Kangxianyixin Granule Improves Myocardial Fibrosis by Suppressing RhoA/ROCK1 Pathway

Shunyu Liu (blues0621@hotmail.com)
Henan University of Traditional Chinese Medicine
Rutao Bian
Henan University of Traditional Chinese Medicine
Yuanshu Liu
Henan University of Traditional Chinese Medicine
Xueping Ren
Henan University of Traditional Chinese Medicine
Hongbo Chang
Henan University of Traditional Chinese Medicine
Zongyao Guo
Henan University of Traditional Chinese Medicine
Hong Wu
Henan University of Traditional Chinese Medicine
Zhentao Wang
Henan University of Traditional Chinese Medicine

Research

Keywords: KXYX, dilated cardiomyopathy, myocardial fibrosis, ROCK1

Posted Date: April 15th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-22531/v1

Abstract

Background: Kangxianyixin granule (KXYX), a traditional Chinese medicine prescription, has been clinically used to treat dilated cardiomyopathy (DCM) for many years. Myocardial fibrosis (MF) is a major pathological feature of DCM and independent predictor of adverse cardiac outcomes. The present study investigated the effect and the possible mechanism of KXYX on myocardial fibrosis.

Methods: Male wistar rats of DCM was induced by furazolidone (Fz) (0.3 mg/g/day, gavage) for 8 weeks and treated with KXYX (3.6, 1.8, 0.9 g/kg/day) or captopril for another 4 weeks. The cardiac function indices were evaluated using ECHO. Myocardial morphology was visualized using H&E and masson staining. Then, the effect of differentiation of Ang II-induced cardiac fibroblasts (CFs) to myofibroblasts was detected using a-SMA and Vimentin immunohistochemical staining. The expression of RhoA, ROCK1, p-MLC were observed using western blot. The mRNA level of RhoA, ROCK1, MLC were assayed by quantitative RT-PCR.

Result: Fz-induced rats had changes in the structure and function of the left ventricle as well as myocardial fibers broken and loosely arranged, cardiomyocytes necrosis in the myocardial tissue. High level expressions of fibrosis were observed in the model rats. The expressions of RhoA, ROCK1, p-MLC were elevated in dilated heart and Ang □-induced CFs, while KXYX can rescued cardiac dysfunction and remodeling, inhibited the process of MF and down-regulated RhoA/ROCK1 signaling which suppressed the differentiation of fibroblasts into myofibroblasts.

Conclusion: Our study demonstrated that KXYX attenuated the development of myocardial fibrosis by down-regulating the expression of RhoA/ROCK1 signaling pathway in vivo and vitro. Thus, we provided an underlying mechanism of KXYX function in DCM therapy.

Background

Dilated cardiomyopathy (DCM) is a cardiomyopathy characterized by ventricular enlargement and systolic dysfunction, which is a common cause of heart failure[1]. Myocardial fibrosis (MF) plays a major role in ventricular remodeling which causes cardiomyopathy progression[2]. Large areas of fibrotic tissue can be detected in the biopsy of patients with DCM[3]. The process of MF mainly includes fibroblasts proliferate and transform to myofibroblasts in the myocardial tissue and collagen fibers excessive deposit in the extracelluar matrix (ECM). These changes lead to ventricular stiffness, and diastolic dysfunction, which exacerbate the heart failure[4]. Therefore, blocking the development of MF may be the key to DCM treatment. Drugs or interventions which can specifically target fibrosis may break through the current clinical treatment limitations.

Rho kinase (ROCK), a downstream effector molecule of RhoA, is a transfer station for a variety of intracellular signaling pathways[5]. Rho kinases, including ROCK1 and ROCK2, promote cytoskeletal migration and recombination in normal physiological conditions. But excessive activation of ROCK1 promotes the transformation and differentiation of myocardial fibroblasts, which leads to the imbalance

of collagen metabolism, and then leads to MF[6]. The RhoA/ROCK1 signaling pathway can induce fibroblasts to release fibrogenic cytokines such as TGF-β1, CTGF and α-SMA[7]. All the cytokines stimulate myofibroblasts to secrete collagen fibers and promote the development of MF[8–10]. The downregulation of RhoA/ROCK signaling pathway may be a new method for the treatment of MF.

Traditional Chinese medicine has a long history in the treatment of heart failure. KXYX is an effective prescription for DCM heart failure. The symptoms in DCM patients include fatigue, palpitations, chest tightness, shortness of breath and edema. Chinese medicine believes that these symptoms are closely related to qi deficiency and blood stasis. Therefore, KXYX combines a variety of herbs responsible for their multiple efficacies such as tonifying qi and invigorating the circulation of blood, Clinical observation showed that KXYX alleviated symptoms, rescued cardiac function and postponed ventricular remodeling in DCM patients[11, 12]. In our previous studies, KXYX had multi-target effects on DCM by means of properties of inhibiting cardiomyocyte apoptosis[13], improving myocardial energy metabolism[14], anti-thrombosis, reducing DCM rat myocardial tissue pathological damage and reducing the expression level of myocardial interstitial collagen[15, 16]. However, the specific therapeutic mechanism of KXYX is still unclear. Therefore, this study used furazolidone-fed rats and angiotensin □ treated fibroblasts, and observe the antifibrogenic effect of KXYX on MF in vitro and in vivo.

Methods

ANIMAL AND GROUP

All animal experiments were approved by the animal care and use committee of Henan hospital of Chinese Medicine. (PZ-HNSZYY-2019-021) one hundred male wistar rats(4-week old) were purchased from Beijing Charles River experimental animal technology Co., Ltd□Beijing,China□. The rats were randomly divided into the normal group (n = 10) and the DCM group (n = 90). All of them were allowed free access to food and water. DCM group received a gavage of furazolidone (Tianjin Lisheng, China) solution (50 mg/ml, 0.3 mg/g for 8 weeks)[17]. Left ventricular end-diastolic diameter (LVEDD) and ejection fraction (LVEF) was measured by echocardiographic examination to confirm the model successful or not[18, 19]. The successful DCM rats (n = 62) were randomly divided into 5 group, including model, KXYX high, KXYX medium, KXYX low and captopril groups. The daily gavage of KXYX was 3.6 g / kg, 1.8 g / kg, 0.9 g / kg, respectively[20]. captopril group was 10.125 mg / kg (Shanghai Sine, China). normal group and model group were given the same amount of normal saline. The indicated treatment was administered orally daily for 4 weeks after furazolidone gavage.

Preparation of KXYX

The KXYX contains the following Chinese medicines: ginseng (*Panax Ginseng* C. A. Mey) 12 g, astragalus (*Astragalus membranaceus* Fisch. Bge.)30 g, poria cocos (*fungus nucleus of Poria cocos* Schw.)15 g, salviae miltiorrhiza(*Salvia miltiorrhiza* Bge. Labiatae)15 g, atractylodes rhizome (*Atractylodes macrocephala* Koidz.)15 g, herba lycopi (*Aconitum gymnandrum* Maxim.) 15 g, leonurus (*Leonurus*

japonicus Houtt)15 g, ophiopogonis □*Ophiopogon japonicus (Linn. f.)* Ker-Gawl.□12 g, cimicifugae Rhizoma (Cimicifuga foetida L.) 9 g. Concentrated granule made from nine Chinese medicinal herbs mentioned above. The batch numbers of the above Chinese herbal formula granules are 18080058, 18060154, 18090064, 18050094, 18040048, 18060048,18010423,18720032,18510233. All herbal materials, dispensing granules, and quality control data of HXWTF were supplied by Sichuan Neo-Green Pharmaceutical Technology Development Co., Ltd (Sichuan, China).

Echocardiography (ECHO)

Rats were anesthetized with intraperitoneal injection of 1% pentobarbital sodium (30 mg/kg). Cardiac function and dimensions were measured by echocardiography with an Acuson Cypress system and a 7L3 probe (Siemens, Germany) after 4 weeks of KXYX treatment for rats, M-mode echocardiography was used to measure the left ventricular end-diastolic diameters (LVEDD)and left ventricular end-systolic diameters(LVESD) on the parasternal long axis views. Next, ejection fraction (EF)and fractional shortening (FS) were calculated. EF was calculated by the Teichholtz method. The mean values were obtained from at least three different cardiac cycles.

H&E and masson staining

After echocardiography examination, the hearts were removed by thoracotomy, the remaining blood was washed in pre-cooled PBS, and fixed in 4% paraformaldehyde solution for 48 hours. After dehydration, heart tissue specimens were embedded in paraffin, and cut into5 μm sections. Hematoxylin eosin staining (HE) and Masson staining were performed according to the standard protocol. The myocardial collagen deposition in each group were observed under optical microscope. 5 fields were randomly selected to observe and record the images.

Cell culture

The ventricles of neonatal rats were cut into pieces under aseptic conditions, digested into single cells in 0.1% collagenase, and collected every 5 min. After centrifugation, all the cells were cultured in DMEM (DMEM, Gibco, USA) medium with 10% calf serum (Gibco, Australia), incubated in incubator of 5%CO_2 at 37℃ (Thermo Fisher Scientific, USA) for 24 h, and myocardial fibroblasts (CFs) were obtained with differential attachment method. Passage as cells grow to a near-fused state. The adherent CFs were spindle-shaped, transparent cytoplasm, large oval shape nucleus under the microscope. the cultured cells identified by SP staining with vimentin. Fibroblasts of passage two were inoculated into a 6-well plate and cultured for 24 h, and the serum containing KXYX or fasudil were added to the culture for 2 h, washed with PBS, and then treated with Ang II for 24 h, and then samples were collected for further test.

Preparation of medicated serum

SD Rats weighing 220 ± 10 g were chosen and randomly divided into KXYX group (KXYX serum) and normal group (normal serum). Rats in KXYX groups were treated gavage with a KXYX solution at a

dosage of 1 ml/100 g/day (n = 10) while the normal group received the same volume of normal saline (n = 10) for 10 days. The blood from abdominal aortic were collected one hour after the final treatment. After that, centrifuged at 3500 rpm for 15 min, and retained the supernatant. Serum was heated at 56 °C for 30 min, filter with a 0.22 μm filter membrane, stored at − 80 °C.

Immunofluorescence

The fibroblasts were fixed in 4% paraformaldehyde for 30 min at room temperature, washed three times with PBS, and permeabilized with 0.3% Triton X-100 for 30 min. After blocking with BSA at room temperature for 30 minutes, slide the slides with anti-αSMA, Vimentin antibodies (Wuhan proteintech Biotechnology Co., Ltd.,) at After overnight incubation at 4℃, washing 3 times with PBS, the slides were incubated with FITC fluorescent secondary antibody (Wuhan proteintech Biotechnology Co., Ltd.,) at 37 ° C, protected from light for 2 h. After counter-staining the nuclei with DAPI, the images were observed and recorded using a fluorescence microscope.

Cell viability

Fibroblasts were grown in 96-well plates, and the cell density in each well was controlled at 1×10^4 cells / well for 24 hours. After that, each well was given different concentrations of KXYX (0–1.0 mg / ml) and cultured for 24 hours. 10 was added to each well. ul of CCK-8, and then detect the absorbance at 450 nm in each well to calculate the growth and viability levels of KXYX cells at different concentrations. The optimal intervention concentrations of angiotensin II and fasudil were measured in the same way.

WB

protein was extracted from heart tissue which was homogenized in RIPA lysis buffer, and quantitated by protein quantification BCA kit (Biyuntian Biological, China). The total protein was separated on SDS-PAGE gel, and then transferred to a PVDF membrane. the membrane was blocked with 5% skim protein powder at 37 ° C for 1 h. Membrane with primary antibody at 4 °C overnight. The primary antibodies against α-SMA,COL-1,RhoA,ROCK1,MLC, CTGF(proteintech),p-MLC□Cell Signaling Technology, USA□□After washing, the membrane was coupled with a specific HRP-conjugated secondary antibody (Wuhan Sewell Biotechnology, China) for 1.5 h. The membrane was subsequently washed. Relative luminescence intensity was analyzed by gel image system (Bio-Rad Laboratories, USA).

RT-PCR

Extract the total RNA solution according to the kit's protocol (Qiagen). The total RNA concentration and purity were measured and reverse transcribed into cDNA. Real-time PCR was performed using cDNA as the template, normal group as the reference group, β-actin as the internal reference, and the relative expression of mRNA was expressed by 2-ΔΔCt, and the relative quantitative analysis of ROCK1, MLC, and CTGF mRNA expression was performed. Data was analyzed with Bio-Rad CFX Manager software.

Statistical analysis

SPSS for windows 22.0 was used for statistical analysis and GraphPad Prism 6.0 software was used for statistical graph. Measurement data are expressed as mean ± standard deviation (SD). analyzed by one-way analysis of variance (ANOVA) test is used for comparison of multi-sample means that follow a normal distribution, and LSD test is used for comparison between groups. A value of $P < 0.05$ was considered statistically significant.

Results

KXYX improve heart function in rats

After 4-week treatment, the Echo of each model rat was measured. LVEDD and LVESD were increased in the model group than normal group, and EF and FS decreased as well. These indicate pathological changes in the left ventricular structure of model rats. Compared with the model group, the KXYX high and KXYX medium group can reduce the effects of LVEDD and LVESD, and improve the effects of EF and FS, but the improvement in the KXYX low group is not significant. The results show that KXYX can improve cardiac function in DCM rats.

KXYX inhibit myocardial fibrosis in DCM rats

H&E and Masson staining showed that cardiomyocytes swell, disorder, necrosis in the myocardial tissue of the model group were increased than the normal. myoglobin arranged disorderly, collagen and α-SMA expression increased can also be found in the model group. Myocardial edema was reduced in myocardial tissues in the KXYX high and middle groups, as well as myoglobin alignment improved. KXYX low group did not show significant effect, and still had a large amount of collagen deposition (Fig. 2A, B, and C).

We use WB and QPCR to determine the expressions of CTGF, COL-1 and α-SMA in myocardial tissues. The expressions in the model group increased, while the myocardial tissues in the rats treated with KXYX were reduced to varying degrees, most obviously in the high-dose group, and the reduction in the low-dose group was not significant (Fig. 2D, E, F). These results indicate that the improvement of cardiac function in DCM rats by KXYX may be achieved by inhibiting the myocardial fibrosis.

KXYX inhibits AngII induced myofibroblast transdifferentiation

Vimentin is a protein that is specifically expressed by fibroblasts. Using immunofluorescence microscopy, we can observe a large number of fibroblasts were isolated from the heart tissue of suckling rats. When the pressure is overloaded, fibroblasts can adopt an “active” state known as “myofibroblast”. Induced by AngII, CFs were transformed into myofibroblasts into round shape, and fibrosis activation marker α-SMA was abundantly expressed in cells (Fig. 3A). AngII did not significantly affect the viability of CFs between 10-10000 nmol / L (Fig. 3B), and the effect of CCK-8 of KXYX on cell viability was measured at different concentrations, the cell viability was optimal when the concentration at 25 mg / ml (Fig. 3C). When induced by different concentrations of AngII, the expression of α-SMA and collagen Ⅰ(COL-1) was the

highest at 10 nmol / L (Fig. 3D, E), so 10 nmol / L was used as the optimal concentration for cell model induction. KXYX can inhibit the expression of myocardial fibrosis factors such as CTGF□connective tissue growth factor□, COL-1 and α-SMA in Ang□-induced CFs (Fig. 4).

KXYX Down-regulate the Expression of RhoA, ROCK1, p-MLC

By detecting the expression of proteins and mRNA in the RhoA / ROCK1 signaling pathway in myocardial tissue, the expression levels of RhoA, ROCK1, and p-MLC in the myocardial tissue of the model group rats were significantly higher than those in the normal group; The reduction effect was seen in the KXYX high and middle dose groups, but the improvement in the low dose group was insignificance (Fig. 5).

Similar results were showed in vitro (Fig. 6A). Then we use fasudil, an inhibitor of the RhoA / ROCK signaling pathway to further evaluate the influence of these signaling pathways. Further validation was performed in the AngII-induced CFs with KXYX or fasudil. It can be seen that both KXYX and Fasudil have the ability to inhibit the expression levels of RhoA, ROCK1, p-MLC, and the combination of KXYX and Fasudil can further inhibit the effect (Fig. 6B, C, D, E).

Discussion

DCM is one of the most common causes of heart failure today, which has become one of the highest mortality cardiovascular diseases and deteriorates the life quality for patients all over the world[21]. This study shows that furazolidone-induced DCM rat models can better replicate the relevant pathological characteristics of human DCM, such as decreased cardiac function, ventricular pathological remodeling and expansion, and the occurrence of myocardial fibrosis[22]. The result is similar to previous studies. When myocardial fibrosis occurs, cardiomyocytes in the myocardial tissue appear swollen, disordered, necrotic, disordered myoglobin arrangement, increased collagen deposition and fibrotic markers such as α-SMA were significantly increased.

KXYX can inhibit the level of myocardial fibrosis in model rats, and at the same time can down-regulate the expression of RhoA, ROCK1, MLC, inhibit the transformation of CFs to myofibroblasts, and the expression of COL-1 and CTGF fibrotic factors. In vitro studies, KXYX show an inhibition of the up-regulation of RhoA / ROCK1 signaling pathway in CFs. This indicates that KXYX may improve the further deterioration of myocardial fibrosis by down-regulating the RhoA / ROCK1 signaling pathway activation.

During the development of DCM, the activation of renin-angiotensin-aldosterone system (RAAS), oxidative stress, and cytokines (such as TGF-β) can promote the proliferation and transdifferentiation of CFs[23]. Inflammatory cells can secrete cytokines and act on CFs, resulting in increased collagen secretion. At the same time, the collagen synthesis and degradation of myocardial tissue are imbalanced, and myocardial fibrosis occurs. The replacement of cardiac muscle by fibrotic tissue, increasing inability of the ventricle to pump blood sufficiently to meet the demands[24, 25]. The transdifferentiation of CFs is the core link of the development of cardiac fibrosis.

In recent years, inhibiting the development of myocardial fibrosis has been a hot issue in clinical treatment and scientific research. A variety of natural and synthetic drugs have been proven to play a protective role for heart by inhibiting the development of myocardial fibrosis. Statins have become the most effective drugs for the prevention and treatment of coronary heart disease. Their main mode of action is to treat atherosclerosis by lowering cholesterol levels. Some statins show the effect in preclinical and clinical studies to inhibit fibrosis and adverse myocardial remodeling[26–28]. Simvastatin show an inhibition for CFs transdifferentiation through TGF-β pathway. Elevation of locally angiotensin II level serves as a potent stimulant for CFs both through direct actions and through TGF-β mediated effects. aldosterone antagonists, angiotensin receptor blockers (ARB) and ACE inhibitors (ACEI) show the good effect on heart failure is closely related to the improvement of cardiac fibrosis[23]. Researches on the pharmacological mechanism of Chinese medicines have gradually increased. Studies have shown that astragaloside IV and astragalus polysaccharides which are the main effective components of astragalus, can inhibit myocardial fibrosis and protect heart[29, 30]. Ginsenoside Re can improve isoprenaline-induced myocardial fibrosis by down-regulating the TGF-β1 / Smad3 pathway[31]. Salvianolic acid which is extracted from salvia miltiorrhiza reduces Ang II-induced cardiac fibrosis in rats by inhibiting the NF-κB pathway[32].

Connective tissue growth factor (CTGF) is a key mediator produced in the extracellular matrix under conditions of pathological fibrosis [33]. The RhoA / ROCK1 signaling pathway can directly or indirectly promote the expression of CTGF [34]. The RhoA / ROCK1 signaling pathway plays an important role in fibroblast proliferation and transdifferentiation, which can directly promote the secretion of collagen by myofibroblasts and also activate other fibrosis-related signaling pathways[35]. KXYX can inhibit the transformation of myocardial fibroblasts to myofibroblasts, inhibit the expression of α-SMA, COL-1, and CTGF, and also down-regulate MF mediated by the RhoA / ROCK1 signaling pathway.

RhoA / ROCK inhibitor fasudil can inhibit the development of myocardial fibrosis and has benefits for cardiac remodeling[36]. ROCK1 can over-activate the phosphorylation of myosin light chains(MLC), which can promote fibroblast migration and MF[37, 38]. Our research shows that KXYX can decrease phosphorylation of MLC, thereby indirectly inhibiting myocardial fibrosis. However, the mechanism of how to inhibit MF by regulating the phosphorylation of myosin light chain needs further research.

Conclusion

To concluded,KXYX inhibit the expression of RhoA, ROCK1, p-MLC in vivo and vitro, it can also directly inhibit expression of myocardial fibrosis factors such as α-SMA, COL-1, CTGF and alleviate MF and improve heart function in DCM rats. Myocardial fibrosis is an important mechanism of DCM. Delaying the development of myocardial fibrosis is a possible way for KXYX to exert cardioprotection.

There are still a few questions in our research that deserve further exploration. We found that KXYX can reduce the apoptosis of cardiomyocytes in DCM myocardial tissue, and we found that KXYX can protect

the mitochondrial morphology of cardiomyocytes under electron microscope. How does KXYX achieve the protective effect on cardiomyocytes? This is the direction of our further research.

Abbreviations

KXYX: Kangxianyixin granule; DCM: dilated cardiomyopathy; MF: myocardial fibrosis; Fz: furazolidone; MLC: myosin light chains; α-SMA: α-smooth muscle actin; COL-1: collagen-1; CTGF: connective tissue growth factor; ECM: extracelluar matrix; TGF-β1: transforming growth factor; ECHO: echocardiography; LVEDD: left ventricular end-diastolic diameters; LVESD: left ventricular end-systolic diameters; EF: ejection fraction; FS: fractional shortening; HE: Hematoxylin eosin staining; DMEM: dulbecco's modified eagle medium; CFs: myocardial fibroblasts; RAAS: renin-angiotensin-aldosterone system

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Shunyu and Rutao designed the study, carried out experiments, Yuanshu and Xueping provided help in feeding rats, Hongbo gave suggestion on experiment design. Zongyao analyzed data, Hong revised the manuscript, Zhentao provided help in designing the study and revised the manuscript. All authors read and approved the final manuscript.

Funding

This study was supported by the National Natural Science Foundation of

China (No. 81573920) and Henan Province Chinese Medicine Scientific Research Project (No. 2019JDZX003)

Availability of data and materials

All data and materials are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The animal experiments were approved by the animal care and use committee of Henan hospital of Chinese Medicine. (PZ-HNSZYY-2019-021).

Consent for publication

This manuscript is approved by all authors for publication.

Conflicts of interest

The authors confirm that there are no conflicts of interest.

References

1. Knowlton KU. Dilated Cardiomyopathy. Circulation. 2019;139(20):2339–41.
2. Gulati A, et al. Association of fibrosis with mortality and sudden cardiac death in patients with nonischemic dilated cardiomyopathy. JAMA. 2013;309(9):896–908.
3. Japp AG, et al. The Diagnosis and Evaluation of Dilated Cardiomyopathy. J Am Coll Cardiol. 2016;67(25):2996–3010.
4. Masci PG, et al. Myocardial fibrosis as a key determinant of left ventricular remodeling in idiopathic dilated cardiomyopathy: a contrast-enhanced cardiovascular magnetic study. Circ Cardiovasc Imaging. 2013;6(5):790–9.
5. Guan R, et al. Advances in the studies of roles of Rho/Rho-kinase in diseases and the development of its inhibitors. Eur J Med Chem. 2013;70:613–22.
6. Liu LJ, et al. The Role of the Rho/ROCK Pathway in Ang II and TGF-beta1-Induced Atrial Remodeling. PLoS One. 2016;11(9):e0161625.
7. Shi J, et al. ROCK1 plays an essential role in the transition from cardiac hypertrophy to failure in mice. J Mol Cell Cardiol. 2010;49(5):819–28.
8. Koitabashi N, et al. Increased connective tissue growth factor relative to brain natriuretic peptide as a determinant of myocardial fibrosis. Hypertension. 2007;49(5):1120–7.
9. Zhu J, et al. Inhibition of RhoA/Rho-kinase pathway suppresses the expression of extracellular matrix induced by CTGF or TGF-beta in ARPE-19. Int J Ophthalmol. 2013;6(1):8–14.
10. Yang X, et al. Mechanism of fibrotic cardiomyopathy in mice expressing truncated Rho-associated coiled-coil protein kinase 1. FASEB J. 2012;26(5):2105–16.
11. Chuiyi Z, Zhentao W, Chen L. Effect of Kangxianyixin granule in patients with dilated cardiomyopathy by echocardiographic evaluation. Modernization of Traditional Chinese Medicine Materia Materia-World Science Technology. 2014;16(01):109–11.
12. Chai S, Zhentao W, Liu S. A cohort study on the treatment of qi-deficiency and blood-stasis syndrome of dilated cardiomyopathy with Kangxianyixin granule. Shaanxi zhongyi. 2019;40(01):49–51.
13. Wang Z, et al. Kangxianyixin granule improves cardiac function and ventricular remodeling in rats with dilated cardiomyopathy. Journal of Emergency in Traditional Chinese Medicine. 2017;26(03):394–6 + 417.
14. Wang Z, et al. Effect of Kangxianyixin granule on BNP and TNF-α in rats with dilated cardiomyopathy. World Journal of Integrated Traditional Western Medicine. 2017;12(05):646–9.
15. Wang Z, Yang F, Zhang Y. Kangxianyixin granule attenuates TGF-β1 expression on dilated cardiomyopathy rats. Lishizhen Medicine Materia Medica Research. 2010;21(06):1430–1.

16. Wang Z, et al. Effect of Kangxianyixin decotion on expression of MMP-1 and its inhibitor in dilated cardiomyopathy rats. J Tradit Chin Med. 2011;52(16):1406–8.
17. Yu Q, et al. Beneficial effects of intramyocardial mesenchymal stem cells and VEGF165 plasmid injection in rats with furazolidone induced dilated cardiomyopathy. J Cell Mol Med. 2015;19(8):1868–76.
18. Zhang M, et al. Calreticulin-STAT3 signaling pathway modulates mitochondrial function in a rat model of furazolidone-induced dilated cardiomyopathy. PLoS One. 2013;8(6):e66779.
19. Hajjar RJ, et al. Pathophysiological and biochemical characterisation of an avian model of dilated cardiomyopathy: comparison to findings in human dilated cardiomyopathy. Cardiovasc Res. 1993;27(12):2212–21.
20. Ren Y, et al. Si-Miao-Yong-An decoction ameliorates cardiac function through restoring the equilibrium of SOD and NOX2 in heart failure mice. Pharmacol Res. 2019;146:104318.
21. Bozkurt B, et al., Current Diagnostic and Treatment Strategies for Specific Dilated Cardiomyopathies: A Scientific Statement from the American Heart Association. Circulation, 2016. 134(23).
22. Glass MG, et al. Differences in cardioprotective efficacy of adrenergic receptor antagonists and Ca2 + channel antagonists in an animal model of dilated cardiomyopathy. Effects on gross morphology, global cardiac function, and twitch force. Circ Res. 1993;73(6):1077–89.
23. Kong P, Christia P, Frangogiannis NG. The pathogenesis of cardiac fibrosis. Cell Mol Life Sci. 2014;71(4):549–74.
24. Moore-Morris T, et al. Cardiac fibroblasts: from development to heart failure. J Mol Med. 2015;93(8):823–30.
25. Bhandary B, et al. Cardiac Fibrosis in Proteotoxic Cardiac Disease is Dependent Upon Myofibroblast TGF -beta Signaling. J Am Heart Assoc. 2018;7(20):e010013.
26. Patel R, et al. Simvastatin induces regression of cardiac hypertrophy and fibrosis and improves cardiac function in a transgenic rabbit model of human hypertrophic cardiomyopathy. Circulation. 2001;104(3):317–24.
27. Bauersachs J, et al. Improvement of left ventricular remodeling and function by hydroxymethylglutaryl coenzyme a reductase inhibition with cerivastatin in rats with heart failure after myocardial infarction. Circulation. 2001;104(9):982–5.
28. Abulhul E, et al. Long-term statin therapy in patients with systolic heart failure and normal cholesterol: effects on elevated serum markers of collagen turnover, inflammation, and B-type natriuretic peptide. Clin Ther. 2012;34(1):91–100.
29. Zhuang Z, et al. Astragaloside IV Exerts Cardioprotection in Animal Models of Viral Myocarditis: A Preclinical Systematic Review and Meta-Analysis. Front Pharmacol. 2019;10:1388.
30. Liu T, et al. Astragalus polysaccharide from Astragalus Melittin ameliorates inflammation via suppressing the activation of TLR-4/NF-kappaB p65 signal pathway and protects mice from CVB3-induced virus myocarditis. Int J Biol Macromol. 2019;126:179–86.

31. Wang QW, et al., Ginsenoside Re Improves Isoproterenol-Induced Myocardial Fibrosis and Heart Failure in Rats. Evid Based Complement Alternat Med, 2019. 2019: p. 3714508.
32. Wang C, et al. Salvianolic Acid B-Alleviated Angiotensin II Induces Cardiac Fibrosis by Suppressing NF-kappaB Pathway In Vitro. Med Sci Monit. 2018;24:7654–64.
33. Frangogiannis NG. Matricellular proteins in cardiac adaptation and disease. Physiol Rev. 2012;92(2):635–88.
34. Pi L, et al. Vascular Endothelial Cell-Specific Connective Tissue Growth Factor (CTGF) Is Necessary for Development of Chronic Hypoxia-Induced Pulmonary Hypertension. Front Physiol. 2018;9:138.
35. Kitamura K, et al. Rho/Rho kinase is a key enzyme system involved in the angiotensin II signaling pathway of liver fibrosis and steatosis. J Gastroenterol Hepatol. 2007;22(11):2022–33.
36. Ocaranza MP, et al. Rho kinase activation in circulating leukocytes is related to hypertensive myocardial remodeling. Clin Sci (Lond). 2018;132(16):1837–53.
37. Shimizu T, Liao JK. Rho Kinases and Cardiac Remodeling. Circ J. 2016;80(7):1491–8.
38. Dai Y, Luo W, Chang J. Rho kinase signaling and cardiac physiology. Curr Opin Physiol. 2018;1:14–20.

Figures

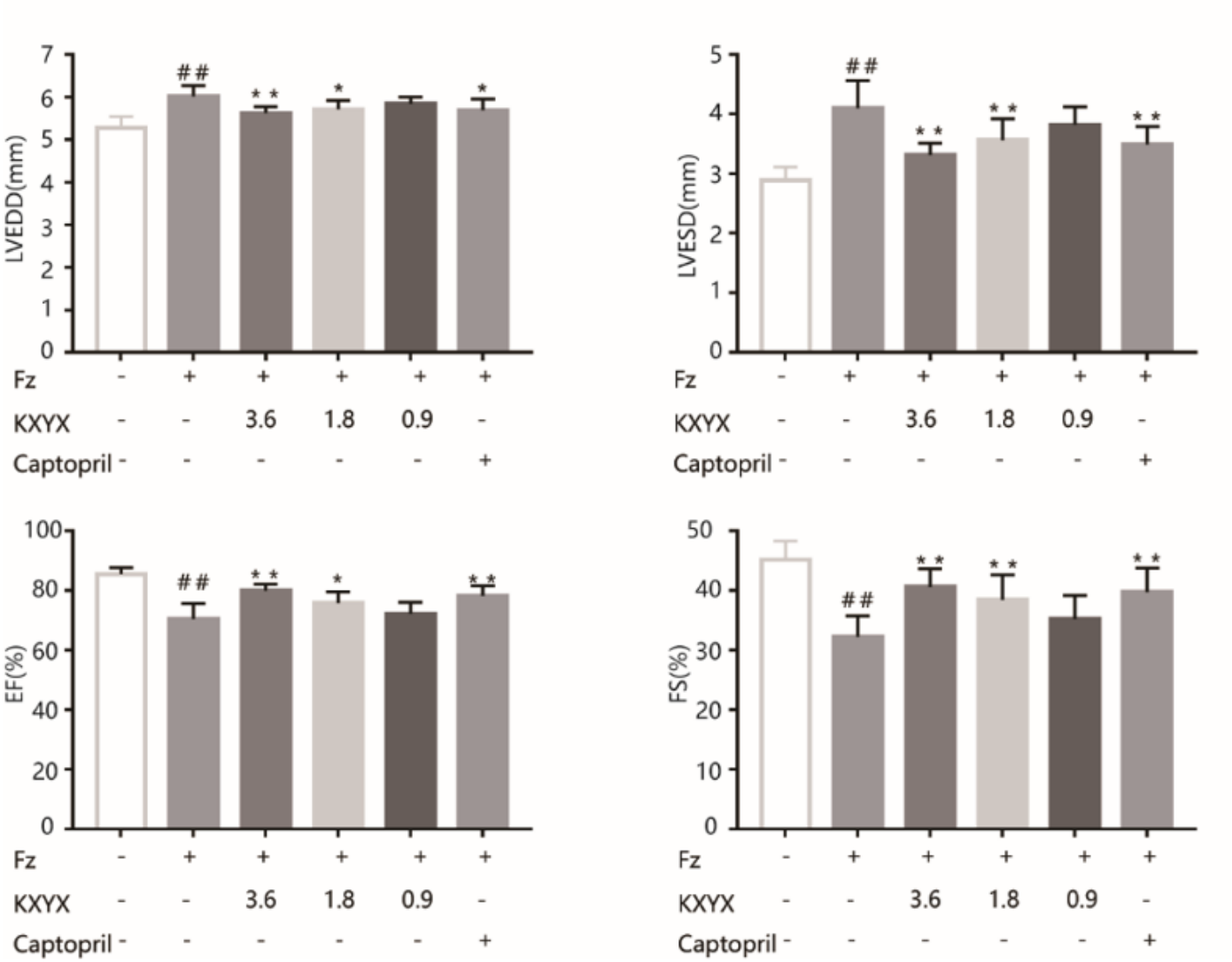

Figure 1

KXYX restored cardiac function in DCM rats. Left ventricular end diastolic diameter（LVEDD）,left ventricular end systolic diameter（LVESD）values, Ejection fraction (EF) values, Fractional shortening (FS) values. Compared with the normal group: ## P <0.01; compared with the model group: *P <0.05, **P <0.01(n=10)

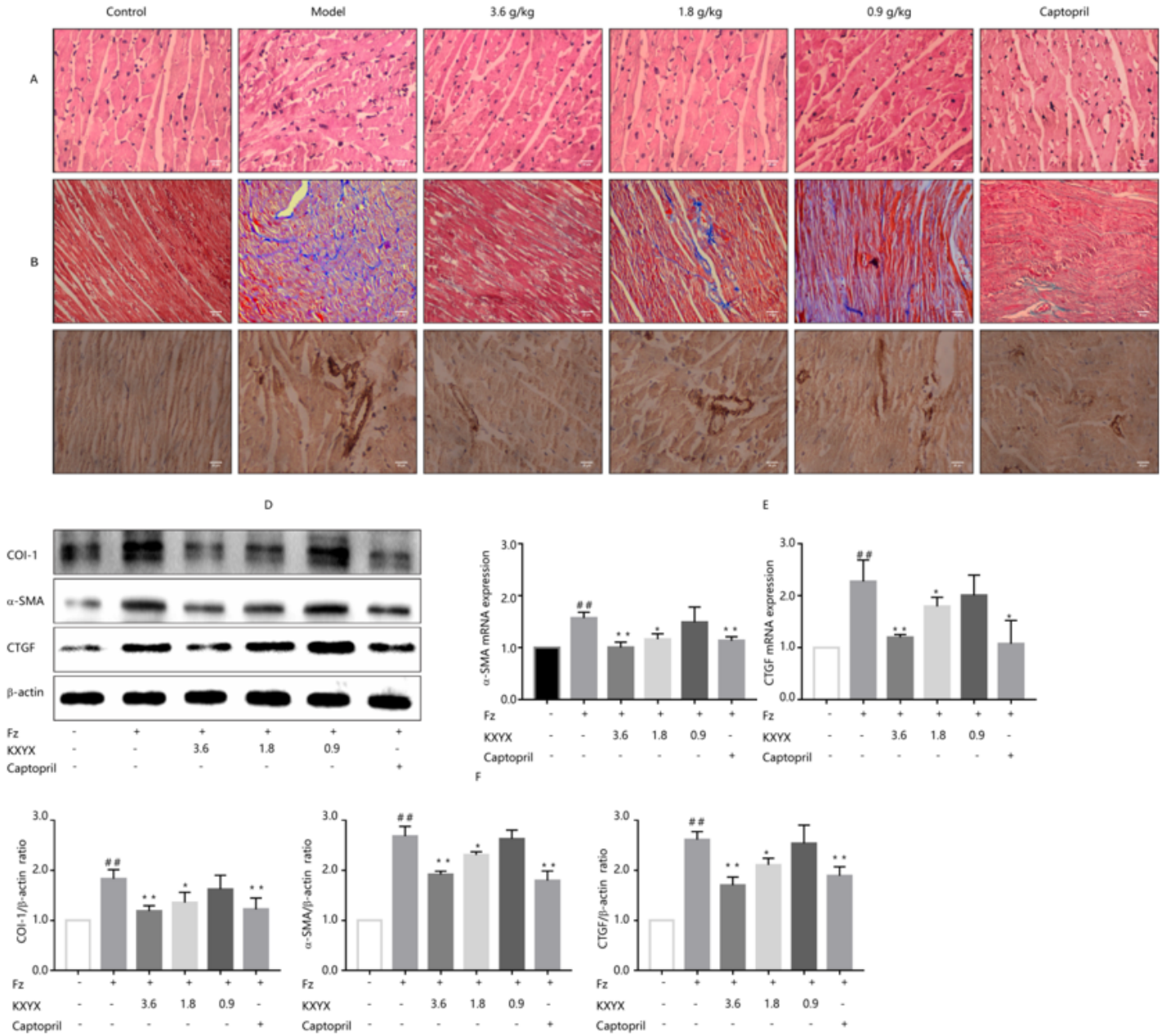

Figure 3

Effect of KXYX on myocardial fibrosis. (A) HE staining was performed to observe the histological changes of the heart tissue, scale bar: 20 μm. (B) Masson staining to observe collagen deposition to observe myocardial fibrosis, scale bar: 20 μm. (C) Immunohistochemical staining to observe the expression of α-SMA in myocardial tissue, scale bar: 20 μm. (D) Effects of KXYX on the expression of COL-1, α-SMA, and CTGF proteins in myocardial tissue of Fz induced rats. (E) Effect of KXYX on the expression of α-SMA and CTGF mRNA in myocardial tissue of furazolidone rat model. (F) Statistical analysis of COL-1, α-SMA, CTGF expression. Compared with the normal group: ## P <0.01; compared with the model group: * P <0.05, ** P <0.01.

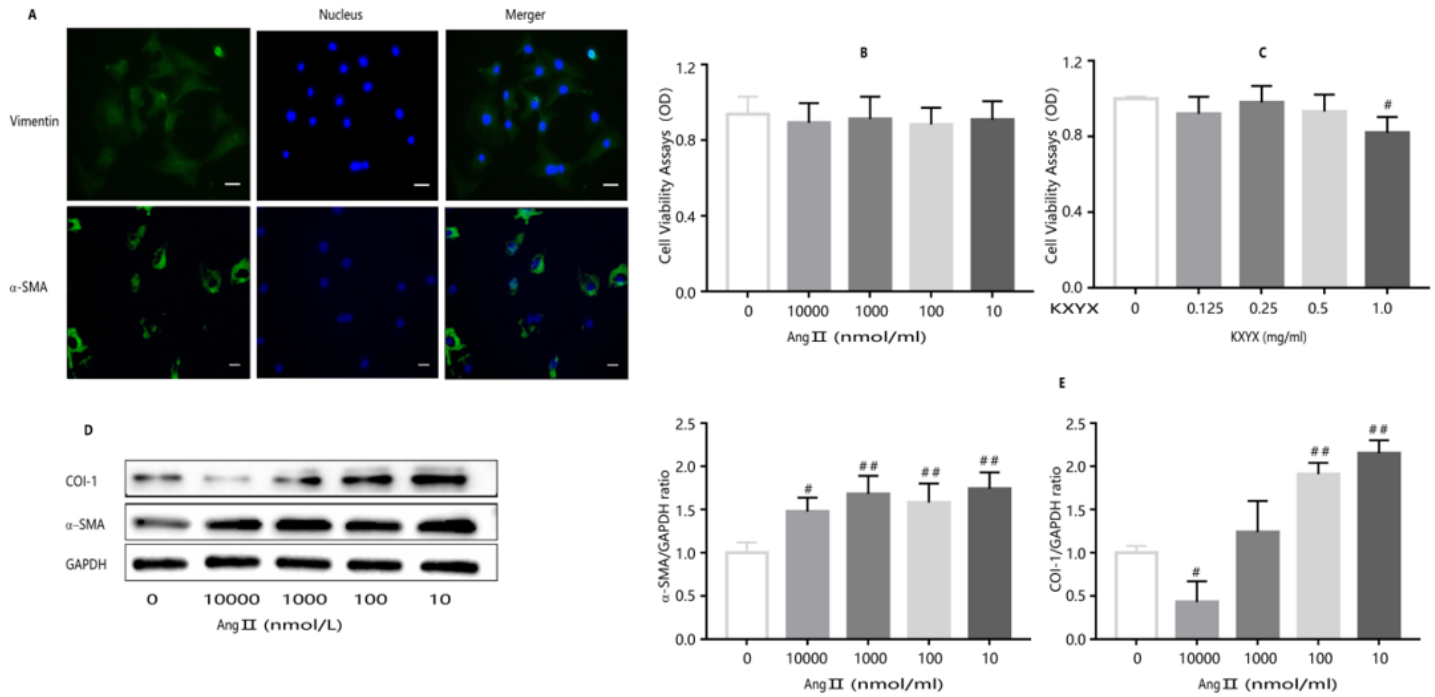

Figure 5

Ang II induces fibroblast transdifferentiation. (A) Cfs were identified by vimentin immunofluorescence staining, and myofibroblast was identified by α-SMA expression, scale bar: 20 μm. (B) Western blotting was used to detect the expression of α-SMA and COL-1 in different concentrations of Ang. (C) Statistical analysis of α-SMA and COL-1 expression. (D) The effects of different concentrations of Ang and KXYX on the viability of fibroblasts were detected by CCK8. Relative to the blank normal group, # P<0.05, ## P<0.01.

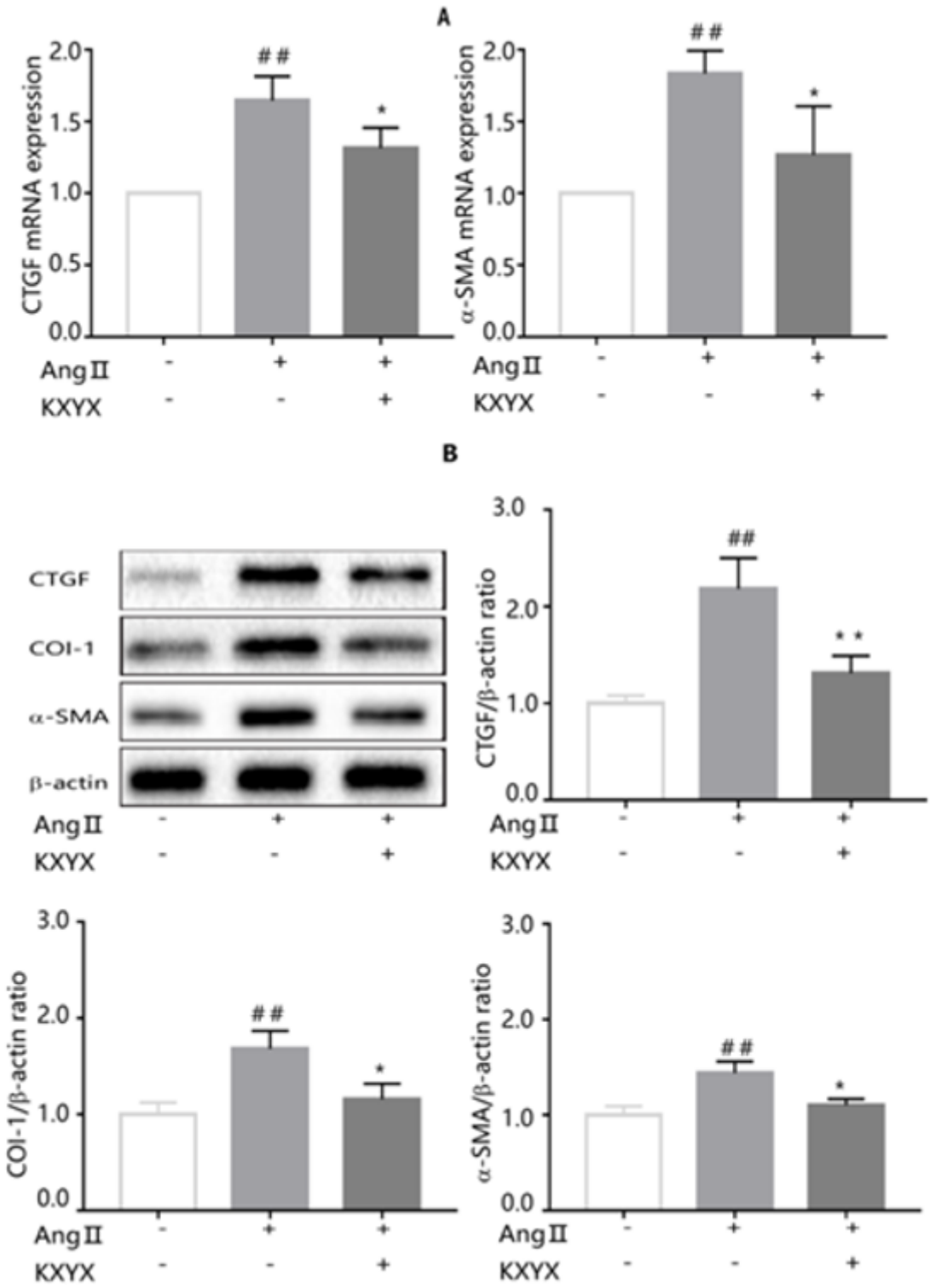

Figure 8

KXYX inhibit CFs transdifferentiation. (A) Effect of KXYX on CTGF and α-SMA mRNA expression in vitro. (B) Effect and statistical analysis of KXYX on the expression of COL-1, CTGF, α-SMA protein in vitro. Compared with the normal group: ## P <0.01; compared with the model group: * P <0.05, ** P <0.01.

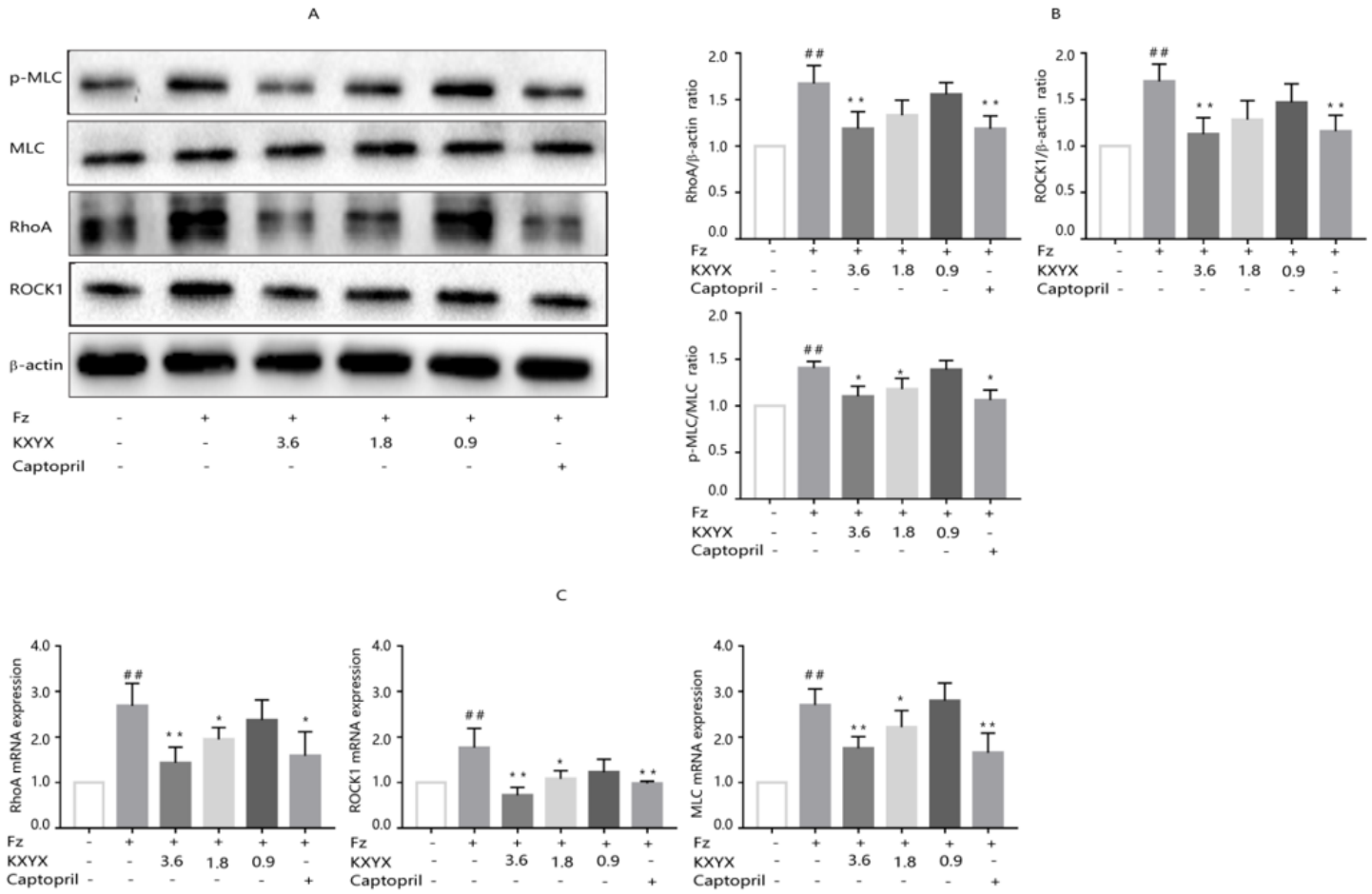

Figure 9

Effect of KXYX on the expression of RhoA, ROCK1, p-MLC in vivo. (A) Western blotting was used to detect the expression of RhoA, ROCK1, and p-MLC in myocardial tissues of furazolidone induced rats. (B) Statistical analysis of RhoA, ROCK1, p-MLC expression. (C) Real-time PCR was used to detect the expression of RhoA, ROCK1, and p-MLC in myocardial tissues of furazolidone induced rats. Compared with the normal group: ## P <0.01; compared with the model group: * P <0.05, ** P <0.01.

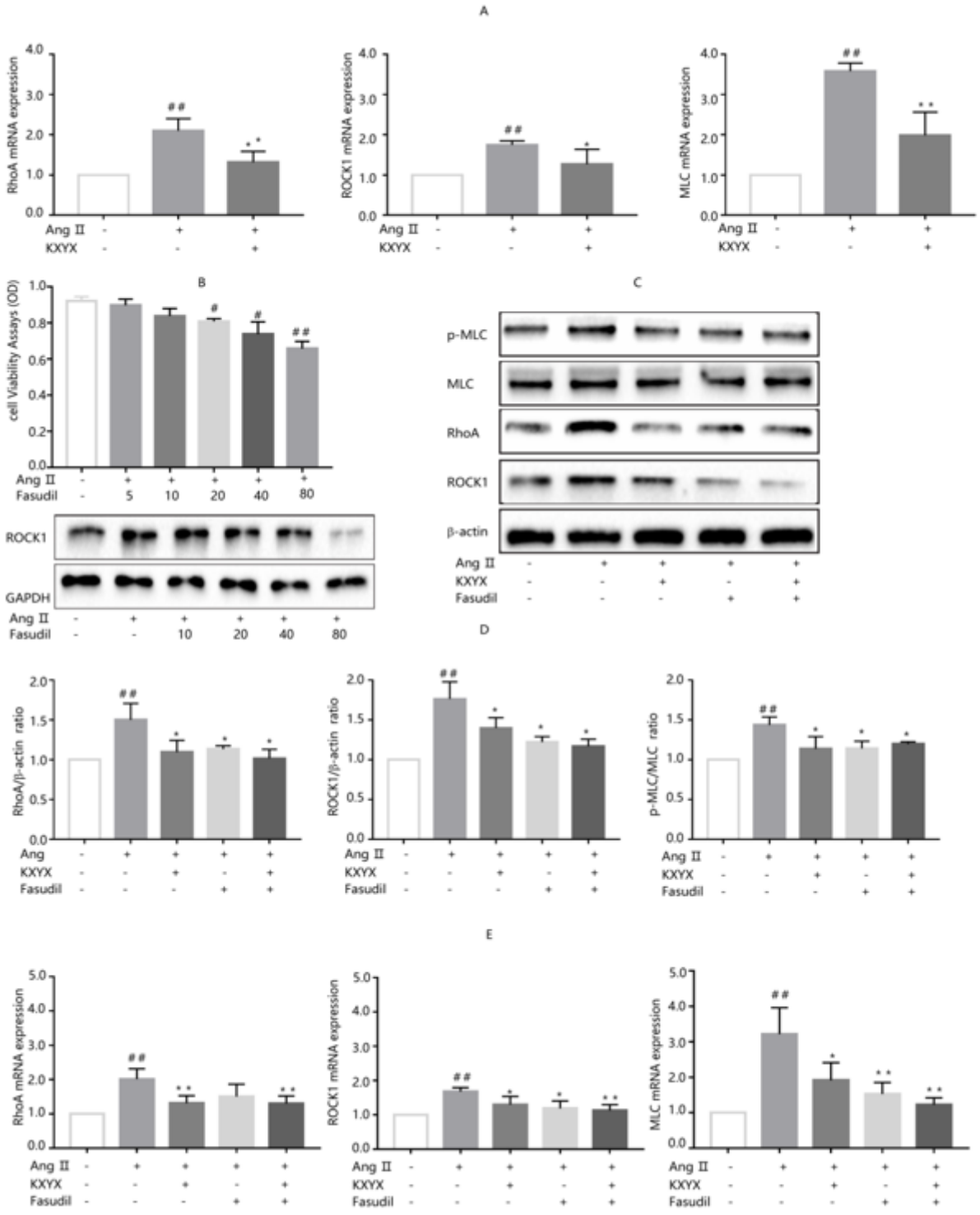

Figure 11

Effect of KXYX on the expression of RhoA, ROCK1, p-MLC in vitro. (A) Real-time PCR was used to detect the expression of RhoA, ROCK1, and p-MLC in CFs. (B) The effect of fasudil at different concentrations on the viability of CFs and the expression of ROCK1 were detected by CCK8. (C) Western blotting to detect the effects of KXYX and fasudil on the expression of RhoA, ROCK1, p-MLC in vitro. (D) Statistical analysis of RhoA, ROCK1, p-MLC expression. (E) Real-time PCR was used to detect the expression of RhoA, ROCK1,

p-MLC in KXYX and fasudil in vitro. Compared with the normal group: ## P <0.01; compared with the model group: * P <0.05, ** P <0.01.