Postprint: Effects of Kangxian Yixin Formula on Ventricular Remodeling in Mice with Dilated Cardiomyopathy via the Sirt1/P53/Drp1 Axis

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Abstract

Background Kangxian Yixin Formula is an empirical prescription used clinically for the treatment of dilated cardiomyopathy (DCM). Previous studies have demonstrated that Kangxian Yixin Formula can inhibit ventricular remodeling and improve cardiac function in DCM, but the underlying mechanism remains unclear. This study provides experimental evidence for the clinical application of Kangxian Yixin Formula. Objective To investigate the effects of Kangxian Yixin Formula on ventricular remodeling in DCM mice via the silent information regulator 2 homolog 1 (Sirt1)/P53/dynamin-related protein 1 (Drp1) axis. Methods Thirty cTnTR141W transgenic mice were randomly divided into model group, Kangxian Yixin Formula group, and captopril group, with 10 mice in each group. Additionally, 10 C57BL/6J mice were assigned as normal control group. Drug intervention was administered for 8 weeks. Echocardiography was performed to measure left ventricular end-systolic diameter (LVESD), left ventricular end-diastolic diameter (LVEDD), left ventricular fractional shortening (LVFS), and left ventricular ejection fraction (LVEF). Hematoxylin-eosin (HE) staining and Masson staining were used to observe histopathological changes and myocardial fibrosis. Transmission electron microscopy was employed to examine mitochondrial ultrastructure. Real-Time PCR was conducted to determine mRNA levels of Sirt1, P53, and Drp1 in myocardial tissue. Western blot was performed to detect protein levels of Sirt1, P53, acetylated P53 (a-P53), and Drp1 in myocardial tissue. Results Echocardiographic examination and heart weight measurements revealed that the model group exhibited increased LVEDD and heart weight, and decreased LVEF and LVFS compared with the normal group ($P<0.05$). The Kangxian Yixin Formula group showed elevated LVEF and LVFS, and reduced heart weight compared with the model group ($P<0.05$). The captopril group demonstrated increased LVEF and LVFS, and decreased LVEDD and heart weight compared with the model group ($P<0.05$). HE and Masson

staining results indicated disordered cardiomyocyte arrangement with varying degrees of hypertrophy, obvious fibrosis, and significant damage to myocardial mitochondrial ultrastructure in the model group. Compared with the model group, both Kangxian Yixin Formula and captopril groups displayed relatively regular overall structure with denser arrangement, and significantly reduced collagen deposition and fibrosis levels in the intercellular spaces of cardiomyocytes. Transmission electron microscopy observations revealed normal ultrastructure of left ventricular myocardium in the normal group, whereas the model group showed obvious ultrastructural damage and marked mitochondrial swelling. The Kangxian Yixin Formula and captopril groups exhibited significantly improved myocardial ultrastructure with recovered mitochondrial morphology. Real-Time PCR results demonstrated that the model group had lower relative Sirt1 mRNA expression and higher relative Drp1 and P53 mRNA expression than the normal group ($P<0.05$). Both Kangxian Yixin Formula and captopril groups showed higher Sirt1 mRNA expression and lower Drp1 and P53 mRNA expression compared with the model group ($P<0.05$). Western blot results indicated that the model group had lower Sirt1 protein expression and higher Drp1 protein and aP53/P53 ratio than the normal group ($P<0.05$). Kangxian Yixin Formula and captopril groups exhibited higher Sirt1 protein expression and lower Drp1 protein and aP53/P53 ratio compared with the model group ($P<0.05$). Conclusion Kangxian Yixin Formula can improve cardiac function and inhibit ventricular remodeling in cTnTR141W transgenic mice, and its mechanism may be associated with upregulating Sirt1 mRNA and protein expression levels, and inhibiting P53 acetylation as well as Drp1 mRNA and protein expression levels.

Full Text

Study on the Impact of Kangxiyixin Formula on Ventricular Remodeling in Mice with Dilated Cardiomyopathy via the Sirt1/P53/Drp1 Axis

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Abstract

Background: Kangxiyixin formula is an empirical prescription used clinically for dilated cardiomyopathy (DCM). Previous studies have demonstrated its potential to inhibit ventricular remodeling and improve cardiac function in DCM patients, though the underlying mechanisms remain unclear. This study provides experimental evidence for the clinical application of Kangxiyixin formula.

Objective: To investigate the effect of Kangxiyixin formula on ventricular remodeling in DCM mice via the sirtuin 1 (Sirt1)/P53/dynamin-related protein 1 (Drp1) axis.

Methods: Thirty cTnTR141W transgenic mice were randomly divided into model, Kangxiyixin formula, and Captopril groups (n=10 each), with 10 C57BL/6J mice serving as normal controls. After 8 weeks of drug intervention, echocardiography was performed to measure left ventricular end-systolic diameter (LVESD), left ventricular end-diastolic diameter (LVEDD), left ventricular fractional shortening (LVFS), and left ventricular ejection fraction (LVEF). Hematoxylin-eosin (HE) and Masson staining assessed myocardial pathology and fibrosis. Transmission electron microscopy examined mitochondrial ultrastructure. Real-time PCR quantified Sirt1, P53, and Drp1 mRNA levels in myocardial tissue, while Western blot analyzed protein expression levels including acetylated P53 (a-P53).

Results: Echocardiography and heart weight measurements revealed that the model group exhibited increased LVEDD and heart weight but decreased LVEF and LVFS compared to the normal group ($P<0.05$). Both Kangxiyixin formula and Captopril groups showed improved LVEF and LVFS and reduced heart weight relative to the model group ($P<0.05$). Histological analysis demonstrated disordered myocyte arrangement, hypertrophy, and significant fibrosis with marked mitochondrial injury in the model group. Treatment groups displayed better myocardial structure, reduced collagen deposition, and diminished fibrosis. Transmission electron microscopy confirmed normal ultrastructure in the normal group, but severe damage and mitochondrial swelling in the model group, with notable improvements in the treatment groups. Real-time PCR showed lower Sirt1 mRNA and higher Drp1 and P53 mRNA levels in the model group versus normal controls ($P<0.05$), while treatment groups exhibited the opposite pattern ($P<0.05$). Western blot revealed decreased Sirt1 protein and increased Drp1 protein and aP53/P53 ratio in the model group ($P<0.05$), with treatment groups showing reversed expression patterns ($P<0.05$).

Conclusion: Kangxiyixin formula improves cardiac function and inhibits ventricular remodeling in cTnTR141W transgenic mice, likely through upregulating Sirt1 expression and downregulating P53 acetylation and Drp1 levels.

Keywords: Cardiomyopathy, dilated; Dilated cardiomyopathy; Ventricular remodeling; Kangxiyixin formula; Sirtuin 1; P53; Dynamin-related protein 1

Introduction

Dilated cardiomyopathy (DCM) is a heterogeneous myocardial disease characterized primarily by left ventricular functional impairment and structural alterations. The disease has an insidious onset with unknown etiology and is often accompanied by malignant arrhythmias, thromboembolism, and can lead to sudden cardiac death. Ventricular remodeling represents the core pathological change in DCM, and abnormal mitochondrial fission-induced mitochondrial dynamics dysfunction is closely associated with this remodeling process. Kangxiyixin formula, developed by Professor Wang Zhentao based on modifications to the classical "Shengxian Decoction," is an empirical prescription for DCM treatment. Our previous research demonstrated that Kangxiyixin formula can improve clinical symptoms, increase ejection fraction, inhibit ventricular remodeling, and enhance cardiac function in DCM patients. This study employs cTnTR141W transgenic mice as a spontaneous DCM model to investigate the formula' s effects through drug intervention, assessing cardiac function, histopathological changes, and ultrastructural alterations in cardiomyocyte mitochondria.

1. Materials and Methods

1.1 Experimental Drugs Kangxiyixin formula granules were provided by Sichuan Xinlv Pharmaceutical Technology Development Co., Ltd., with each gram of granule equivalent to 8 grams of crude herbs. The formula composition includes: Astragalus membranaceus 30 g (Lot No. 20100015), Red Ginseng 12 g (Lot No. 19080058), Salvia miltiorrhiza 15 g (Lot No. 20040120), Ophiopogon japonicus 12 g (Lot No. 20060012), Atractylodes macrocephala 15 g (Lot No. 20090042), Lycopus lucidus 15 g (Lot No. 19110126), Leonurus cardiaca 15 g (Lot No. 20040219), Poria cocos 15 g (Lot No. 20100186), and Cimicifuga racemosa 9 g (Lot No. 20060046), totaling 138 g. Captopril tablets were purchased from Shanghai Sine Pharmaceutical Co., Ltd. (National Drug Approval No. H31021832, Lot No. 187280), 25 mg/tablet, with an intragastric dose of 6.39 $mg \cdot kg^{-1} \cdot d^{-1}$.

1.2 Experimental Animals cTnTR141W transgenic mice were obtained from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (Animal License No. 1103261911000021). C57BL/6J mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Animal License No. 1102861914000028). All mice were housed in the Laboratory Animal Center of Henan Provincial Hospital of Traditional Chinese Medicine (License No. SYXK(Yu)2021-0018) at 4 per cage under controlled conditions: humidity 50$±5±$2°C, 12/12-hour light/dark cycle, with ad libitum access to food and water. All animal procedures were approved by the Ethics Committee of Henan Provincial Hospital of Traditional Chinese Medicine (Ethics Approval No. PZ-HNSZYY-2022-055).

1.3 Reagents Masson trichrome staining kit and hematoxylin-eosin (HE) staining kit (Beijing Solarbio Science & Technology Co., Ltd., Cat. Nos. G1340, G1120); 25% glutaraldehyde (Shanghai Macklin Biochemical Co., Ltd., Cat. No. G849973); Tri-color pre-stained protein marker (Yazyme Biomedical Technology Co., Ltd., Cat. No. WJ103); QuickBlock™ Western primary antibody dilution buffer (Shanghai Beyotime Biotechnology Co., Ltd., Cat. No. P0256); ECL Plus hypersensitive luminescent solution (Beijing Solarbio Science & Technology Co., Ltd., Cat. No. PE0010); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Mouse Monoclonal antibody (Wuhan Sanying Biotechnology Co., Ltd., Cat. No. CL594-66009); Sirt1 Rabbit mAb (Cell Signaling Technology, Cat. No. 3931S); P53 antibody (Wuhan Sanying Biotechnology Co., Ltd., Cat. No. 60283-2-Ig); Acetyl-P53 (Lys382) Antibody (Cell Signaling Technology, Cat. No. #2525); Anti-dynamin-related protein 1 (Drp1) antibody (Abcam, Cat. No. ab184247); HRP-labeled goat anti-rabbit secondary antibody and HRP-labeled goat anti-mouse secondary antibody (Wuhan Sanying Biotechnology Co., Ltd., Cat. No. ab184247); Column-type animal tissue total RNA extraction and purification kit (Sangon Biotech Co., Ltd., Cat. No. B518651); Amplification kit and qPCR reverse transcription kit (Takara Bio, Cat. Nos. RR820B, RR047A).

1.4 Instruments VISUALSONICS small animal ultrasound imaging system (Fujifilm, Model Vevo1100); IVC system (Feng' s Laboratory Animal Equipment Co., Ltd., Model PSU touch type); Thermal cycler (Bio-Rad, Model 185-2148); Biological tissue intelligent dehydrator, cryostat microtome, tissue flotation workstation, paraffin embedding station (Huida Instruments Co., Ltd., Models HD-300D, HD-1800, HD-330, HD-310B); Protein vertical electrophoresis apparatus, electrotransfer apparatus, chemiluminescence gel imaging system (Bio-Rad, Models 164-5070, 170-4070, ChemiDoc XRS+); Ultra-micro nucleic acid/protein analyzer (One Drop, Model OD-2000+); Thermal cycler (Bio-Rad, Model DNA Engine); Real-time PCR system (Thermo Fisher Scientific, Model 7500Fast).

1.5 Experimental Methods 1.5.1 Animal Grouping and Drug Administration: Following genotyping identification using the method described in reference [5], cTnTR141W transgenic mice were randomly divided into model, Kangxiyixin formula, and Captopril groups (n=10 each). Ten C57BL/6J mice served as the normal control group. Previous studies [6] and our preliminary experiments have demonstrated that the cTnTR141W transgenic mouse model is stable and exhibits pathological and echocardiographic features consistent with human DCM. After genotyping confirmation via PCR, drug administration commenced. The conversion from human to mouse dosage was calculated using the body surface area method (mouse dose = human dose/weight × conversion factor). Based on a human dose of 138 g crude herbs for a 70 kg individual and a conversion factor of 9, the calculated intragastric dose for Kangxiyixin formula was 2.22 $g \cdot kg^{-1} \cdot d^{-1}$. Captopril was administered at 6.39 $mg \cdot kg^{-1} \cdot d^{-1}$. All

treatments were given once daily at a fixed time for 8 weeks, while the normal and model groups received equivalent volumes of normal saline.

1.5.2 Echocardiographic Examination: After the final administration and a 12-hour fasting period, mice were anesthetized with isoflurane inhalation. In the supine position with the precordial area depilated, echocardiography was performed to record left ventricular end-diastolic diameter (LVEDD), left ventricular ejection fraction (LVEF), and left ventricular fractional shortening (LVFS).

1.5.3 Myocardial Histopathology: Following cardiac function assessment, hearts were excised and rinsed with ice-cold normal saline to remove residual blood. After blotting dry, left ventricular myocardial tissue was fixed in 4% paraformaldehyde for sectioning. HE and Masson staining were performed to evaluate left ventricular histopathology under light microscopy.

1.5.4 Transmission Electron Microscopy for Mitochondrial Ultrastructure: Approximately 1 mm^3 of left ventricular apical tissue was fixed in 2.5% glutaraldehyde for 4 hours, washed three times with PBS, post-fixed in 1% osmium tetroxide for 2 hours, dehydrated through a graded acetone series (30%, 50%, 70%, 80%, 90%, 100%), sectioned, and stained with saturated uranyl acetate and lead citrate solutions for transmission electron microscopy examination.

1.5.5 Real-Time PCR for Sirt1, P53, and Drp1 mRNA Expression: Total RNA was extracted from 50 mg of myocardial tissue according to kit instructions. RNA purity and concentration were measured using an ultramicro nucleic acid/protein analyzer, followed by reverse transcription to cDNA. Real-time PCR was performed using TB Green™ Premix Ex Taq™ II with the following conditions: pre-denaturation at 95°C for 90 s, 40 cycles of denaturation at 95°C for 10 s, annealing/extension at 60°C for 30 s, and slow heating from 65°C to 95°C for 60 s. Amplification and melting curves were verified post-reaction. mRNA expression levels were calculated using the 2^(-ΔΔCt) method. Primer sequences are listed in Table 1 .

1.5.6 Western Blot for Sirt1, P53, Acetylated P53 (a-P53), and Drp1 Protein Expression: Fifty mg of myocardial tissue was lysed and centrifuged to collect supernatant. Protein concentration was determined using BCA assay. Samples were mixed with 4× loading buffer, heated at 100°C for 5 min, separated by gel electrophoresis, transferred to membranes, blocked at room temperature for 1 hour, incubated with primary antibodies overnight, washed three times with TBST (10 min each), incubated with secondary antibodies at room temperature for 1 hour, and visualized using ECL chemiluminescence. GAPDH served as internal control, and protein expression was quantified using ImageJ software. The a-P53/P53 ratio was calculated.

1.6 Statistical Analysis: Data were analyzed using IBM SPSS 21.0 software. Normally distributed continuous variables are expressed as mean ± standard deviation ($\bar{x}\pm s$). Inter-group comparisons were performed using one-way

ANOVA, with pairwise comparisons conducted using Student-Newman-Keuls test. $P<0.05$ was considered statistically significant.

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2. Results

2.1 PCR Genotyping of Hybrid Mice Eleven mice were genotyped. Lanes 1, 2, 3, 6, 7, 9, and 10 corresponded to cTnTR141W transgenic mice, while lanes 5, 8, and 11 represented wild-type C57BL/6J mice. Lane 4 showed low gene expression and required repeat PCR for confirmation (Figure 1 [Figure 1: see original paper]).

2.2 Echocardiographic Parameters and Heart Weight Echocardiography and heart weight measurements demonstrated significant differences among groups in LVEDD, LVEF, LVFS, and heart weight ($P<0.05$). Pairwise comparisons revealed that the model group exhibited increased LVEDD and heart weight but decreased LVEF and LVFS compared to the normal group ($P<0.05$). Both Kangxiyixin formula and Captopril groups showed elevated LVEF and LVFS and reduced heart weight versus the model group ($P<0.05$). The Captopril group additionally demonstrated decreased LVEDD ($P<0.05$). Results are summarized in Table 1 and Figure 2 [Figure 2: see original paper].

2.3 Cardiac Appearance and Histopathology Gross examination revealed enlarged hearts in the model group compared to normal controls, with reduced heart size observed in both treatment groups (Figure 3 [Figure 3: see original paper]). HE staining showed regularly arranged, densely packed myocardium with centrally located nuclei and uniform cytoplasm in the normal group. The model group exhibited myocardial fiber rupture, dissolution, disarray, interstitial edema, inflammatory cell infiltration, and nuclear pyknosis. Both treatment groups demonstrated relatively regular myocardial architecture with reduced edema and inflammation (Figure 4 [Figure 4: see original paper]). Masson staining revealed uniform myofiber distribution without fibrosis in the normal group, whereas the model group showed marked myocyte disarray, increased collagen deposition, and significant fibrosis. Both Kangxiyixin formula and Captopril groups exhibited substantially reduced interstitial collagen deposition and fibrosis (Figure 5 [Figure 5: see original paper]).

Transmission electron microscopy demonstrated normal left ventricular ultrastructure in the normal group. The model group showed severe ultrastructural damage, coexisting hypertrophied and atrophied cardiomyocytes, marked mitochondrial swelling with rarefied matrix and vacuolar changes, sarcomere disruption, and interstitial fibrosis. Both treatment groups exhibited significantly improved ultrastructure, with restored mitochondrial morphology and relatively orderly myofilament arrangement (Figure 6 [Figure 6: see original paper]).

2.4 Myocardial Sirt1, Drp1, and P53 mRNA Expression Real-time PCR revealed significant inter-group differences in Sirt1, Drp1, and P53 mRNA expression ($P<0.05$). The model group showed decreased Sirt1 and increased Drp1 and P53 mRNA levels compared to normal controls ($P<0.05$). Both Kangxiyixin formula and Captopril groups exhibited elevated Sirt1 and reduced Drp1 and P53 mRNA expression versus the model group ($P<0.05$) (Table 2).

2.5 Myocardial Sirt1, Drp1, aP53, and P53 Protein Expression Western blot analysis demonstrated significant differences in Sirt1, Drp1 protein, and aP53/P53 ratio among groups ($P<0.05$). The model group exhibited decreased Sirt1 protein and increased Drp1 protein and aP53/P53 ratio compared to normal controls ($P<0.05$). Both treatment groups showed opposite expression patterns with increased Sirt1 and decreased Drp1 and aP53/P53 ($P<0.05$) (Table 3 , Figure 7 [Figure 7: see original paper]).

Discussion

DCM is a heterogeneous myocardial disease characterized by unilateral or bilateral ventricular dilation, thinning of ventricular walls, and consequent impaired contractility with reduced ejection fraction, culminating in non-ischemic heart failure. The disease has an insidious onset with unknown etiology, and patients often present at middle-to-advanced stages with extremely poor prognosis, with 5-year mortality reaching up to 50%. Ventricular remodeling constitutes a critical pathological process in DCM development, frequently accompanied by arrhythmias. Overactivation of the sympathetic nervous system and renin-angiotensin-aldosterone system plays a key role in ventricular remodeling, leading to clinical use of ARNI, ACEI/ARB, β-blockers, and aldosterone receptor antagonists to control remodeling and arrhythmias, thereby blocking disease progression and improving survival and quality of life.

The pathological basis of ventricular remodeling lies in cardiomyocyte alterations that are intimately linked to mitochondrial dysfunction. Mitochondria adapt their morphology and function to maintain cellular homeostasis in response to changing intracellular environments. Conversely, mitochondrial dysfunction impairs the ability of cells to respond to environmental changes, causing structural, functional, and metabolic damage that can lead to cell death. Research has shown that mitochondrial dysfunction contributes to the pathogenesis of various cardiovascular diseases. Notably, KEDA et al. demonstrated that Drp1-specific knockout induces excessive mitochondrial fusion, suppresses mitophagy, triggers mitochondrial dysfunction, and causes cardiac dysfunction and heart failure. LIANG et al. showed that inhibiting Drp1-mediated mitochondrial fission in doxorubicin-induced DCM mice could suppress mitochondrial fragmentation, mitophagy, and oxidative stress, thereby improving mitochondrial and cardiac function. WANG et al. found that cardiac-specific knockout of SDHAF4 caused dilated cardiomyopathy with markedly increased Drp1-mediated mito-

chondrial fission, mitochondrial fragmentation, impaired mitophagy, metabolic dysfunction, and heart failure, which could be ameliorated by targeting mitochondrial fission. These studies collectively indicate that suppressing Drp1 expression can improve mitochondrial dynamics and cardiac function.

Sirt1 is an NAD^+-dependent deacetylase that functions as a cytoprotective factor; its deficiency induces mitochondrial abnormalities and DCM. Studies have shown that Sirt1 can regulate mitochondrial function and improve cardiac function by deacetylating P53 and thereby modulating Drp1 expression. Although Traditional Chinese Medicine does not have a disease category specifically named "dilated cardiomyopathy," its clinical manifestations align with descriptions of "heart distention" and "heart water" in ancient texts, corresponding to heart failure symptoms such as fatigue, dyspnea, and respiratory distress. DCM is considered to have both internal and external etiologies, primarily involving congenital deficiency of heart qi, stagnation of qi-blood and fluids, or external pathogenic heat-toxins causing deficiency of heart qi-blood-yin-yang and toxin accumulation. The condition is characterized as root deficiency with branch excess, with heart yang deficiency as the root and blood stasis, water retention, and phlegm obstruction as the branch manifestations. Treatment principles focus on supplementing qi, warming yang, activating blood, and promoting diuresis.

Professor Wang Zhentao, drawing from classical literature and extensive clinical experience, proposes that DCM represents root deficiency with branch excess, with core pathogenesis being ancestral qi deficiency. The degree of ancestral qi depletion and associated pathological products determine disease stage and clinical manifestations. He established the treatment principle of supplementing qi, ascending the sunken, activating blood, and promoting diuresis, creating Kangxiyixin formula for DCM treatment. Previous clinical studies have confirmed that Kangxiyixin formula can improve TCM syndrome scores, clinical efficacy, cardiac function, and inhibit ventricular remodeling, though its mechanisms remained unclear. This animal study demonstrates that cTnTR141W transgenic mice exhibited cardiac chamber enlargement, myocardial damage, myofiber rupture and disarray, interstitial edema, inflammatory infiltration, severe ultrastructural damage, mitochondrial swelling, reduced Sirt1 mRNA and protein expression, elevated Drp1 mRNA and protein levels, increased P53 mRNA expression, and enhanced P53 acetylation. Kangxiyixin formula intervention attenuated these pathological changes, increased Sirt1 expression, decreased Drp1 and P53 expression, and reduced P53 acetylation. These findings suggest that Kangxiyixin formula may inhibit DCM ventricular remodeling via the Sirt1/P53/Drp1 axis.

In summary, Kangxiyixin formula can upregulate Sirt1 mRNA and protein expression, reduce P53 deacetylation, and consequently decrease Drp1 expression, thereby inhibiting mitochondrial fission, improving cardiac function, and suppressing ventricular remodeling.

Author Contributions: KONG Guixuan designed the study and wrote the

manuscript; YU Xiaoyang and CHANG Hongbo collected and organized data; LI Kang performed statistical analysis; QIU Yueqing and WANG Suyun revised the manuscript; WANG Zhentao and WU Hong supervised quality control and take overall responsibility for the article.

Conflict of Interest: The authors declare no conflicts of interest.

References: [References 1-22 are preserved as in the original text]

Note: Figure translations are in progress. See original paper for figures.