Mitophagy Regulation by Kangxian Yixin Granule in a Mouse Model of Dilated Cardiomyopathy

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Abstract

Objective Kangxian Yixin granule (KXYXG) has been found to be effective in the clinical treatment of dilated cardiomyopathy (DCM). We aim to explore the effect of KXYXG and the underlying mechanism in a mouse model of DCM.

Methods Thirty specific pathogen-free (SPF) male $cTnT^{R141W}$ mice with DCM were randomly divided into the model group, KXYXG (20.4 g/kg/d) group and coenzyme Q10 (CoQ10) (1.5 mg/kg/d) group; 10 SPF male C57BL/6J mice were included to form the normal group. The mice in KXYXG group and CoQ10 group were administered by oral gavage for 8 weeks. M-echocardiography was used to evaluate the cardiac function in mice, and hematoxylin and eosin staining and transmission electron microscopy were performed to observe morphological characters. The colocalization and expression levels of mitophagy-related proteins were observed using immunofluorescence and western blot.

Results Compared with the normal group, the model group showed ventricular remodeling, cardiac insufficiency, disordered arrangement of cardiomyocytes, as well as disordered mitochondria and irregular and diffuse swelling. Furthermore, the model group had lower mitophagy protein colocalization and autophagy flux. Furthermore, PINK1 and Parkin expression levels decreased in the mice with DCM ($p < 0.05$). KXYXG could decrease the left ventricular end-diastolic and end-systolic diameters and mitochondrial injury, rescue cardiac dysfunction and remodeling, and protect against myocardial ultrastructure changes in the mice with DCM. KXYXG also increased the colocalization levels of mitophagy-related proteins and PINK1 and Parkin expression levels compared with those in the model group ($p < 0.05$).

Conclusion KXYXG can protect against heart injury by possibly activating the PINK1/Parkin pathway and mitophagy in mice with DCM.

Keywords
- dilated cardiomyopathy
- mitophagy
- Kangxian Yixin Granule
- PINK1/Parkin pathway
- Chinese medicine
- heart failure

Dilated cardiomyopathy (DCM) is a heterogeneous myocardial disease with ventricular enlargement and reduced myocardial systolic function, which is one of the main causes of heart failure.[1] The etiology of DCM is diverse and includes genetic and nongenetic causes. At present, the known genetic causes are mainly related to the mutations of cytoskeletal and sarcomeric proteins.[2] DCM has a high fatality rate; however, an effective treatment plan for this disease is lacking, which poses a heavy burden to families and society.[3]

Mitophagy can selectively eliminate impaired or depolarized mitochondria, which is an important mechanism in the maintenance of cardiac homeostasis.[4] In response to various

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environmental stresses, dysfunctional mitophagy results in the accumulation of damaged and dysfunctional mitochondria and excessive mitochondrial oxidative stress, leading to energy metabolism disorder and a decrease in cardiac contractile function.[5] A deficiency of mitophagy-related proteins in the heart can directly affect cardiac function or lead to fatal cardiomyopathy in mouse models.[6–8] Several studies have shown that mitophagy plays a protective role in DCM heart failure.[9] Therefore, restoring the level of mitophagy may be a potential treatment for DCM.

Traditional Chinese medicine has long been used in the treatment of DCM. Invigorating qi and activating blood circulation is considered a core method in the treatment of DCM.[10] Kangxian Yixin granule (KXYXG) is an effective clinical treatment for DCM. In our preliminary clinical study, we confirmed that KXYXG could effectively relieve the clinical symptoms and improve the left ventricular remodeling and improve the cardiac function of DCM patients.[11] We were also able to confirm that the therapeutic effect of KXYXG in cases of DCM is related to reducing myocardial fibrosis and myocardial cell apoptosis and restoring myocardial energy metabolism in vivo.[12] Hence, this study aimed at exploring the underlying mechanism of the therapeutic effect of KXYXG on DCM.

Methods

Animals

The α-myosin heavy chain $cTnT^{R141W}$ transgenic mice were purchased from the Institute of Laboratory Animals Science, Chinese Academy of Medical Sciences and Peking Union Medical College (certificate no.: 1103261911000021). The mice developed the characteristics of human DCM within 4 months[13]; C57BL/6J mice were purchased from Beijing Charles River Company (certificate no.: 1102861914000028). All mice were raised at the Experimental Animal Center of Henan Province Hospital of Chinese Medicine at a temperature of (22+2)°C and humidity of (50± 5)%. This study was approved by the Ethics Committee of Henan Province Hospital of Chinese Medicine (approval no.: PZ-HNSZYY-2019-004). M-echocardiography was used to identify the phenotype of the $CTNT^{R141W}$ mice. The phenotype of cardiac dilation was confirmed if the left ventricular end-diastolic diameter (LVEDD) was >2.64 mm at the age of 2 months.[14]

Drugs

KXYXG contains the following Chinese herbs: Renshen (Ginseng Radix et Rhizoma) 12 g, Huangqi (Astragali Radix) 30 g, Fuling (Poria) 15 g, Danshen (Salviae Miltiorrhizae Radix et Rhizoma) 15 g, Cangzhu (Atractylodis Rhizoma) 15 g, Gouqizi (Lycii Fructus) 15 g, Yimucao (Leonuri Herba) 15 g, Maidong (Ophiopogonis Radix) 12 g, Shengma (Cimicifugae Rhizoma) 9 g. Concentrated granules are prepared from these nine Chinese medicinal herbs. The batch numbers of the above Chinese herbal formula granules were as follows: 19080058, 20100015, 20040120, 19110126, 20040219, 20100186, 20090042, 20060046, and 20060012. All herbal materials, dispensing granules, and quality control data of KXYXG were supplied by Sichuan Neo-Green Pharmaceutical Technology Development Co., Ltd (Sichuan, China). A dose of KXYXG was dissolved in sterile distilled water and heated to prepare the medicine solution. Coenzyme Q10 (CoQ10) tablets (10 mg/tablet; Eisai Pharmaceutical Co., Ltd, China, batch no.: 1912001) were crushed, ground, and dissolved in 50 mL of sterile distilled water to prepare the medicine solution. The solution was divided into 1.5 mL sterile Eppendorf tubes and stored at −20°C. The dosages of KXYXG (20.4 g/kg/d) and CoQ10 (1.5 mg/kg/d) were determined according to the equivalent human dosages.

Groups

Thirty specific pathogen-free (SPF) male $cTnT^{R141W}$ mice with DCM were identified and randomly divided into the model group, KXYXG group, and CoQ10 group; 10 SPF male C57BL/6J mice formed the normal group. The normal and model groups were administered normal saline, the KXYXG group was administered the KXYXG solution, and the CoQ10 group received the CoQ10 solution, once a day, for 8 weeks.

Echocardiography

M-mode echocardiography was performed with an animal echocardiography analysis system (VEVO1100; VisualSonics Inc., Canada). In brief, the mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium solution after weight measurement and fixed in the supine position; the animals' precardiac region was shaved. M-mode echocardiography was performed with a 30 MHz transducer. LVEDD and left ventricular end-systolic diameter (LVESD) were measured, and the left ventricular ejection fraction (EF) and left ventricular axis shortening rate [fractional shortening (FS)] were calculated by echocardiography.

Hematoxylin and Eosin Staining

Myocardial samples from each group were fixed with 4% paraformaldehyde solution and embedded in paraffin after decalcification and dehydration. The sections (5 μm) were stained using hematoxylin and eosin, and morphological characters were observed using an image autoanalysis system.

Transmission Electron Microscopy

The tissues obtained were cleaned in normal saline at 4°C, and pieces of myocardial tissues at the apex of the left ventricle were cut and divided into 1 mm^3 square samples. The segmented myocardial samples were quickly placed into a centrifugation tube containing precooled 2.5% glutaraldehyde solution for 4 hours. Thereafter, they were rinsed with 0.1 mol/L phosphate-buffered saline (PBS) 3 times for 15 minutes each. Then, 1% osmium was added, stewing for 2 hours. Gradient dehydration was performed using acetone of different concentrations. The samples were then embedded in epoxy resin overnight, and gradient curing was performed in the oven at 37°C (12 h), 45°C (12 h), and 60°C (36 h), after which they were sliced using an ultrathin slicer. Finally, the samples were dyed with saturated uranium

acetate solution and lead citrate solution, after which transmission electron microscopy was performed.

Measurement of Immunofluorescence Colocalization

Section preparation: Paraffin-embedded sections of the myocardium were prepared as described previously. The sections were dewaxed, and antigen repair was performed. Then, after allowing for natural cooling, the slices were placed in PBS solution and washed 3 times. They were sealed with a 3% (BSA:PTST) solution for 30 minutes, followed by incubation overnight with a primary antibody at 4°C. The slides were washed and covered with fluorescent secondary antibody (1:400) drops under dark conditions, and incubation was performed at 25°C under dark conditions for 1 hour. After that, an antifade mounting medium with DAPI was dropped onto the slides, and the slides were incubated at 25°C for 30 minute in the dark. The sections were observed under a fluorescence microscope and images were obtained. Fluorescence images were retained using an imaging system and processed using image-Pro Plus Image analysis software. The position coincidence of COX4, Parkin, and LC3 was determined, and the Pearson correlation coefficient was used for quantitative analysis.

Western Blot

Protein was extracted from heart tissue, which was homogenized in RIPA lysis buffer, and quantitated using the BCA kit (Biyuntian Biological, China). 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 hour. The membrane was incubated with the primary antibody at 4°C overnight. Primary antibodies against p62, PINK1, Parkin (1:1000 dilution Proteintech, United States), GAPDH (1:2000 dilution Proteintech, United States), and LC3 (1:1000 dilution Cell Signaling Technology, United States) were used. After washing, the membrane was treated with a specific horseradish peroxidase–conjugated secondary antibody (1:2000 dilution Wuhan Sewell Biotechnology, China) for 1.5 hour and subsequently washed. Relative luminescence intensity was analyzed using a gel imaging system (Bio-Rad Laboratories, United States).

Statistical Analysis

All data are expressed as means ± standard error of the mean ($\bar{x} \pm$ standard error). SPSS 22.0 statistical software was used for data analysis. If the measurement data showed normal distribution and homogeneity of variance, one-way analysis of variance was used with Tukey's post hoc test. If the data distribution was not normal, a nonparametric test was used. $p < 0.05$ was considered statistically significant.

Results

Cardiac Function of Mice Treated with Kangxian Yixin Granule

The cardiac structure and function in DCM mice were measured and calculated using Vevo1100. As shown in ►**Fig. 1**, the model group showed obvious left ventricular enlargement, left ventricular wall thinning, decreased ventricular wall movement, decreased systolic function, and a

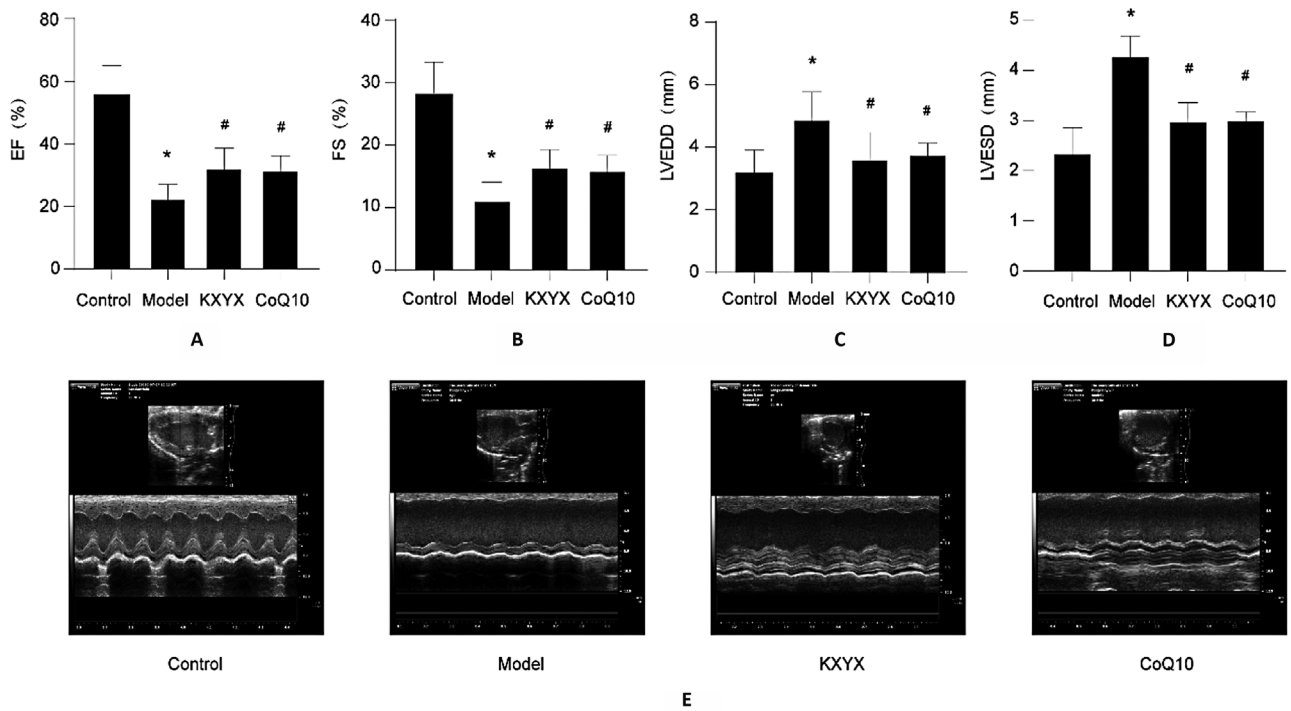

Fig. 1 Kangxian Yixin granule (KXYXG) restored cardiac function in DCM mice. Ejection fraction (EF) values (**A**). Fractional shortening (FS) values (**B**). Left ventricular end-diastolic diameter (LVEDD) values (**C**). Left ventricular end systolic diameter (LVESD) values (**D**). Representative M-mode echocardiographic images (**E**). Values are presented as mean ± standard deviation ($n = 10$ per group); comparison with the control group. $^{*}p < 0.05$; comparison with the model group, $^{\#}p < 0.05$.

spherical heart. In comparison with the normal group, the model group showed significantly higher LVESD and LVEDD and significantly lower FS and LVEF ($p < 0.05$). In comparison with the corresponding values in the model group, LVESD and LVEDD significantly decreased after treatment with KXYXG or CoQ10 ($p < 0.05$), whereas LVEF increased significantly ($p < 0.05$) and FS was higher ($p < 0.05$).

Myocardial Histopathological Findings of Mice Treated with Kangxian Yixin Granule

As shown in ►**Fig. 2**, the ventriculus cordis of mice in the model group was significantly enlarged and spherical-shaped and showed a significantly thin ventricle wall. In comparison with the model group, the KXYXG group showed decreased volume of the left ventricle and increased relative thickness of the ventricle wall. The reversal effect of KXYXG on ventricular remodeling was more obvious than that of CoQ10.

The cardiomyocytes in the normal group were arranged neatly, with clear stripes and uniform cytoplasm staining. The nucleus was oval, located in the center of the cell, and the cells showed no obvious interstitial fiber hyperplasia and edema. In the model group, the arrangement of cardiomyocytes was relatively disordered and myocardial tissue was seriously damaged, with irregular hypertrophy of cardiomyocytes and irregular arrangement of nuclei. In addition, some nuclei were pyrotic and fragmented, and interstitial edema and interstitial fibrous hyperplasia were observed. In contrast, the KXYXG group showed a relatively regular arrangement of cardiomyocytes and a relatively clear texture. Some cardiomyocytes showed irregular hypertrophy, a few cells showed nuclear pyrosis and fragmentation, and interstitial fiber hyperplasia and edema were not obvious. In the CoQ10 group, cardiomyocytes were arranged in a relatively regular and clear texture, and some cardiomyocytes showed irregular hypertrophy.

Myocardial Ultrastructure of Mice Treated with Kangxian Yixin Granule

In comparison with the normal group, the model group showed a significantly altered ultrastructure of myocardial fibers, with dissolved and broken myocardial myofilaments, obviously swollen cardiomyocytes, blurred M-line and myofilaments, and destroyed myofibrils. The mitochondria were disordered and showed irregular and diffuse swelling. However, the KXYXG and CoQ10 groups showed significantly reduced damage to the myocardial ultrastructure. Mitochondria in the KXYXG group were mostly round, arranged in an

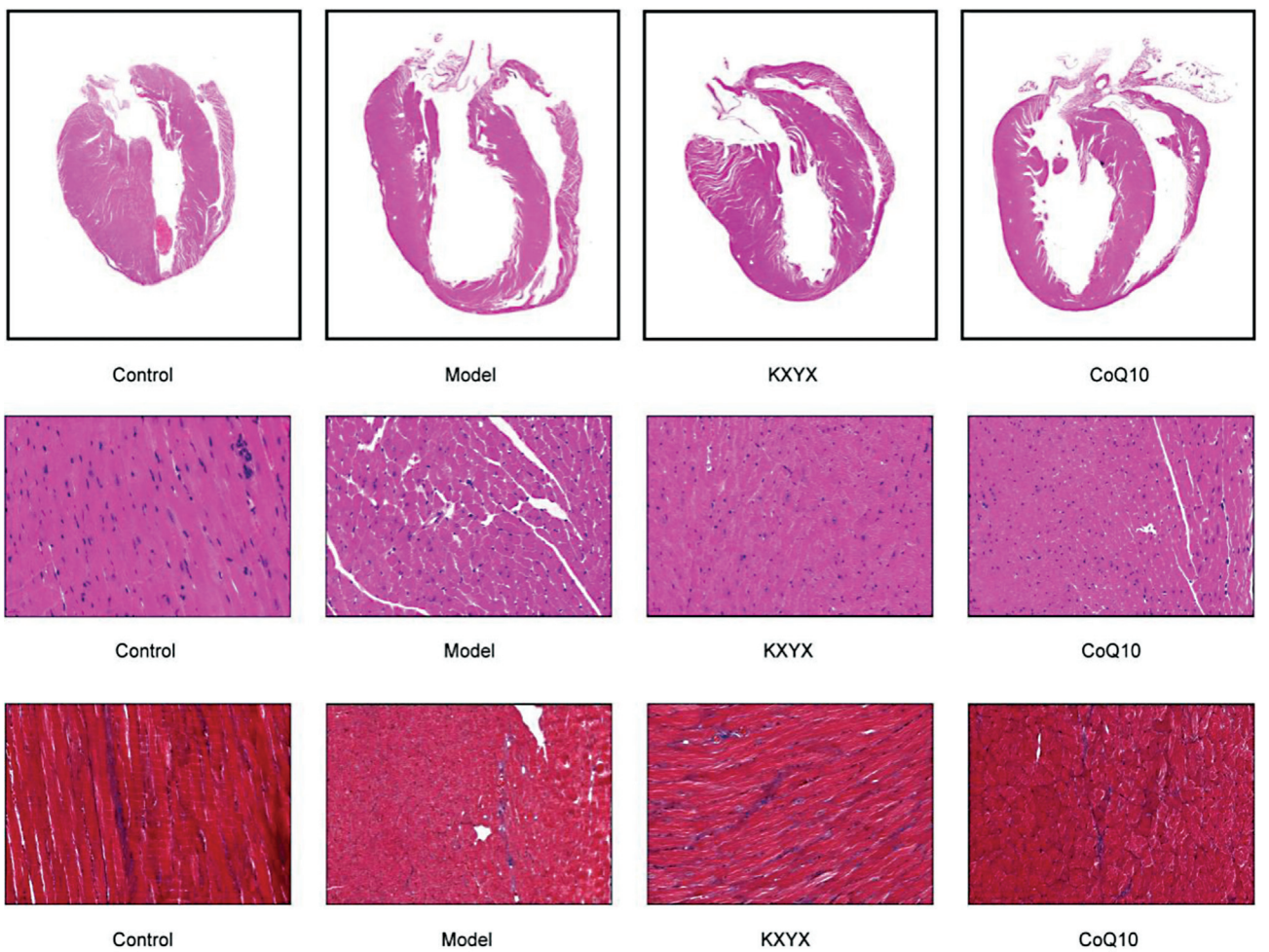

Fig. 2 Kangxian Yixin granule (KXYXG) attenuated ventricular remodeling in dilated cardiomyopathy mice. Representative images of hematoxylin and eosin (×1) and Masson staining (×40).

orderly manner, and significantly greater in number. In the CoQ10 group, some myocardial myofilaments were dissolved and broken and myocardial cells were swollen. However, the arrangement of mitochondria was orderly, and most of them were oval-shaped (►**Fig. 3**).

Immunofluorescence Colocalization of Mice Treated with Kangxian Yixin Granule

Mitochondrial inner membrane COX4 was used to mark the mitochondrial position, and Parkin and LC3 were used to mark the autophagosome position. DAPI was used to label the nuclear location. LC3 and COX4 colocalization as well as Parkin and COX4 colocalization were observed in the same field. Fluorescence images were captured with a camera. As shown in ►**Fig. 4A** and **C**, in comparison with the normal group, the model group showed no significant difference in the colocalization of LC3 and COX4 ($p > 0.05$). However, in comparison with the model group, both KXYXG group and CoQ10 group showed a greater degree of colocalization of LC3 and COX4 ($p < 0.05$). As shown in ►**Fig. 4B** and **D**, in comparison with the normal group, the model group showed reduced degree of colocalization of Parkin and COX4 ($p < 0.05$). However, in comparison with the model group, both KXYXG group and CoQ10 group showed greater degree of colocalization of Parkin and COX4 ($p < 0.05$).

Mitophagy Protein Expression of Mice Treated with Kangxian Yixin Granule

The LC3 II/I ratio is the primary measure for evaluation of autophagy. P62 is a ubiquitin-binding protein that can target autophagosomes to respond to the elimination of ubiquitinated proteins. As shown in ►**Fig. 5A**, the LC3 II/I ratio in the myocardial tissue of the model group was lower than that in the normal group ($p > 0.05$), with no significant difference. In comparison with the model group, the KXYXG group showed significantly greater LC3 II/I ratio in the myocardial tissue ($p < 0.05$), whereas the CoQ10 group only showed a slightly increased LC3 II/I ratio ($p > 0.05$). As shown in ►**Fig. 5B**, in comparison with the normal group, the model group showed significantly increased expression of P62 protein ($p < 0.05$). However, in comparison with the model group, the KXYXG group and CoQ10 group showed decreased expressions of the P62 protein ($p < 0.05$).

The PINK1/Parkin pathway is a classic pathway of mitophagy. As shown in ►**Fig. 5C** and **D**, in comparison with the normal group, the model group showed reduced expression of PINK1 protein in the myocardial tissue ($p < 0.05$). In comparison with the model group, both KXYXG group and CoQ10 group showed increased PINK1 protein expression ($p < 0.05$). In comparison with the normal group, the model group showed reduced expression of the Parkin protein ($p < 0.05$). However, in comparison with the model group, the KXYXG group showed increased expression of the Parkin protein ($p < 0.05$), whereas the CoQ10 group showed slightly increased expression of the same protein ($p > 0.05$).

Discussion

DCM, as a myocardial disease defined by pathological features, is mainly diagnosed based on ventricular dilatation and decreased systolic function of the left ventricle and exclusion of etiologies of ischemic heart disease, hypertension, and valvular disease.[15] DCM is currently the most common cause of heart failure worldwide and the most common indication for heart transplant surgery. Globally, the prevalence of DCM is approximately 40/100,000, and the average annual incidence is approximately 7/100,000.[16] This condition can seriously endanger the health of patients. Recent studies have suggested that traditional Chinese medicine is effective in the prevention and treatment of DCM.[17] Some records related to diseases with similar clinical symptoms as DCM have provided new ideas for the prevention and treatment of DCM. Our team has long been committed to clinical and basic research on the prevention and treatment of DCM. We believe that qi deficiency and blood stasis are at the core of the disease, and KXYXG can be used to treat DCM by invigorating qi and promoting blood circulation. In clinical studies, KXYXG was found to delay the progression of DCM and improve the cardiac function of patients.[18] Our previous study found that mice treated with KXYXG for 8 weeks improved significantly compared with 4 weeks.

With advancements in molecular biology research, the diagnosis rate of familial dilated cardiomyopathy (FDCM) has also increased.[19] About 30% to 48% of patients with DCM carry DCM-related gene mutations. Moreover, the pathogenic genes underlying DCM are complex and diverse, with more than 40 genes reported to be related to DCM.[20] The cardiac troponin T (cTnT) mutation is a common genetic marker in FDCM patients. The $cTnT^{R141W}$ DCM mouse model we adopted was mainly characterized by severe DCM.[21] In

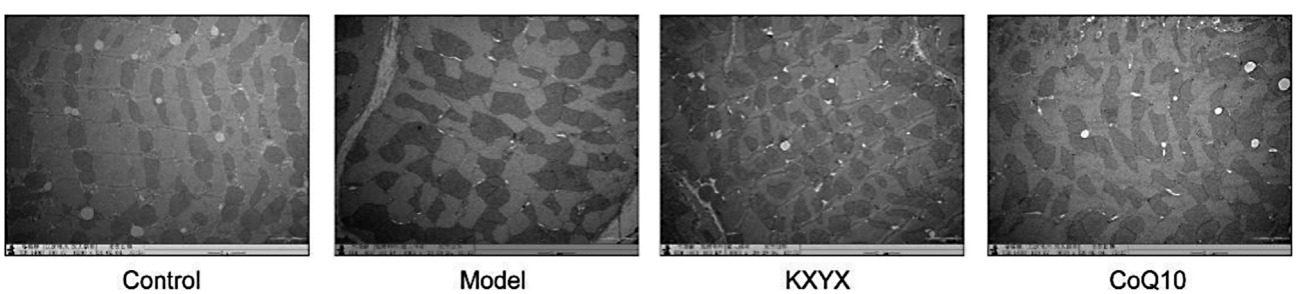

Fig. 3 Kangxian Yixin granule (KXYXG) reduced mitochondrial injury in the hearts of dilated cardiomyopathy mice. Representative images of electronic speculum (×8000).

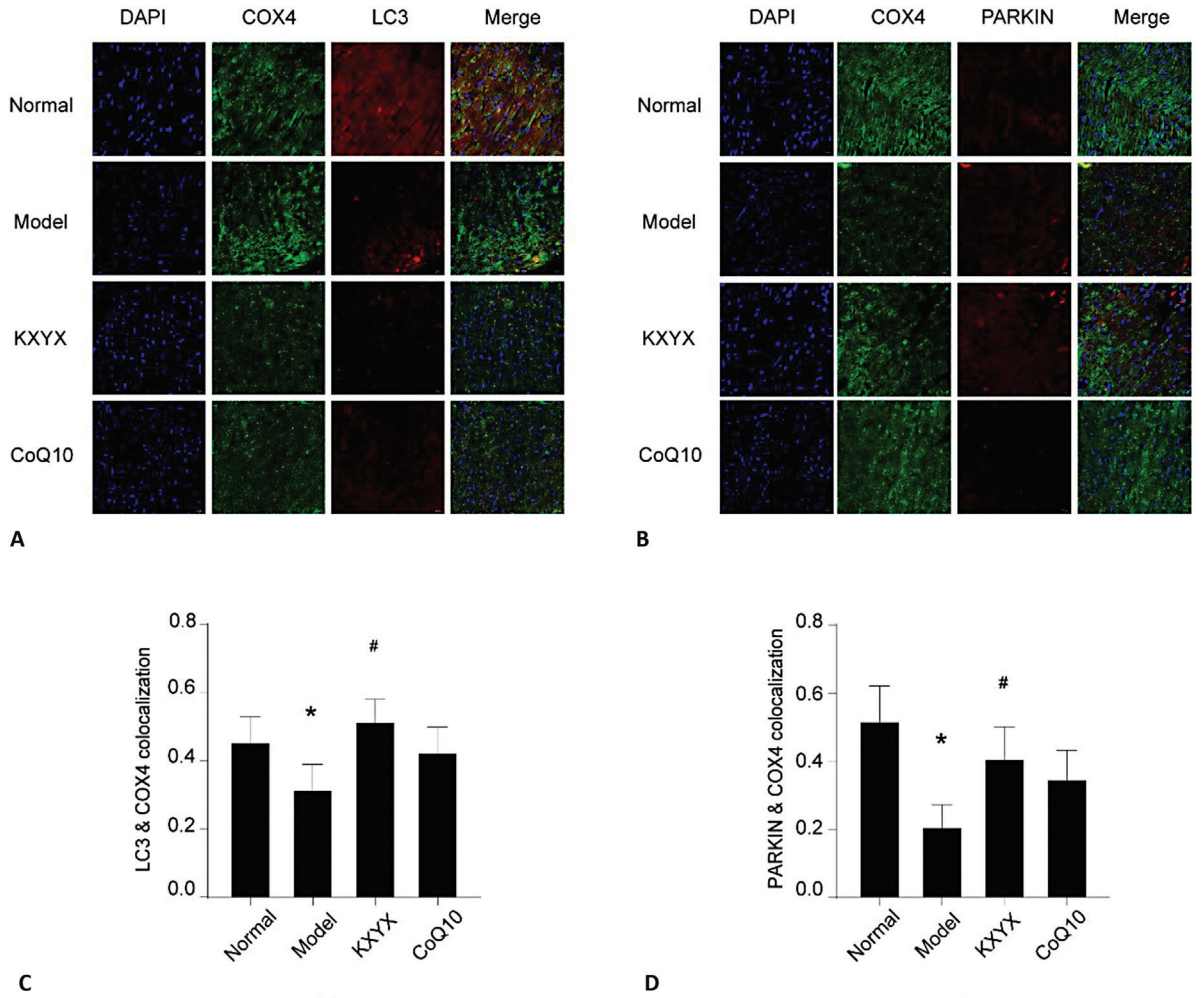

Fig. 4 Kangxian Yixin granule (KXYXG) promoted mitophagy in the hearts of dilated cardiomyopathy mice. Representative images showing immunofluorescence colocalization of COX4 and LC3 (**A**). Representative images showing immunofluorescence colocalization of COX4 and Parkin (**B**).The ratio of colocalization of COX4 and LC3 in each group (**C**). The ratio of colocalization of COX4 and Parkin in each group (**D**) ($n = 6$ per group). Comparison with the control group, $^{*}p < 0.05$; comparison with the model group, $^{\#}p < 0.05$.

comparison with wild mice, 4-month-old $cTnT^{R141W}$ mice showed obvious pathological manifestations such as whole-heart enlargement, ventricular wall thinning, reduced cardiac systolic function, and cardiomyocyte hypertrophy, which were similar to the pathological phenotypes of multiple FDCM patients. Ginsenoside-RB1 and tetramethylpyrazine phosphate have been previously confirmed to improve ventricular remodeling in the $cTnT^{R141W}$ DCM mouse model, and their mechanism may be closely related to improving myocardial energy metabolism and inhibiting myocardial fibrosis.[14] In this study, we found that KXYXG could effectively improve cardiac EF in DCM, postpone ventricular remodeling, and alleviate disorders of myocardial cell arrangement, interstitial fibrosis, and abnormal myocardial ultrastructure.

Mitochondria are important two-membranous organelles in eukaryotic cells. They play important roles in cell homeostasis, including the generation of energy through oxidative phosphorylation, maintenance of calcium homeostasis, and regulation of signals leading to a programmed cell death. CoQ10 is a commonly used drug for cardiovascular diseases in clinical conditions[22] and is responsible for transferring electrons from complex I and complex II to complex III in the mitochondrial respiratory chain, thereby promoting cardiac ATP production. The results of the Q-SYMBIO trial showed that CoQ10 could significantly improve the symptoms of heart failure in patients and reduce the occurrence of adverse cardiovascular events.[23] Therefore, CoQ10 was selected as the control drug in this study. We found that KXYXG caused a more obvious improvement in ventricular remodeling than CoQ10. Both KXYXG and CoQ10 had protective effects on DCM myocardial mitochondrial injury. However, there were significant differences in the mitochondrial morphology between the two groups after treatment.

Mitophagy is a process in which damaged mitochondria are specifically isolated by autophagosomes and bound with lysosomes to remove them, and it serves as an important mechanism of mitochondrial quality control.[24] In general, mitophagy preserves mitochondrial function by removing dysfunctional mitochondria of the heart.[25] However, both inadequate and aggravated mitophagy can lead to cardiomyopathy. For example, in diabetic cardiomyopathy, a reduction in mitophagy may contribute to a beneficial adaptive response

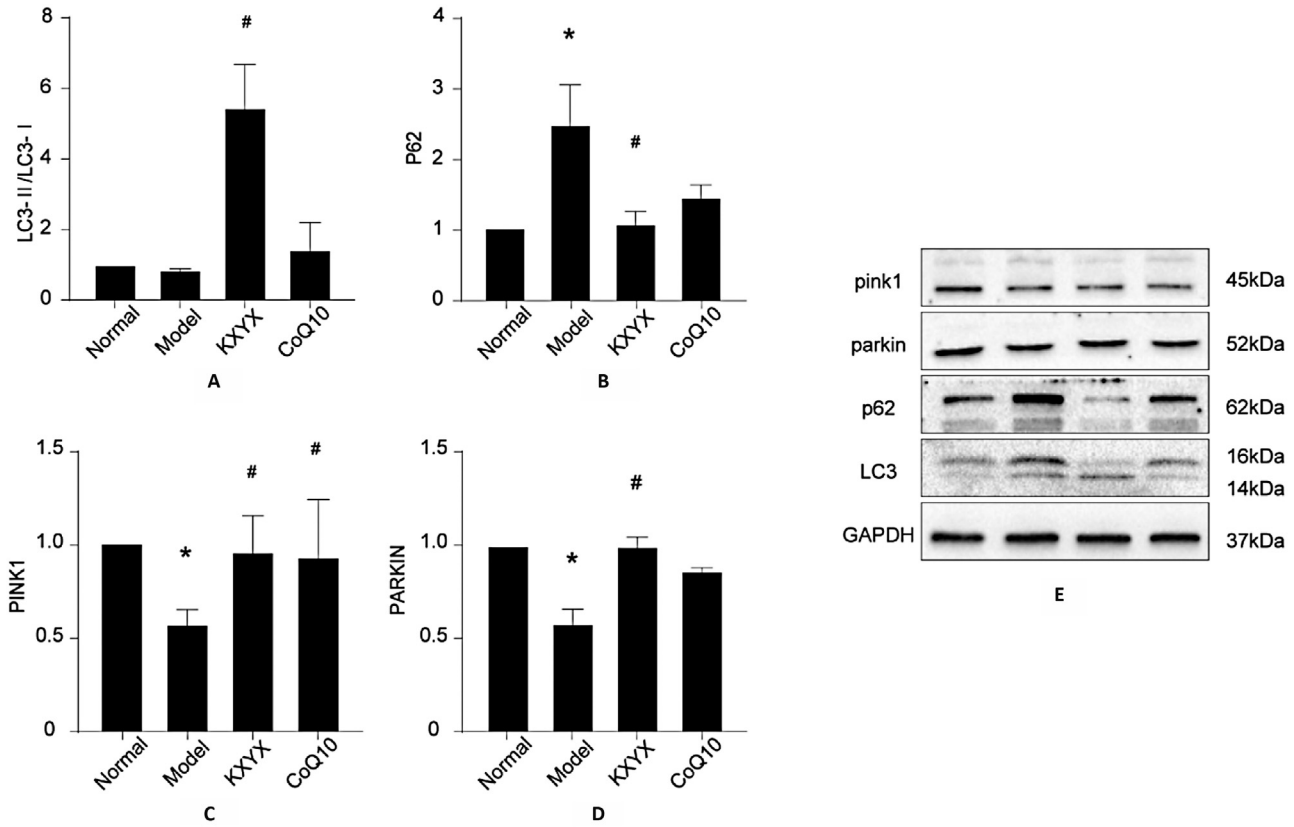

Fig. 5 Kangxian Yixin granule (KXYXG) promoted dilated cardiomyopathy heart mitophagy via PINK1/Parkin pathway. Western blotting was used for the analysis (**A**–**D**). Expression of LC3II/LC3I, P62, PINK1, and Parkin protein (**E**). Comparison with the control group, $^{*}p < 0.05$; comparison with the model group, $^{\#}p < 0.05$.

to diabetic heart injury.[26] Mitophagy can be divided into receptor-dependent and nonreceptor-dependent categories. The PINK/Parkin pathway is the most widely studied regulatory pathway for nonreceptor-dependent mitophagy. Qiliqiangxin improves the symptoms of heart failure by inhibiting the PINK1/Parkin pathway.[27] The protein expression of PINK1 has also been confirmed to be significantly reduced in heart failure.[28] $ZnCl_2$ can clear damaged mitochondria by inducing autophagy and improve myocardial ischemia–reperfusion injury.[29] In this study, we found that KXYXG could improve the fusion of mitochondria and autophagosomes in DCM mouse myocardial tissue through immunofluorescence colocation. There is no significant difference in LC3 II/I ratio between the control group and the model group. As a chronic progressive disease, the changes in the level of autophagy may be moderate or insignificant in the pathological development of DCM, but after KXYXG treatment, the level of autophagy is significantly improved. Western blot analysis confirmed that KXYXG could significantly increase the LC3II/LC3I ratio and decrease P62 expression in the DCM heart, which was significantly different from the findings in the CoQ10 group. Simultaneously, the expression level of the PINK1/Parkin protein significantly increased, indicating that KXYXG could significantly improve the expression of mitophagy in the DCM heart, thus promoting the clearance of damaged mitochondria and preventing myocardial injury in DCM.

Conclusion

This study demonstrated that KXYXG has a significant therapeutic effect on DCM and can inhibit ventricular remodeling and myocardial fibrosis development in DCM mice. Its therapeutic effect may be to enhance the mitophagy level of myocardial mitochondria by regulating PINK1/Parkin pathway. This study preliminarily explained the mechanism of antiventricular remodeling of KXYXG and provided a direction for further research.

CRediT Authorship Contribution Statement

S.L.: conceptualization, data curation, formal analysis, writing—original draft. X.H.: data curation, resources. H.W.: writing—review and editing. Z.W.: conceptualization, data curation, software, project administration.

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Conflict of Interest

The authors declare no conflict of interest.

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