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MiR-195 inhibits myocardial fibrosis in hypertensive rats by regulating TGFβ1-Smad3 signaling pathway

Q. XU, X.-X. LIN, P. LIU, W. ZHANG, K. TANG, Y.-S. ZHAI, L.-[illegible]U, W.-[illegible]

Department of Cardiology, East Hospital, The First Affiliated Hospital, S[illegible]at-sen[illegible]versity, Guangzhou, China

Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of micro-ribonucleic acid-195 (miR-195) on myocardial fibrosis in hypertensive rats through the transforming growth factor beta 1 (TGFβ1)-Smad3 signaling pathway.

MATERIALS AND METHODS: Spontaneously hypertensive rats (SHRs) were selected in this study to establish the animal model. The content of miR-195 in the model group and control group was measured, respectively. Arterial blood pressure, liver function and myocardial function in the two groups were detected and examined. Pathological changes in rat myocardial t[illegible] were detected via hematoxylin-eosin (H[illegible]ing. After that, myocardial fibroblasts we[illegible]ol-lected and added with miRNA inhibitors[illegible]d mimics to suppress and overexpress miR-Thereafter, Reverse Transcription-Polymera[illegible] Chain Reaction (RT-PCR) and W[illegible]n blottin[illegible] were employed to detect the[illegible] protein expression levels of checkp[illegible]kinas[illegible](Chek1) and alpha-smooth muscle[illegible]n (α-SM[illegible]import-ant molecules for proli[illegible]n an[illegible] tion of myocardial fibr[illegible]bla[illegible]as the re-lated pathway TGFβ[illegible]nad3[illegible]ermore, the effects of miR-195[illegible]myocardi[illegible]sis in hy-pertensive rats [illegible]e TGFβ1-Sm[illegible]naling pathway were [illegible]sively obser[illegible]d.

RESULTS: [illegible]m a[illegible]phosphatase (ALP), glutamic p[illegible]c amin[illegible]ferase (ALT) and creatine [illegible]se (CK) level[illegible]e SHR group were s[illegible]icantly higher th[illegible] those of the norma[illegible]oup. [illegible]rdiac function examination sho[illegible] that [illegible] group had significantly re-duc[illegible]ncti[illegible] short[illegible]g (FS, %) and ejec-tion fr[illegible], %) in[illegible]mparison with the nor-mal grou[illegible]weve[illegible]ystolic blood pressure, [illegible]ic blo[illegible]e, left ventricular end-di-ast[illegible]dimens[illegible]LVEDd) and left ventricu-lar e[illegible]systolic d[illegible]ension (LVESd) were mark-edl[illegible]in the SHR group. In addition, the m[illegible]ssion level was remarkably re-[illegible]ed in hypertensive rats. Histopathological [illegible]ges in rat myocardial tissues were detect-e[illegible]ugh HE staining. The results showed that the [illegible]al group had orderly arranged myocar-dial cells, [illegible] SHR group [illegible]owed disor-derly arr[illegible]ed [illegible]rdial cells, thickened myo-cardial f[illegible]ers and [illegible]rdial fibrosis. RT-PCR assay results revealed [illegible] the mRNA levels of Col[illegible]Chek1, α-SMA[illegible]Fβ1 and Smad3 in r[illegible]yocardial fibroblasts were significantly re-[illegible]ed in Mimics group ($p<0.05$) and increased [illegible]nhibitors gro[illegible]($p<0.05$). Western blotting re-[illegible]s demonstra[illegible]that, compared with the con-[illegible]roup, the [illegible]ein levels of α-SMA, TGFβ1 an[illegible]d3 i[illegible] myocardial cells decreased signi[illegible]Mimics group ($p<0.05$). Oppo-site results were observed in Inhibitors group ([illegible]<0.05). The above results suggested that [illegible]ression of miR-195 inhibited the ex-[illegible]s of TGFβ1-Smad3 signaling pathway [illegible]nd related molecules, further repressing myo-cardial fibrosis.

CONCLUSIONS: MiR-195 participates in the development and progression of myocardi-al fibrosis in hypertensive rats through the TG-Fβ1-Smad3 signaling pathway. Furthermore, this can inhibit the development of myocardial fibro-sis in hypertensive rats and prevent myocardi-al diseases.

Key Words:
MiR-195, TGFβ1-Smad3 pathway, Rat, High blood pressure, Myocardial fibrosis.

Introduction

High blood pressure (HBP), the increase of arterial systolic and/or diastolic pressure(s) (≥140/90 mmHg) in resting state, is the most important cause of myocardial fibrosis. Myocardial fibrosis is pathological damage caused by various cardiovascular diseases[1-3]. The mechanism of hypertensive myocardial fibrosis is relatively complex, which is regulated by a variety of cell growth factors. At present, a few studies have paid much attention to hypertensive myocardial

Corresponding Author: Weiyi Mei, MD; e-mail: meiweiyi@163.com

fibrosis. Myocardial fibroblasts maintain the systole and diastole of a healthy heart. However, after activation, abnormally proliferated and differentiated myocardial fibroblasts will cause myocardial fibrosis. Meanwhile, this may affect the connection and contraction of myocardial cells, leading to the occurrence and development of cardiovascular diseases, including coronary arteriosclerosis, arrhythmia and cardiomyopathy[4,5].

MiRNAs are a group of small non-coding RNAs with about 22 nucleotides in length, which can regulate the gene expression. Multiple studies have manifested that micro-ribonucleic acids (miRNAs) are involved in the differentiation, proliferation and apoptosis of cells[6,7]. They participate in the specific regulation of protein-coding and -non-coding genes mainly *via* binding to the 3'-untranslated region (3'-UTR) of target gene messenger RNAs (mRNAs)[8,9]. Previous studies have revealed that miRNAs bind to the 3'-UTR of target mRNAs through base pairing, leading to instability of mRNAs. Recent researches have discovered that miRNAs are capable of modulating and participating in various cellular processes during normal development and disease occurrence. This may eventually c... ib... ute to the progression of many diseases[10]. M... can modulate 1/3 human genes. Meanwhi... ey play important roles in physiological homeos... health and many diseases, including cell cyc... development, metabolism, variou... immune r... sponses[12] and other processes[13]. ... of miRNAs in the pathogenesis of d... ent d... es have been widely studied. Curr... ndings ... shown that they are important r... rs of ... sion in many diseases. Mor... regulatory networks have attra... much a... n in recent years[14]. For exam... miR-195 par... s in the systole and dia... yocardium a... myocardial fibrosis t... gh ... signaling pathways. Another st... y has found ... iRNAs, including miR-129... -21, miR-13 and ... 195, are specifically e... ssed in myocardial fibrosis and other disea... . Rese... evidence has suggested that the ... form... growth ... ctor beta 1 (TGFβ1)/Smad ... pathwa... involved in myocardial r...odeli... Additi... tudies have revealed that ...5 can ... he TGFβ1/Smad pathway, with ...nificantly ...proved myocardial hypertrophy ... fibrosis, in which TGF-β1 plays a crucial ro... , the regulatory role of miR-195 in ... -β1/Smad and its effect on myocardial fibrosis ...ot been fully elucidated. Moreover, the mech... f activating myocardial fibroblasts remains uncl... so far.

MiR-195 is an important regulator of various diseases. However, a few studies h... gated its role in the pathogenesis ... yocar... fibrosis in hypertensive rats. Th... re, the aim of this work was to investigate ... otential role of miR-195 in myocardial fibrosis ... rtensive rats through *in-vivo* and *in-... o* exper... Ou... findings might help to im... e the theor... sis for the influences ... iR-195 ... n myoca... fibrosis and TGF-β1/... d3 sig... g pathway in hypertensive rats.

M... ials and M... s

Experi... tal A... ls and Grouping

A total of 20 ma... ntaneously hypertensive ... SHRs) aged ... eks and 10 Wistar r... eighing 250 g were enrolled in this study ... subjected to ... daptive feeding. All rats were ... domly divide... to two groups, including the ... group (n=1... each group) and normal control ... up (Wis... ats, n=10). This study was approv... imal Ethics Committee of Sun Yat-sen University Animal Center. All operations ... animals were carried out according to relevant ... in the NIH Guide for the Care and ...se of ... aboratory Animals.

Culture and Grouping of Myocardial Fibroblasts

SHRs were disinfected and dissected under aseptic conditions. The heart was taken out, washed, cut into pieces and fully digested with collagenase. Then, they were homogeneously digested into cell suspension using a constant temperature water bath shaker. After that, the cell suspension was repeatedly centrifuged, and the supernatant was discarded. Subsequently, the cells were inoculated into a cell culture flask. Cells in the logarithmic growth phase were collected and divided into negative control group (NC group), miR-195 inhibitors group (Inhibitors group) and miR-195 mimics group (Mimics group). After starvation treatment, the cells were transfected, followed by 36 h of continuous culture.

Examination of Liver Function and Myocardial Function

To predict myocardial fibrosis in hypertensive rats in the clinic and provide important references for early diagnosis, liver function indicators, including glutamic pyruvic aminotransferase (ALT), alkaline phosphatase (ALP), and myocardial function index

creatine kinase (CK) were examined in this study. Blood samples were routinely collected from rats in the two groups of rats, followed by centrifugation to isolate the serum. Finally, detection was performed using an automatic biochemical analyzer.

Determination of Arterial Blood Pressure and Cardiac Physiological Function Indexes in Rats

Left ventricular function, including left ventricular end-diastolic dimension (LVEDd) and left ventricular end-systolic dimension (LVESd), fractional shortening (FS, %) and ejection fraction (EF, %), was examined *via* magnetic resonance imaging (MRI) and echocardiography (ECG). Each rat was subjected to electrocardiography using with a 10 MHz probe. Systolic blood pressure and diastolic blood pressure (mmHg) of the rat tail artery were measured in accordance with relevant instructions.

Changes in MiR-195 Content in Rats of Model Group and Control Group

Blood samples were first collected from rats in each group, followed by centrifugation to [illegible] the serum. Collected samples were then [illegible] ed to pre-treatment. Reverse Transcription [illegible]merase Chain Reaction (RT-PCR) assay was [illegible] formed to detect the content of miR-195 in e[illegible] group, and the original data were recorded.

Detection of Pathologic[illegible] in Rat Myocardial Tissues Th[illegible]gh He[illegible]oxylin-Eosin (HE) Stainin[illegible]

Rats were sacrifi[illegible]d [illegible]ation, and heart samples were [illegible]parated [illegible]reated with 4% paraformald[illegible]e at 4°C fo[illegible]. After washing with [illegible]water, the ti[illegible]s were dehydrated w[illegible] diffe[illegible]oncentrations of alcohol, and [illegible]mbedded wi[illegible]affin (5 μm). After depar[illegible]ization, the ti[illegible]were hydrated with 9[illegible]90%, 80%, 75% and 50% ethanol, respe[illegible]ely, fo[illegible]ed by HE staining (Boster, Wu[illegible] Chi[illegible] Finally, [illegible]athological changes in my[illegible] tissue[illegible]cture were observed [illegible]der a l[illegible]icros[illegible]e.

De[illegible]ination[illegible] MRNA Expression Lev[illegible]of Checkpoint Kinase 1 (Chek1) a[illegible]ooth Muscle Actin [illegible]MA) and Related Pathway TGFβ1-Smad3 [illegible]RT-PCR

[illegible]RNA was extracted from collected cells and [illegible]thesized into complementary deoxyribose nucleic acids (cDNAs) in strict accordance with PrimeScriptTM Kit (TaKaRa, [illegible] Japan). Subsequently, single-stra[illegible]cDN[illegible] were amplified using a conventio[illegible]eaction system and stored at -20°C for [illegible]erase Chain Reaction (PCR) amplification re[illegible] Primer sequences for target gene[illegible]nd gly[illegible]hyde 3-phosphate dehydrogen[illegible](GAPDH) [illegible] signed based on the se[illegible]ces on GenBank[illegible] expression levels of [illegible]et gen[illegible]ere measured by quantitative RT[illegible]ass[illegible]Specifi[illegible]imer sequences were [illegible]wn[illegible]e I. T[illegible]elative expression lev[illegible]of relate[illegible]s i[illegible]yocardial cells of rats [illegible]calculated by [illegible]$^{-\Delta\Delta CT}$ method.

Determ[illegible]tion[illegible]otein Expression Levels of α-SMA a[illegible]lated Pathway TG[illegible]ad3 throug[illegible]estern Blotting A[illegible]

[illegible]he culture s[illegible]tion was discarded, and the [illegible]s were wash[illegible]with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) 3 ti[illegible] Lysis bu[illegible]was added, followed by centrifu[illegible]ect the supernatant. The protein concentration was determined according to [illegible]e instructions of the bicinchoninic acid (BCA) [illegible], Waltham, MA, USA). Total protein [illegible]s e[illegible]acted from myocardial cells, followed by water bath for 8 min and centrifugation at 1000 g for 5 min. Extracted protein was separated by electrophoresis and transferred onto a membrane using a semi-dry transfer method. After blocking, the membranes were incubated with primary antibodies overnight. On the next day, the membranes were incubated with the corresponding secondary antibody. Finally, protein band scanning and

Table I. Primer sequences.

Target gene	Primer sequence (5'–3')
GAPDH	GACATGCCGCCTGGAGAAAC AGCCCAGGATGCCCTTTAGT
Collagen I	TCAGCCCAAACCCCAAGGAGA CGCAGGAAGGTCAGCTGGATAG
Collagen II	TGATGGGATCCAATGAGGGAGA GAGTCTCATGGCCTTGCGTGTTT
α-SMA	GTCCCAGACATCAGGGAGTAA TCGGATACTTCAGCGTCAGGA
Chek1	GGCAGTGCCTTTTGTGGAAG TCTATGGCCCGCTTCATGTC
TGF-β1	TGT GGC TCC TAG TGT TGA CG GCA GTT TGG ACA GGA TCT GG
Smad3	GCTTCTTGACGAGAGAGTCTACGG TACTAACACTGGTGGCAGCACTGG

quantification was detected by an Odyssey scanner (Lincoln, NE, USA). GAPDH was used as an internal reference.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 analysis software (IBM, Armonk, NY, USA) was used for all statistical analysis. GraphPad Prism 5.0 (La Jolla, CA, USA) was applied for image plotting. Experimental results were expressed as mean ± standard deviation ($\bar{x}$±SD). $p<0.05$ was considered statistically significant.

Results

Liver Function and Myocardial Function

To provide important references for early diagnosis in clinical practice, liver function indexes (ALT and ALP) and myocardial function index CK were examined. The results (Table II) showed that the levels of serum ALP, ALT and CK in the SHR group were overtly higher than those of the normal group ($p<0.05$). These results implied that liver function and myocardial function indexes increased markedly in the development and pr... sion of myocardial fibrosis in hypertensive...

Arterial Blood Pressure and Cardiac Function Indexes in Rats of Model Gro... and Control Group

Arterial blood pressure an... function... indexes in rats of the two ...ups ... shown in Table III. The results i...ated th... SHR group had significantly ...d FS... elevated systolic bloo... pre... tolic blood pressure, LVEDd ar... ESd i... arison with normal group ($p<$... This sugge... uccessful establishme... HR model i... s.

Changes ...n MiR-195 ...nt in Model Group ... Control Grou...

The ...ent of serum miR-19... in rats of each grou... s dete... hrough RT-PCR. The results

Table II. ...of serum ...hemical examination.

	...L)	ALT (U/L)	CK (U/L)
No... group	102.3±0.4	55.2±0.5	80.5±0.6
S...	...6±0.1[a]	125.5±0.3[a]	198.9±0.4[a]

Serum ALP, ALT and CK levels in SHR group are ...antly increased compared with those in the normal group ([a]p... ndicating that liver function and myocardial function index... abnormal ([a]$p<0.05$).

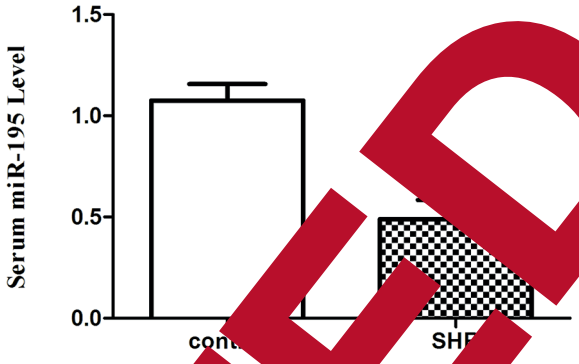

Figure 1. MiR-... conten... R grou... control group. The conte... miR-195 in ... ...s markedly lower than tha... control group (...

revealed that miR-19... ent in the SHR group was ...cantly lower ... that of the control g... (p...0.05). This implied that the expression ...l of miR-195 ...ecreased in hypertensive rats ...ure 1).

P...logical ...anges in Rat Myocardial Tiss... Staining

The morphology and damage of rat myocardial ...sues in each group were detected through HE ... The results (Figure 2) showed that myo...rdia... ells were arranged in order in the normal group (Figure 2A). However, they were disorderly arranged in the SHR group, with thickened muscle fibers and significant myocardial fibrosis (Figure 2B).

MRNA Expression Levels of Collagen, Chek1 and α-SMA and Related Pathway TGFÐ1-Smad3 via RT-PCR

The results of RT-PCR assay showed that the mRNA expression levels of Collagen, Chek1, α-SMA, TGFβ1 and Smad3 decreased significantly in Mimics group when compared with the control group ($p<0.05$). However, their expressions were all remarkably up-regulated in Inhibitors group ($p<0.05$). The above findings suggested that miR-195 overexpression suppressed the expressions of TGFβ1-Smad3 signaling pathway and related molecules, further inhibiting myocardial fibrosis (Figure 3).

Protein Expression Levels of α-SMA and Related Pathway TGFβ1-Smad3 through Western Blotting

To further determine the key molecules in the fibrosis of myocardial fibroblasts and the effect of miR-195 on the TGFβ1-Smad3 signaling

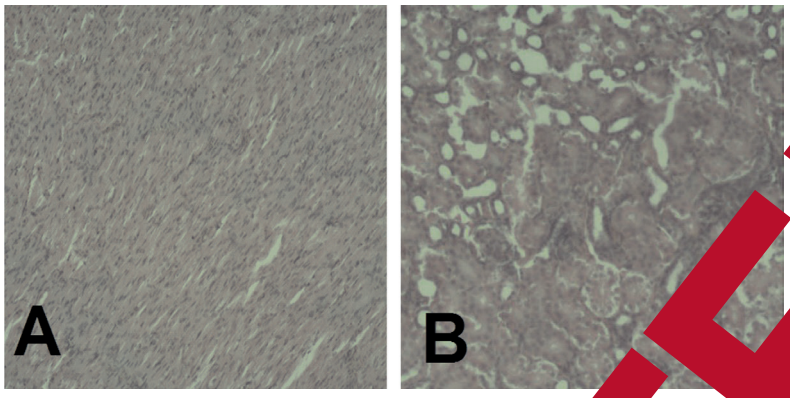

Figure 2. Pathological changes in rat myocardial tissues. Myocardial cells were ...ged in orde... group ***(A)***, while was disorderly arranged in the SHR group (***B***), with thickened muscle fibers ...ocardial fibrosis ...cation: 100×).

pathway, Western blotting was performed. The results (Figure 4) revealed that compared with the control group, the protein expression levels of α-SMA, TGFβ1 and Smad3 were significantly down-regulated in Mimics group ($p<0.05$). However, they increased markedly in Inhibitors group ($p<0.05$). These results indicated that overexpression of miR-195 inhibited the e...s-sions of the TGFβ1-Smad3 signaling ... and related molecules, further repressing ...o-cardial fibrosis.

Discussion

MiRNAs are able to r...ate the ...erentiation, proliferation and a...is of ... Therefore, they play impo...t r... physiological homeostasis and he... as well ...ious diseases[18]. The roles of ...NAs in the p...nesis of different diseas... been widely in...tigated. MiRNAs hav... c...d as important gene expression ...gulators in ... diseases. For example, m...95 plays a key r... many processes of m...ardial fibrosis in hyp...tensive rats. In this ... we revealed ... the content of miR-1... as significantly reduced in the SHR group. ...s indicated tha... the expression of miR-195 was ...ibited. More... the results of HE staining ...ed that my...dial cells were arranged or-d... the no... group. However, myocardial cells ...d disorderly, muscle fibers were thickened, and myocardial fibrosis was detected ... the SHR group. These results suggested that ... of miR-195 expression promoted the ...velopment and progression of myocardial fibrosis. To provide important references for early diagnosis in clinic, liver function indexes (ALP and GPT) and myocardial function index (CK) were examined as well. The results showed that serum levels of ALP, GPT and CK in the SHR group were overtly higher than those of the normal group ($p<0.05$). These results implied that liver function and myocardial function indexes increased significantly in the development and progression of myocardial fibrosis in hypertensive rats, which might provide an important reference for early diagnosis. Cardiac hemodynamic indexes and arterial blood pressure were also observed in this study. It was found that the SHR group

Table ... ...rial blo...ssure and cardiac function indexes determined via MRI & ECG ($\bar{x}\pm s$).

G...	...Dd (mm)	LVESd (mm)	EF (%)	FS (%)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)
N...	...29±0.46	3.21±0.33	59±5.2	52.8±2.0	105.4±2.6	79.9±3.9
...group	8.12±0.51[a]	6.28±0.52[a]	48±2.6[a]	38.4±2.8[a]	151.3±4.9[a]	115±5.8[a]

...Blood pressure and cardiac function indexes. Compared with those in the normal group, the FS and EF in SHR group are ev... reduced, while the systolic blood pressure, diastolic blood pressure, LVEDd and LVESd are remarkably increased ($p<0.$... [a]$p<0.05$).

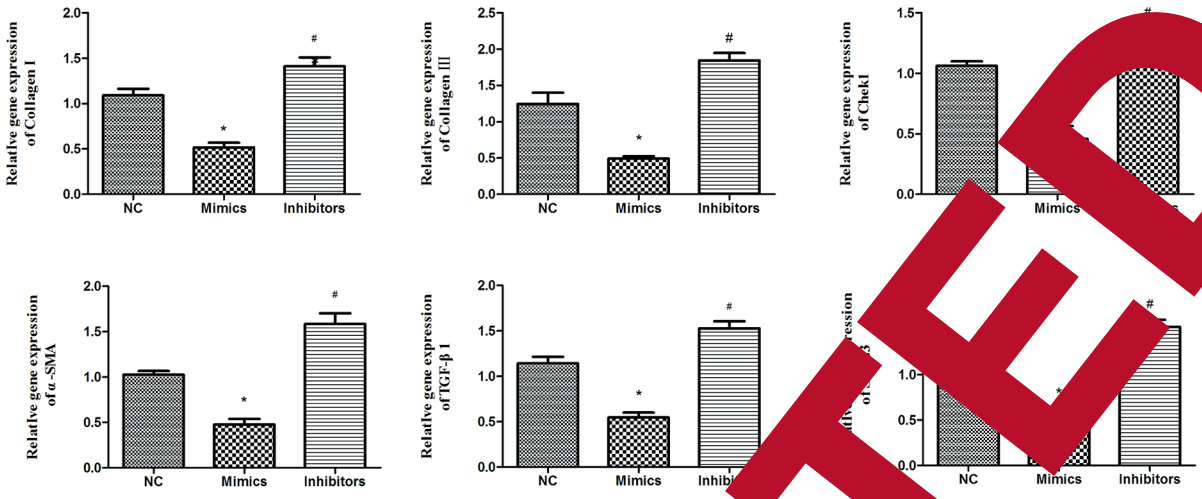

Figure 3. MRNA expression levels of Collagen, Chek1 and α-SMA and related pathway ... Smad3 via RT-PCR. The expression levels of Collagen, Chek1, α-SMA, TGFβ1 and Smad3 d... significantly in M... group when compared with the control group ($p<0.05$). However, they were remarkably el... in Inhibitors group compared with the control group ($p<0.05$) ($^{*}p<0.05$, $^{\#}p<0.05$).

showed evidently reduced FS and EF and markedly increased systolic blood pressure, di... blood pressure, LVEDd and LVESd in c... son with normal group ($p<0.05$). This in... ed that the selection of SHR model was a suc... Our results met the requirements for experime... animals, and further studies could be performe... In summary, *in-vivo* results s... t inhibition of miR-195 expression p... oted ... evelopment of myocardial fibrosi... hich wa... sistent with the findings of prev... udies...

In addition, miR-19... ta... genes and proteins important ... myoca... fibrosis, including Collage... hek1 and α... Chek1 is a key molec... ulating the ... sis and proliferation ... myo... l cells. Meanwhile, its increas... expression ... itates myocardial fibrosis. ...MA, Collagen ... Collagen III are cru... comp...nents of myocardial fibroblast expr...n. Th... xpression levels have been fo... mar... incr...ed once myocardial fibros...-23]. Th... study discovered that C...llagen... k1 a... -SMA were lowly ex...d in M... oup. The results suggested t... overexp...on of miR-195 inhibited the exp...on of genes related to the proliferation a...on of myocardial fibrosis, there... epressing myocardial fibrosis development. ...eover, our findings indicated that miR-195 ... d multiple steps of myocardial fibrosis, affe... its development and progression. To further ... s effect, the protein expression level of α-SMA was detected by Western blotting. The results were consistent with those of ...ession levels, further confirming the ...y role of miR-195 in myocardial fibrosis. In addition, these data were similar to the findings of predecessors[24,25].

TGF-β1 acts as a regulator in the differentiation, proliferation and apoptosis of fibroblasts. The activation and binding of TGF-β1 to its receptor can further trigger the phosphorylation of its downstream protein Smad[26]. Researches have found that the activation of the TGF-β1/Smad signaling pathway promotes the proliferation and migration of myocardial cells. Ultimately, this may increase cardiovascular resistance and induce cardiovascular remodeling[27]. This study revealed that the expression levels of TGF-β1 and Smad3 in rat tissues in Mimics group decreased significantly. Increased expression of miR-195 in myocardial cells reduced myocardial fibrosis in hypertensive rats. Meanwhile, it markedly lowered the expression levels of TGF-β1 and Smad3 in myocardial cells. These results suggested that the activation of the TGF-β1/Smad3 signaling pathway might be a leading cause of myocardial fibrosis in hypertensive rats. Furthermore, miR-195 might regulate myocardial fibrosis in rats by inhibiting the TGF-β1/Smad3 signaling pathway, which was consistent with the findings reported by Lal H and Melchior-Becker A[28,29].

Conclusions

Overexpression of miR-195 suppresses the progression of myocardial fibrosis in hypertensive rats by inhibiting the TGF-β1/Smad3 signaling pathway. Meanwhile, the miR-195/TGF-β1/Smad3 axis regulates myocardial cells, playing an important role in the pathogenesis of myocardial fibrosis in hypertensive rats. Therefore, the miR-195/TGF-β1/Smad3 axis can be used for the treatment of patients and the evaluation of therapeutic effect and prognosis.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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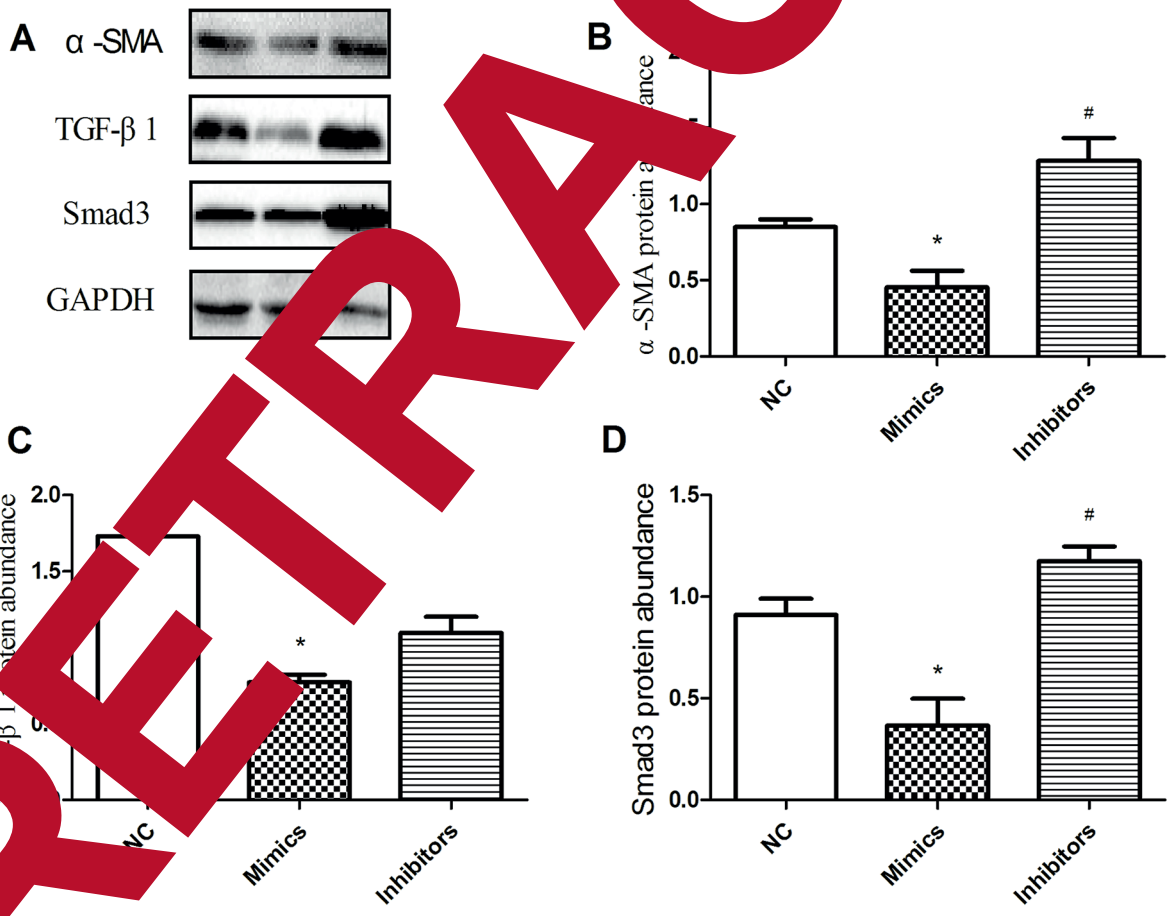

Figure 4. Protein expression levels of α-SMA and related pathway TGFβ1-Smad3 through Western blotting. The protein expression levels of α-SMA, TGFβ1 and Smad3 decreased significantly in Mimics group ($p<0.05$), whereas increased markedly in Inhibitors group ($p<0.05$). $^{*}p<0.05$, $^{\#}p<0.05$.

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