

miRNA-130a improves cardiac function by down-regulating TNF- α expression in a rat model of heart failure

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Abstract. – OBJECTIVE: To investigate the effect of MicroRNA (miRNA)-130a on cardiac function and the expression of tumor necrosis factor- α (TNF- α) in rats with heart failure.

MATERIALS AND METHODS: The rat heart failure model (n = 30) were established, then divided into miRNA-130a group, phosphate-buffer saline (PBS) group, rAAV9 group, and sham group (n = 10 in each group). Four weeks after the operation, the cardiac ultrasound and hemodynamic determination were performed. Blood endothelin-1 (ET-1) content was measured by enzyme-linked immunosorbent assay (ELISA). Hematoxylin and eosin (HE) staining was used to observe the morphology of myocardium. The expression levels of miRNA-130a and TNF- α were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). And the expression of TNF- α protein was determined by immunohistochemistry and Western blotting.

RESULTS: The rat heart failure model was successfully constructed. The miRNA-130a expression was decreased in rats with heart failure, and miRNA-130a transfection was successful. miRNA-130a improved left ventricular ejection fraction in the rat with heart failure. The blood ET-1 in miRNA-130a group was significantly lower than that of PBS group and rAAV9 group (p < 0.05). RT-PCR, Immunohistochemistry and Western blotting results showed that compared with the sham group, the expression of TNF- α in the model group was increased. And the expression of TNF- α in miRNA-130a group was significantly lower than that of PBS and rAAV9 group.

CONCLUSIONS: miRNA-130a could improve cardiac function of heart failure rat by down-regulating TNF- α .

Key Words:

miRNA-130a, Heart failure, TNF- α , ET-1, Left ventricular ejection fraction (LVEF), Cardiac function.

Introduction

Heart failure, a complex clinical symptom, is the final outcome of various heart diseases¹. It can cause cardiac structure or dysfunction resulting in ventricular filling or impaired ejection function². Heart failure has high morbidity and mortality, but there is no ideal treatment. Bo et al³ {Bo, 2017 #2461} revealed that TNF was significantly increased in heart failure, it indicated that heart failure was associated with inflammation. Previous researches found that the expression of tumor necrosis factor- α (TNF- α) increased in patients with heart failure, and TNF- α could promote the expression of endothelin-1 (ET-1)^{4,5}. As a direct inflammatory cytokine associated with heart failure, TNF- α plays a role in the process of heart failure by influencing cardiac contractility, mediating cell apoptosis, participating in ventricular remodeling, and promoting the development of cardiogenic cachexia⁶. Also, ET-1, as a strong vasoconstrictor, may lead to vasoconstriction, myocardial ischemia, metabolic disorder, and cell proliferation, and further induce disease related to vascular injury⁷. A related study⁸ found that increased ET-1 level could induce cardiomyocyte hypertrophy, and ET-1 also had direct toxicity effect on cardiomyocytes.

MicroRNA (miRNA) is endogenous noncoding RNA, which contains 18-25 nucleotides. And it is highly conserved in evolution, which can regulate gene expression and participate in many biological processes through targeting gene sequence specialization interaction⁹. Various miRNAs, such as miRNA-30e, miRNA-133, miRNA-21,

miRNA-132, miRNA-130a, had been known as key factors for the pathogenesis of heart failure¹⁰⁻¹⁴. Li et al¹⁵ found that low expression of miRNA-130a could increase the expression of TNF- α , and promote the development of osteoarthritis. And Zhang et al¹⁶ confirmed that miRNA-130a could down-regulate TNF- α in cervical cancer. At present, the mechanism of miRNA-130a on heart failure has not yet been studied clearly.

Therefore, we hypothesize that miRNA-130a can improve heart failure by regulating TNF- α . We also studied the correlation between miR-130a and cardiac function, and discussed the expression of ET-1 in plasma-mediated by miR-130a/TNF- α .

Materials and Methods

Animals and Grouping

Forty clean grade healthy Wistar rats (sex in half, 12-16 weeks, 320-350 g body wt) were fed in a cage at 25°C and relative humidity of 40%. The drinking water was provided by an automatic water supply system, and the feed was prepared by the experimental center of our school. Four weeks after the operation, the rats' hearts were divided into two parts: half of the rats' hearts was freshly taken from each group, and the other half was fixed for Hematoxylin and eosin (HE) staining and immunohistochemistry.

All animal experiments have been approved by our hospital's Medical Ethics Committee.

Construction of rAAV9-miRNA-130a Vector

After linearization, the vector was cut by endonuclease Hind III and treated with CIP for 1 h. The carrier fragment of 5451 bp was recovered and amplified. miRNA-130a: forward: 5'-GCTCTTTTCA-CATTGTGCTACT-3'; reverse: 5'-CAGTGCAAT-GTTAAAAGGGCATA-3; Colony PCR primers: F: 5'-CATGGTCCTGCTGGAGTTCGTG-3'; R: 5'-CATAGCGTAAAAGGAGCAACA-3'. The miRNA-130a and the linearized vector were mixed at 2:1 (mole ratio) and incubated at 42°C for 30 min. Ten μ l reaction liquid was inoculated into the competent cells, then added 10 μ l connect fluid and incubated at ice for 30 min, 42°C for 90 s. 900 μ l LB culture liquid was added to the bacterial fluid, then incubated at 37°C for 1 h. The transformed bacteria liquid was coated on LB agar plate (containing the corresponding antibiotic of the expression vector) at 37°C for 16 h. The colony PCR was identified and sequenced; then, the plasmid was extracted.

Construction of Heart Failure Rat Model And Grouping

Coronary artery ligation was performed in rats which fed for one week. The rats were anesthetized with 2.5% pentobarbital sodium (35 mg/kg), and tracheotomy and tracheal intubation were performed. Positive pressure ventilation was performed by the ventilator (TKR – 200C animal ventilator, Jiangxi Teli anesthesia breathing equipment company, Nanchang, Jiangxi, China) with a tidal gas of 30 ml/100 mg. The left margin of the sternum was opened and the rat heart was exposed. 10 rats were randomly selected to suture (sham group, and the other rats' left ventricular cavities were randomly injected with phosphate buffer saline (PBS), blank AAV plasmid vector and rAAV9-miRNA-130a (N=10 in each group). Then the rats were divided into 4 groups: sham group, PBS group, rAAV9 group, and miRNA-130a group. The left anterior descending branch of coronary artery was ligated at 5 mm away from the root of the aorta with 6-0 noninvasive ophthalmic suture silk.

Color Doppler Echocardiography

Four weeks after operation, the rats in each group (N = 5) were anesthetized and fixed in the supine position, then left ventricular posterior wall thickness (LVPWT) and interventricular septal thickness (IVST) during diastolic and systolic period, left ventricular end-diastolic (LVEDd) and end-systolic (LVESd) dimension were measured by echocardiography. Left ventricular ejection fractions (LVEF) was calculated. The recorded values of each index were an average of three consecutive cardiac cycles. All echocardiographic studies were performed in the supine position using Vevo 770TM (High resolution imaging system, VisualSonics Inc, Toronto, Ontario, Canada) with an 8.0-12.0 MHz by an experienced investigator.

Determination of Hemodynamic Indexes

Four weeks after the operation, the rats in each group (N=5) were anesthetized with 35 mg/kg chloral hydrate. Then, heart rate (HR), left ventricular end-systolic pressure (LVESP), left ventricular end-diastolic pressure (LVEDP), maximum rate of left ventricular pressure rise, and drop (LV + dp/dtmax, LV-dp/dtmax) were measured by BL-420F biological function experiment system recorder (Chengdu Taimeng Technology Co., Ltd., Chengdu, Sichuan, China).

Enzyme-Linked Immunosorbent Assay (ELISA)

Four weeks after the operation, 10 ml fasting venous blood of rats in each group were taken from the right carotid artery under the aseptic condition. After serum isolation, serum ET-1 level was determined in strict accordance with the instruction of the ELISA kit (Beijing Huaxia Peilagic Technology Co., Ltd., Beijing, China). Optical density (OD) values were measured at 450 nm with the microplate reader. The standard curves of OD value and concentration were drawn, and the concentration of the sample was calculated according to the standard curve.

HE Staining

The sample of annular myocardium was taken from the central part of the left ventricle, which was fixed in polyoxymethylene for 24 hours, dehydrated in ethanol, permeabilized in PBST (PBS + 0.5% Triton X-100), and embedded in paraffin. Then, paraffin sections (3–4 μ m) were dewaxed by xylene for 10 minutes, and hydrated by different concentration of ethanol (100%, 95%, 90%, 85%) for 1 minute, and washed with water for 2 minutes. These sections were stained with hematoxylin for 4 minutes, differentiated in a hydrochloric acid-ethanol mixture for 3–5 minutes, and then stained with eosin for 2 minutes. Then, gradient alcohol from low concentration to high concentration was used for dehydration treatment, xylene infiltration, and neutral balata seal. The myocardial tissue morphology changes were observed under the microscope.

Real-Time Fluorescence Quantitative PCR (qRT-PCR)

Total RNA was isolated from myocardium tissue by GenElute™ Mammalian Total RNA Miniprep Kit (Seebio, Shanghai, China). The cDNA template was synthesized by reverse transcriptase. qRT-PCR was done by using the TaqMan miRNA detection kit. U6 snRNA and GAPDH were used as a reference gene for miRNA-130a and TNF- α respectively. The relative gene expression was analyzed with the $2^{-(\Delta\Delta CT)}$ method. The primers used for qPCR were as follows: TNF- α , forward: 5'-CAGAC-CCTCACACTCAGATCATC-3'; reverse: 5'-AGCCTTGTCCTTGAAGAGAAC-3'; U6, forward: 5'-CTCGCTTCGGCAGCACA-3'; reverse: 5'-AACGCTTCACGAATT-TGC-GT-3'; GAPDH, forward: 5'-CGGAGTCAAC-GGATTTGGTCGTAT-3'; reverse: 5'-AG-CCTTCTCCATGGTGGTG-AAGAC-3'.

Western Blot

Total protein was extracted from myocardium tissue, then quantified by BCA Protein Assay Kit, subjected to SDS-PAGE, and transferred onto the membranes. GAPDH gene was used as a reference gene for TNF- α . After blocked by with 5% defatted milk powder, the membrane was incubated with the TNF- α antibody (1:1000, 3707, CST, Danfoss, MA, USA) at 4°C overnight. Then it was washed by TBST for 3 times, incubated with horseradish peroxidase-labeled secondary antibody (1:5000, Beijing Zhong Shan Biotechnology Co., Ltd., Beijing, China) at room temperature for 1 hour, and washed with TBST again. Proteins were visualized with enhanced chemiluminescence (ECL) kit and gel imaging system. The results were analyzed by Image Tools.

Immunohistochemistry

After dewaxed and hydrated, paraffin sections were placed in the boiling 0.01 M citrate buffer, then cooled down after heating for 90 s. These sections were incubated with 3% hydrogen peroxide at room temperature for 10 minutes; then, they were incubated with the TNF- α antibody (1:1000, 3707, CST, Danfoss, MA, USA) at room temperature for 2 hours. After added Max Vision™ 30 minutes at room temperature, these sections were incubated with goat anti-rabbit-HRP at room temperature for 30 minutes and visualized with DAB substrate.

Statistical Analysis

The terms of mean values $\pm$ standard deviation was used to express experimental data. The comparison of data between groups was analyzed by Student-Newman-Keuls test using SPSS19.0 (SPSS Inc, Chicago, IL, USA). $p < 0.05$ was considered as significant.

Results

Successful Construction of the Rat Heart Failure Model

Four weeks after operation, HE staining was used to detect whether the rat heart failure model was successfully constructed. The results showed that the myocardial fiber structure was clear and arranged orderly in the sham operation group. In model groups, the necrosis of myocardial cells in the infarct area was replaced by fibrous tissue, and the fibers were arranged in disorder with a large number of proliferating small vessels invol-

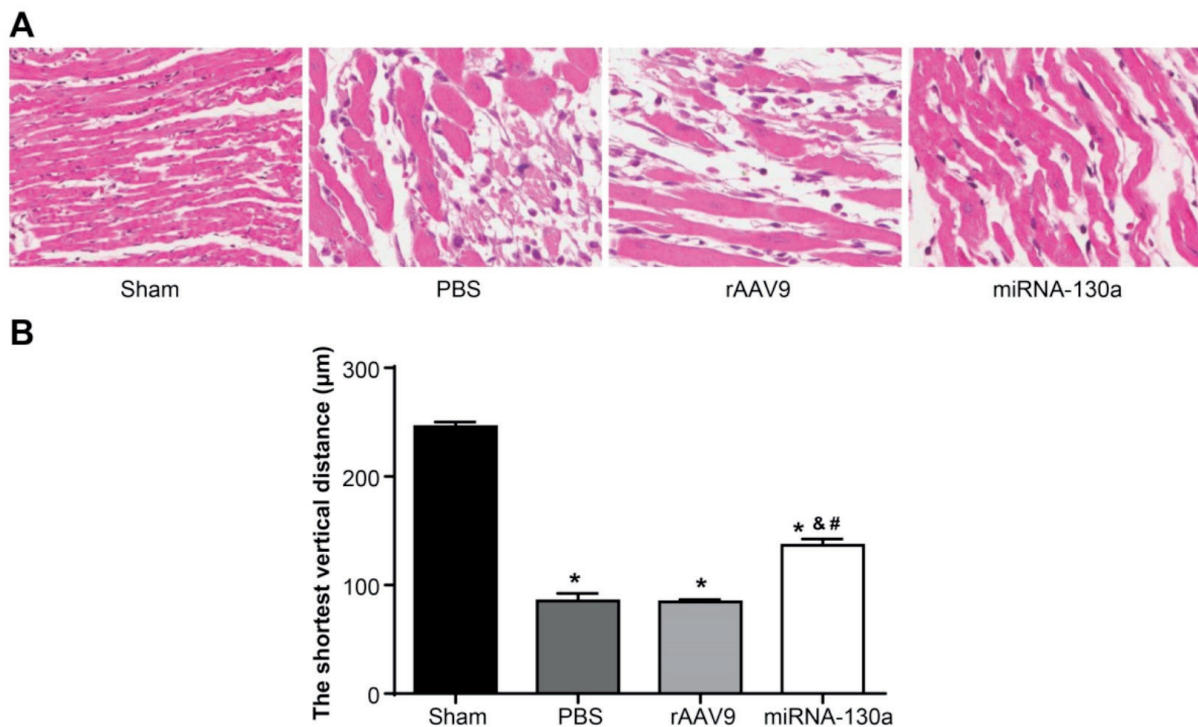


Figure 1. Successful construction of the rat heart failure model. **A**, HE staining was used to detect the degree of myocardial infarction in rats. **B**, the shortest vertical distance from apex to infarct site. $N = 5$ in each group; * $p < 0.05$ compared with sham group; # $p < 0.05$ compared with PBS group; & $p < 0.05$ compared with the rAAV9 group.

ved. And some hypertrophy cardiomyocytes were encapsulated and separated by fibrous tissue with a large number of inflammatory cell infiltration. The myocardial cells in the non-infarction area were compensatory hypertrophy and irregular (Figure 1A). It showed that the model of myocardial failure constructed successfully. There was no significant difference in the degree of myocardial infarction between the PBS group and rAAV9 group. The degree of myocardial infarction in the group miRNA-130a was smaller than that in PBS and rAAV9 group ($p < 0.05$) (Figure 1B).

The Expression of MiRNA-130a

Four weeks after surgery, compared with the control group, the miRNA-130a expression in PBS and rAAV9 group was reduced, while increased in miRNA-130a group ($p < 0.05$). It suggested that the miRNA-130a expression was decreased in rats with heart failure, and miRNA-130a transfected successfully in miRNA-130a group (Figure 2).

MiRNA-130a Inhibited Heart Failure in Rats

Color Doppler echocardiography results showed that there was no significant difference among the

four groups in left ventricular systolic phase. The left ventricular diastolic wall of the rats in the PBS group and rAAV9 group was thickened ($p < 0.05$ versus sham group). It indicated that the left ventricular diastolic function was weakened, the heart was decompensated, and the rat heart failure was constructed successfully. There was no significant difference in the diastolic wall between the sham

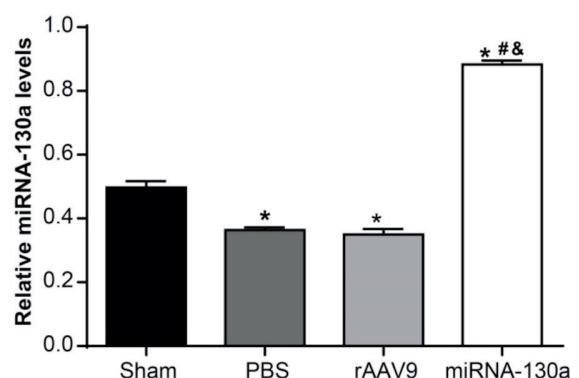


Figure 2. The relative expression of miRNA-130a in each group. $N = 5$ in each group; * $p < 0.05$ compared with sham group; # $p < 0.05$ compared with PBS group; & $p < 0.05$ compared with the rAAV9 group.

Table I. The diastolic and systolic index in left ventricular.

Groups	Diastolic (left ventricle)			Systole (left ventricle)		
	IVST (mm)	LVPWT (mm)	LVEDd (mm)	IVST (mm)	LVPWT (mm)	LVESd (mm)
Sham	1.772 ± 0.306	1.9 ± 0.449	5.667 ± 0.749	3.275 ± 0.614	2.738 ± 0.554	2.777 ± 0.411
PBS	2.487 ± 0.578**	2.667 ± 0.282	6.762 ± 0.613	4.033 ± 0.721	3.413 ± 0.615	3.429 ± 0.479
rAAV9	2.378 ± 0.425*	2.682 ± 0.343	7.323 ± 0.804*	4.037 ± 0.601	3.355 ± 0.549	3.478 ± 0.859
miRNA-130a	1.92 ± 0.21#&	2.147 ± 0.154	6.303 ± 0.525	3.443 ± 0.482	3.047 ± 0.166	2.825 ± 0.717

Note: N = 10 in each group; IVST: Interventricular septal thickness; LVPWT: left ventricular posterior wall thickness; LVEDd: left ventricular end-diastolic dimension; LVESd: left ventricular end-systolic dimension; * $p < 0.05$ compared with sham group; # $p < 0.05$ compared with PBS group; & $p < 0.05$ compared with the rAAV9 group.

group and miRNA-130a group. It suggested that miRNA-130a could effectively inhibit the process of heart failure in rats (Table I).

miRNA-130a Improved LVEF in Rats

LVEF data showed that compared to the sham group, the LVEF decreased significantly in PBS and rAAV9 groups ($p < 0.05$), indicating heart failure could reduce the LVEF of the rat. LVEF in miRNA-130a group reduced ($p > 0.05$ versus sham group), which was significantly higher than that in other model groups, suggesting that miRNA-130a could effectively improve LVEF in rats (Figure 3).

Hemodynamic Indexes

Hemodynamic indexes results showed that HR of rats in each group were at a normal level. There was no significant difference in HR among the four groups of rats. The LVESP, LVEDP, and LV $\pm dp/dt_{max}$ in PBS and rAAV9 groups were significantly decreased ($p < 0.05$), but there was no significant difference between miRNA-130a and sham group ($p > 0.05$) (Table II).

miRNA-130a Inhibited the Expression of ET-1 in Blood

The expression of ET-1 in plasma was detected by ELISA. The results showed that the ET-1

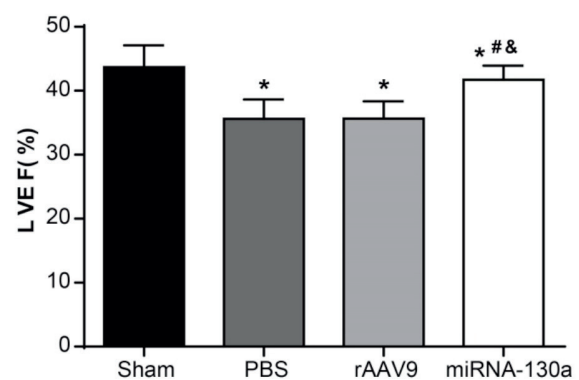


Figure 3. LVEF in rats of each group. LVEF: left ventricular ejection fraction; N = 10 in each group; * $p < 0.05$ compared with sham group; # $p < 0.05$ compared with PBS group; & $p < 0.05$ compared with the rAAV9 group.

expression in rats with heart failure was increased ($p < 0.05$ versus sham group). It indicated that heart failure in rats promoted the expression of ET-1. There was no significant difference in ET-1 expressions between the PBS group and rAAV9 group, and the ET-1 expression in miRNA-130a group was significantly decreased ($p < 0.05$ versus PBS group and rAAV9 group). It indicated that miRNA-130a inhibited the expression of ET-1 in rats with heart failure (Figure 4).

Table II. Hemodynamic index.

Groups	HR (beat/min)	LVESP (mmHg)	LVEDP (mmHg)	+dp/dt _{max} (mmHg/s)	-dp/dt _{max} (mmHg/s)
Sham	419 ± 19	132.61 ± 6.07	3.98 ± 1.05	8.42 ± 1.26	-5.405 ± 1.12
PBS	338 ± 55	83.93 ± 7.42*	21.89 ± 9.44*	1.14 ± 0.24	-1.072 ± 0.16
rAAV9	396 ± 35	109.95 ± 9.13*	19.89 ± 3.81*	3.59 ± 0.42	-3.313 ± 0.37
miRNA-130a	401 ± 25	119.95 ± 7.13	10.89 ± 3.56	5.89 ± 0.86	-4.313 ± 0.57

Note: N = 10 in each group; HR: heart rate; LVESP: left ventricular end-systolic pressure; LVEDP: left ventricular end-diastolic pressure; * $p < 0.05$ compared with sham group.

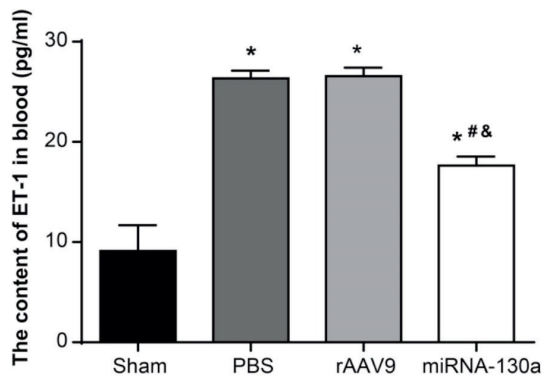


Figure 4. The expression of ET-1 in rats plasma. N = 10 in each group; * $p < 0.05$ compared with sham group; # $p < 0.05$ compared with PBS group; & $p < 0.05$ compared with the rAAV9 group.

miRNA-130a Inhibited the Expression of TNF- α

The expression of TNF- α was detected by qRT-PCR and Western blotting. The results showed that the expression of TNF- α in the heart failure model group was increased ($p < 0.05$ versus sham group). It indicated that heart failure increased the expression of TNF- α . Compared with PBS group and rAAV9 group, the expression of TNF- α in miRNA-130a group was decreased ($p < 0.05$), indicating that miRNA-130a inhibited TNF- α expression (Figure 5A, B). Immunohistochemical results showed that the number of TNF- α positive cells in the heart failure model group was significantly increased ($p < 0.05$ versus sham group). Compared with the PBS group and rAAV9 group, the number of TNF- α positive cells in miRNA-130a group was significantly decreased ($p < 0.05$). It indicated that miRNA-130a inhibited the expression of TNF- α (Figure 5C).

Discussion

In our study, we established the rat heart failure model and regulated miRNA-130a expression by injecting rAAV9-miRNA-130a into left ventricular cavities of the rat heart failure model. The results revealed that miRNA-130a was down-regulated in rat heart failure model, and overexpression of miRNA-130a could improve cardiac function and LVEF in rat heart failure model, reduce the expression TNF- α and plasma ET-1.

MiRNA-130a is widely known because its different expression in many malignant tumors,

which could inhibit the proliferation, invasion, and metastasis of cancer cells^{17,18}. Jakob et al¹⁹ found that the miRNA-130a function was a loss in the angiogenesis-EOCs and circulating CD34 (+) in the process of heart failure. Low expression of miRNA-130a in coronary heart disease could inhibit the early growth cells of vascular repair²⁰. Calway et al¹⁴ also found that miRNA-130a increased in end-stage heart failure. In our work, the expression of miRNA-130a in the rat heart failure model was decreased, indicating that miRNA-130a played a certain role in heart failure. Jakob et al¹⁹ reached the same conclusion, but did not observe the role and mechanism of miRNA-130a. In our study, up-regulation of miRNA-130a in the rat with heart failure could inhibit the process of heart failure, reduce the area of myocardial infarction, and improve the LVEF and hemodynamic indexes.

Scholars^{15,16} have showed that miRNA-130a could inhibit the TNF- α expression. TNF- α was an inflammatory factor, which closely related to heart failure²¹. Up-regulation of TNF- α in myocardial tissue could induce Cx43 reconfiguration, and TNF- α could lead to ventricular arrhythmia in rats with heart failure²². In our study, miRNA-130a overexpression was carried out in the rat model of heart failure, and the expression of TNF- α in myocardial tissue was decreased, indicating that miRNA-130a could inhibit the development of heart failure by reducing the inflammatory response. At present, some researches had improved heart failure by reducing TNF- α . For example, Liu et al²³ found that Danhong injection could reduce the expression of TNF- α and improve the clinical symptoms and quality of life in elderly patients with heart failure. Coenzyme Q (10) combined with rosuvastatin could decrease the concentration of serum TNF- α , and further significantly improved the cardiac function of patients²⁴.

Endothelin could mediate vasoconstriction and maintain vascular tension in heart failure²⁵. However, increasing endothelin could induce cardiomyocyte hypertrophy⁸. In this report, the expression of ET-1 was increased in rat heart failure model, and hypertrophy of myocardial cells was observed. Overexpression of miRNA-130a in rat heart failure model reduced the expression of ET-1 significantly, and it reduced the degree of myocardial infarction in miRNA-130a group. The role of ET-1 in promoting heart failure was fully demonstrated. Clinical researches had been done to reduce heart failure by reducing ET-1 in

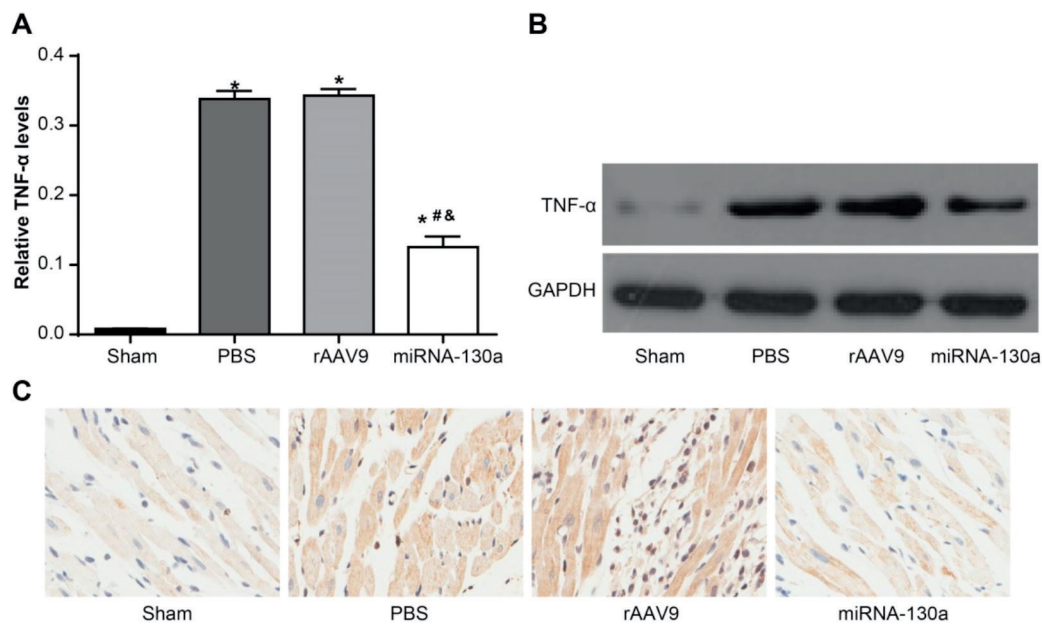


Figure 5. The expression of TNF- α in myocardial tissue of each group rats. **A**, RT-PCR detection of TNF- α expression; **B**, Western blot detection of TNF- α expression; **C**, Immunohistochemical detection of TNF- α expression. N = 5 in each group; * p < 0.05 compared with sham group; # p < 0.05 compared with PBS group; & p < 0.05 compared with the rAAV9 group.

plasma. For example, RhBNP improved the heart function in patients with heart failure by decreasing plasma ET-1 level²⁶. Xuebijing injection could improve endothelial function and cardiac function by decreasing ET-1 level, and enhance patients' endurance²⁷. Shinagawa et al⁵ revealed that TNF- α could promote the expression of ET-1. Similarly results were obtained in our study, miRNA-130a could inhibit ET-1 by mediating TNF- α , and improve cardiac function by reducing the expressions of TNF- α and ET-1 in rats with heart failure. It is worth noting that, as a vasoconstrictor, the expression of ET-1 was increased in rats with heart failure, but there was no statistical difference in IVST, LVPWT, and LVESd of left ventricular systolic phase in our work. This may be because the sample size of this study is limited, so further research is needed in expanded samples.

Conclusions

We showed that the miRNA-130a expression was down-regulated in rats with heart failure. Overexpression of miRNA-130a could decrease the expressions of TNF- α and ET-1 in heart failure rats, and improve heart failure and heart function in rats with heart failure. miRNA-130a may

be used as a potential biomarker for the prevention and treatment of heart failure.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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