiomyocytes to detect changes in luciferase activity, as could be seen in Figure 3B. After co-transfection with wild-type and mutant SERCA2a expression vectors and NC group, there were no significant differences in relative luciferase activity in cells. There were no significant changes in the relative luciferase activity of the SERCA2a expression vector after co-transfection with miR-214-3p mimic (P>0.05). The relative luciferase activity in cells co-transfected with wild-type SERCA2a expression vector and miR-214-3p mimic was significantly higher than in other groups (P<0.01).

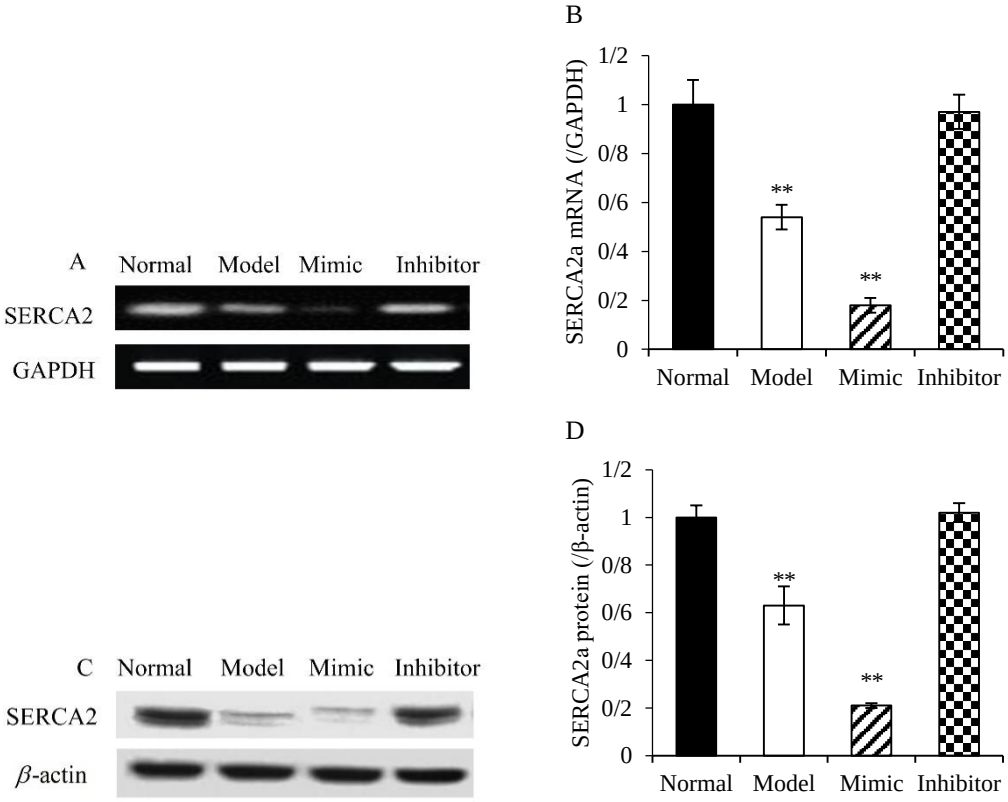

Figure 2. Effects of miR-214-3p on SERCA2a mRNA and protein expression levels in HF rat cardiomyocytes. Notes: Compared with the normal group (**P<0.01), A was the PCR map of SERCA2a mRNA expression in cells. B was the relative expression level of SERCA2a mRNA in cells. C: SERCA2a protein expression western blot. D: the relative expression level of SERCA2a protein in cells

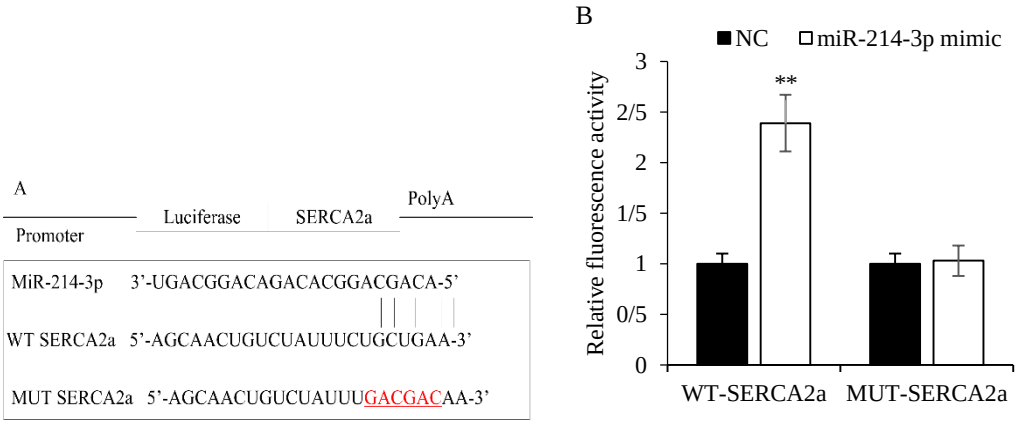

Figure 3. Results of miR-214-3p targeting double luciferase reporter gene at SERCA2a. Notes: Compared with NC and wild-type SERCA2a vector co-transfection group, **P<0.01, A was the prediction of miR-214-3p targeted binding SERCA2a binding sites. B: The result of relative luciferase activity after the dual-luciferase reporter gene test.

Effect of miR-214-3p on the systolic function of cardiomyocytes in rats with HF

The effect of miR-214-3p transfection on the systolic function of cardiac myocytes in the rat model of HF was detected, and the results are shown in Figure 4. First, the contraction of individual heart muscle cells was plotted. As could be seen from Figure 4A, the contraction range of cells in the normal group was significantly larger than those in the model group and mimic group and slightly larger than that in the inhibitor group, among which the contraction range of cells in the mimic group was the least. The contractile function of each group was quantitatively analyzed. As could be seen from Figure 4B, the percentage of maximum contraction amplitude in the normal group, model group, mimic group, and inhibitor group was 3.15 ± 0.43%, 1.03 ± 0.15%, 0.52 ± 0.10%, and 2.53 ± 0.48%, respectively. The percentage of maximum contraction in the mimic group was significantly lower than those in other groups (P<0.05). There were no significant differences between the normal and inhibitor groups (P>0.05).

As could be seen from Figures 4B and 4C, TTP and R50 of normal group, model group, mimic group, and inhibitor group were 0.29 ± 0.03s and 0.34 ± 0.02s, 0.13 ± 0.01s and 0.26 ± 0.03s, 0.05 ± 0.01s and 0.16 ± 0.01s, and 0.23 ± 0.02s and 0.32 ± 0.04s, respectively. TTP and R50 in the mimic group were significantly lower than those in other groups (P<0.05). TTP and R50 in the model group were significantly lower than those in the normal group and inhibitor group (P<0.05), but there were no significant differences between the normal group and inhibitor group (P>0.05).

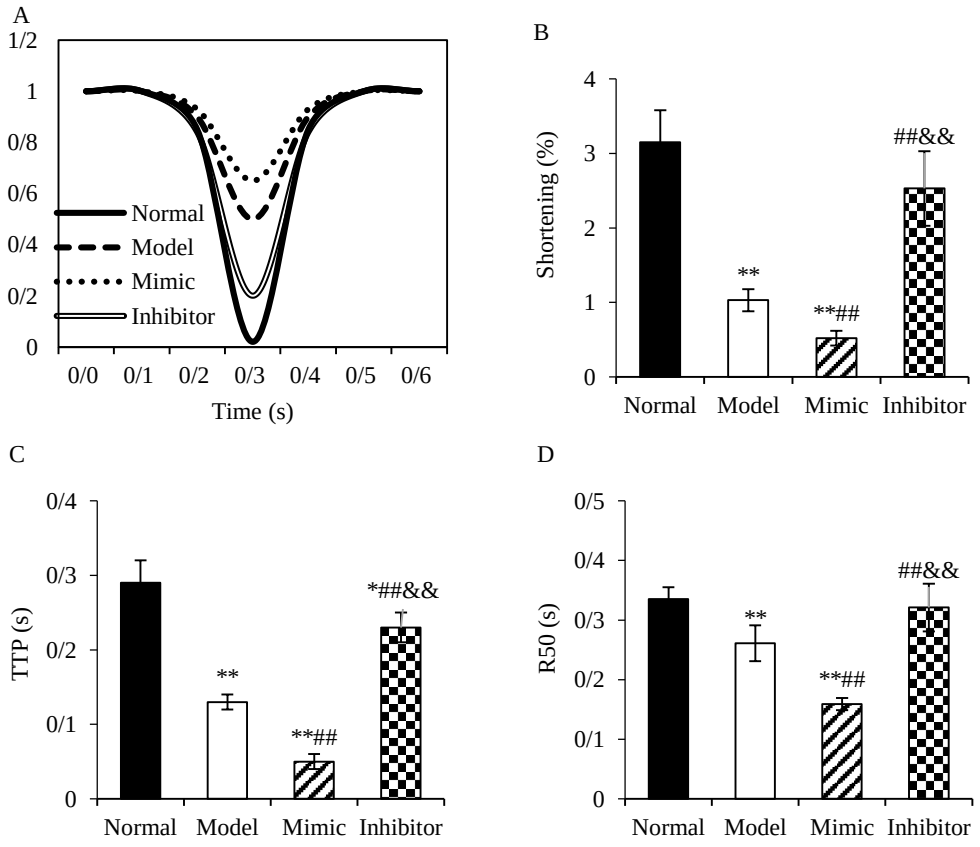

Figure 4. Effect of miR-214-3p on myocardial systolic function in rats with HF. Notes: Compared with (normal group **P<0.01, model group, ##P<0.01, and mimic group, &&P<0.01), A was cell contraction curve. B was the maximum percentage of cell contraction. C was the time when cell contraction reached the maximum TTP. D was 50% diastolic time of cell contraction (R50).

The excitation or contraction coupling is the basis for regulating contraction and relaxation. Ca^{2+} plays an important role in the biological process of excitation or contraction coupling of cardiomyocytes

(15). Ca^{2+} in the cytoplasm of cardiomyocytes is mainly taken up by the SERCA2a gene into the sarcoplasmic reticulum. Some Ca^{2+} can be excreted through membrane exchange. At this time, the decrease of intracellular Ca^{2+} concentration can promote the dissociation of troponin and further cardiac diastole (16). Previous studies have confirmed that SERCA2a can positively regulate the muscle strength and diastolic effect of the myocardium, thereby improving the contractility of the myocardium and cardiac function (17).

MicroRNA is a class of non-protein-coding RNA molecules with a length of about 22 nucleotides, which participate in the regulation of embryo development, cell proliferation, and angiogenesis (18). Melo et al. (2015) (19) showed that microRNA-214 was involved in regulating cardiac function, and microRNA-214 and SERCA2a expressions were significantly changed in rats with myocardial infarction. This study aimed to explore the regulatory effect of microRNA-214 on SERCA2a and its mechanism of action in the pathological process of HF. A rat model of HF was prepared by injection of isoproterenol, and myocardial cells were extracted to explore the effect of microRNA-214 targeting SERCA2a and its effect on cell systolic function in this study. The results showed that SERCA2a mRNA and protein expression levels were significantly decreased in cardiac myocytes of the HF model, which was basically consistent with the results of Guo et al. (2020) (20). After infection with microRNA-214-3p mimic, SERCA2a mRNA and protein expression levels were significantly inhibited. Moreover, TargetScan and other software predicted that the detection results of the dual-luciferase reporter gene showed that microRNA-214-3p could target SERCA2a and inhibit the expression of SERCA2a.

SERCA2a has been shown to be significantly down-regulated in HF. When SERCA2a function was limited, the sarcoplasmic reticulum's Ca^{2+} uptake ability was significantly reduced, which affected the release of Ca^{2+} in the systolic period of the myocardium and led to the dysfunction of myocardial systolic function (21,22). Therefore, this study further quantitatively evaluated the effects of microRNA-214 targeting SERCA2a on cell systolic function by using indicators such as percentage of maximum systolic amplitude, peak systolic time, and 50% diastolic period. The results showed that inhibition of microRNA-214 significantly upregulated SERCA2a mRNA and protein expression levels and significantly prolonged the percentage of maximal contraction amplitude, peak contraction time, and 50% diastolic period. This suggested that microRNA-214's ability to bind and inhibit SERCA2a expression was weakened after inhibition. Therefore, the ability to recycle Ca^{2+} into the sarcoplasmic reticulum was significantly enhanced. The cardiomyocytes reached full diastole and R50 was prolonged (23). In addition, increased calcium storage in the sarcoplasmic reticulum could promote the contraction of myocardial cells (24).

Conclusions

This study investigated the expression of microRNA-214-targeted SERCA2a and its effect on the contractility of cardiomyocytes in HF models. The results showed that SERCA2a expression was significantly decreased in cardiac myocytes in the HF model, while microRNA-214-3p was able to target and inhibit SERCA2a expression. Inhibition of microRNA-214-3P expression promoted the contraction of cardiomyocytes in HF models. In this study, specific regulatory mechanisms are only verified in vitro cell models. The mechanism of microRNA-214-targeted SERCA2a on HF should be further studied in pathological animal models. In conclusion, the results of this study can lay a theoretical foundation for the development of gene transduction therapy in HF.

Acknowledgments

Not applicable.

Interest conflict

The authors declare that they have no conflict of interest.

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