# Tongmai Yangxin Pills Improves Cardiac Function by Enhancing the Expression of SIRT3 and PGC-1α in a Rat Model of Isoproterenol-Induced Cardiomyopathy

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# Abstract

**Background:** Mitochondrial biosynthesis regulated by the PGC-1α-NRF1-TFAM pathway is closely related to mitochondrial function and mitochondrial reactive oxygen generation, and is considered to be a new possible therapeutic target for the treatment of heart failure.

**Methods:** Tongmai Yangxin Pills (TMYXP) is a Chinese patent medicine for the treatment of ischemic heart disease. It has the ability to scavenge oxygen free radicals and improve the antioxidant capacity of the heart muscle, thereby protecting cardiomyocytes, however, its mechanism is not well established. In this study, to investigate the mechanisms of TMYXP in cardiac protection, a rat model of cardiomyopathy was established by continuous isoproterenol (ISO) stimulation. Changes in the rat body weight, heart weight index (HWI), echocardiography, histological staining and biochemical indicators were examined.

**Results:** Our results suggested that TMYXP reduced myocardial remodeling, inhibited deterioration of cardiac function, and delayed HF onset in rats with ISO-induced cardiomyopathy. Meanwhile, TMYXP markedly reduced ROS production, increased the levels of antioxidant enzymes, improved function of the mitochondrial respiratory chain, and promoted ATP production in myocardial tissues. In addition, TMYXP inhibited cytochrome C (Cyt C) release in mitochondria and caspase-3 activation in cytosol, thereby reducing the apoptosis of myocardial cells. Finally, TMYXP remarkably up-regulated the mRNA and protein expression levels of SIRT3, PGC-1α, NRF1 and TFAM in myocardial cells.

**Conclusions:** Taken together, our results suggest that TMYXP regulates mitochondrial biosynthesis mediated enhancing the expression of SIRT3 and PGC-1α, thereby improving the cardiac function in rats with ISO-induced cardiomyopathy.


# Background

Heart failure (HF) is a continuously developing disease, and delaying the progress of HF is an issue that cannot be ignored in modern medical research (1). When the myocardial diastolic function is impaired or the heart is overloaded, a variety of complex interacting compensation mechanisms can be activated, leading to cardiac remodeling. Studies have shown that cardiac remodeling not only causes abnormal myocardial contraction and diastolic function, uncoordinated activity of various parts of the myocardium, but also leads to irreversible changes in myocardial energy metabolism and structure (2).

Previous studies (3,4) has found that oxidative stress (OS) injury plays an important role in the process of cardiac remodeling and heart failure. When heart failure occurs, cardiomyocytes show obvious mitochondrial dysfunction, and decreased ATP energy supply leads to calcium pump dysfunction, causing dysfunction of the cytochrome oxidase system and damage to the electron transfer chain, resulting in increased production of reactive oxygen species (ROS) (5)[5. The rapid accumulation of ROS causes oxidative damage to membrane lipids, proteins, nucleic acids and chromosomes, which leads to oxidative stress reactions, eventually leading to damage to cells and tissues and causing disease (6).

Sirtuins is a type of nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylase. Its deacetylation is non-specific. In addition to histone substrates, it can also deacetylate non-histone substrates (7). SIRT3 (Sirtuin3) is one of the most active members of this family, mainly existing in mitochondria. SIRT3 has the strongest deacetylase activity of all mitochondrial Sirtuins, and through its deacetylation activity, participates in most activities in the mitochondria (8). Studies have shown that SIRT3 can improve mitochondrial function by activating peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). Mitochondrial biosynthesis is essential for maintaining the structure and function of mitochondria, and it is regulated by the PGC-1α-NRF1 (nuclear respiratory factor 1)-TFAM (mitochondrial transcription factor A) signaling pathway (9). Decreased PGC-1α expression can be observed in various HF models with oxidative stress (OS) and mitochondrial dysfunction (10,11).

Tongmai Yangxin Pill (TMYXP) is a traditional Chinese medicine composed of 11 Chinese herbal medicines, including Radix Scutellariae, Glycyrrhiza uralensis, Radix Glycyrrhizae, Schisandra chinensis, Radix Polygonum multiflorum, etc. It has the effect of nourishing the heart and nourishing the blood, tongmai analgesia, and is used for the treatment of chest pain, heartache, angina pectoris, arrhythmia, etc (12). Studies (13) have shown that the myocardial protective effect of TMYXP may be related to antioxidant effects, but its mechanism has not yet been elucidated. Many active ingredients of TMYXP such as glycyrrhizic acid can inhibit ROS production through SIRT1/PGC-1α pathwayα (14). SIRT3 is a deacetylase homologous to SIRT1 in myocardial tissue. Therefore, we speculate that TMYXP can promote mitochondrial biosynthesis and inhibit oxidative stress by up-regulating SIRT3 and PGC-1α, thereby improving cardiac remodeling and delaying HF processes.

# Materials And Methods

### Animal model and treatment protocols

Our animal experimental protocol was approved by the Animal Care and Use Committee of Tianjin Union Medical Center. A total of 70 male adult Sprague-Dawley (SD) rats (weight, 200-250 g) were used in this study, which were provided by the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animals were adapted to the new environment for 1 week prior to experiments, and they had free access to foods and water under laboratory conditions. All animals were randomized into two groups, namely, the ISO-induced cardiomyopathy group (n=60) and the control group (n=10). Typically, the ISO-induced cardiomyopathy group was given subcutaneous injection of isoproterenol (ISO) (Purty: 98%, Beijing Solarbio Science & Technology Co., Ltd, China) dissolved in normal saline (NS) at 10 mg/kg/d for once daily for the initial 2 consecutive weeks. The control group was given injection of NS at identical dose for the initial two weeks consecutively. Afterwards, 50 surviving animals from ISO-induced cardiomyopathy group were further classified into 5 groups, namely, low-dose TMYXP was given TMYXP (0.5 g/kg) administration for 6 weeks,middle-dose TMYXP was given TMYXP (1 g/kg) administration for 6 weeks, high-dose TMYXP was given TMYXP (2 g/kg) administration for 6 weeks and The BIS group was given bisoprolol (BIS, 0.5 mg/kg) for 6 weeks as a positive control group, while those in ISO and control groups were given NS gavage at identical volume once a day for six consecutive weeks.

At the end of week 8, animals were euthanized, and blood samples were collected into tubes. At the same time, fresh cardiac tissues were rinsed with cold saline, of them, a fraction was utilized for mitochondrial protein extraction, while another part was used for monoplast suspension preparation to determine ROS and MMP, and the rest tissues were preserved at -80 °C for subsequent use. Moreover, the intact heart had been fixed using 4% paraformaldehyde, followed by paraffin embedding prior to pathological and TUNEL staining.

### Echocardiography

At weeks 2 and 8, all animals were injected with 3% sodium pentobarbital solution at 10.0 mg/kg for anesthesia before echocardiographic measurements. Typically, data on the left ventricular end diastolic diameter (LVESD), left ventricular end diastolic diameter (LVEDD), left ventricular fractional shortening (LVFS%), and left ventricular ejection fraction (LVEF%), were determined based on short-axis M-mode echocardiography using the Vevo2100 imaging system (VisualSonics, Toronto, ON, Canada).

### HWI assessment

The excised cardiac tissues were washed with ice-cold PBS; later, the adipose tissue, aorta and atria were separated, and the heart weight (HW, mg) was measured. The heart weight index (HWI, mg/g) was defined as the ratio of HW-to-body weight.

### Histopathological analysis

Ventricular tissues were fixed with 4% paraformaldehyde, followed by paraffin embedding, sectioning (5 μm in thickness), and staining using Masson's trichrome as well as hematoxylin-eosin (H&E) solution. Finally, the collagen contents and morphological changes of cardiac tissues were assessed using the light microscope (DS-Ri2, Nikon, Japan).

### Determination of serum BNP

After standing for 30 min, blood samples were centrifuged for 15 min at 3000 g to separate the serum. Thereafter, the serum levels of B-type natriuretic peptide (BNP) were determined using the ELISA kits (Elabscience Biotechnology Co., Ltd, Wuhan, China).

### Assessment of oxidase/antioxidase activities

Cardiac tissues (10% w/v) were homogenized using cold saline, followed by 10 min of centrifugation at 3000 g to collect the supernatant. Then, the contents of malondialdehyde (MDA), glutathione peroxidase (GSH-Px) and manganese superoxide dismutase (Mn-SOD) were measured in accordance with reagent protocols (Nanjing Jiancheng Bioengineering Institute, China)

### Determination of the mitochondrial complexes I, II, III and IV activities

Myocardial mitochondria were extracted, and the mitochondrial respiratory chain complexes I, II, III and IV activities were measured using the Mitochondrial respiratory chain complexes I, II, III and IV Activity Assay kits (Beijing Solarbio Science & Technology Co., Ltd, China) following the manufacturer protocols.

### Determination of ATP contents

Cardiac tissues (10% w/v) were homogenized using boiling double distilled water: The samples were then placed in a boiling water bath for 10 min, followed by 10 min of centrifuged at 3500 g to collect the supernatant. Thereafter, the ATP level was measured according to the reagent protocols (Nanjing Jiancheng Bioengineering Institute, China).

### 2.10 Flow cytometry

Cardiac tissues were cut into 1 $mm^3$ pieces by ophthalmology, digested with trypsin in the constant temperature water bath (37 ° C) for 30 min, and filtered with the 300-mesh nylon filter. Afterwards, the filtrate was centrifuged at 600×g for 5 min at 4 °C, the supernatant was discarded, and the sediment was re-suspended with cold PBS for several times. The above-mentioned steps were repeated for three times, and finally the sediment was suspended with cold PBS. Then, the monoplast suspension was loaded using the DCFH-DA probes and JC-1 probes in accordance with the active oxygen detection kit (Nanjing Jiancheng Bioengineering Institute, China) and the Mitochondrial membrane potential assay kit with JC-1(Beyotime Biotechnology, Shanghai, China). The monoplast was washed with PBS for three times after incubation at 37 °C; finally, the DCF and JC-1 fluorescence intensity were determined through FC using the Guava EasyCyte flow cytometer (EMD Millipore Corporation, Hayward, Calif).

### TUNEL assay

The apoptosis level was determined according to the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay performed using the One Step TUNEL Apoptosis Assay Kit (Dalian Meilun Biotechnology Co., LTD, China) following the manufacturer protocol. Cells with bright blue nuclei were deemed as positive for apoptosis. Five representative fields were randomly selected at ×200 magnification.

### Protein extraction and Western blotting

Myocardial tissues were cut into pieces and homogenized within the lysis buffer containing 100×phosphatase inhibitor, 100 mM PMSF and 1000× protease inhibitor (KeyGen Biotech. Inc, Nanjing, China). Later, the homogenization solution was transferred into the ep tube to centrifuge at 12000 g for 5 min at 4 °C. Concentration of the supernatant was determined using the BCA protein assay kit (Beijing Solarbio Science & Technology Co., Ltd, China). Moreover, cytoplasmic protein was extracted according to operation procedure from KeyGen Biotech. Inc (Nanjing, China). In brief, tissues were homogenized, placed on ice for 30 min, and centrifuged at 3000 g for 10 at 4 °C, and the supernatant was cytoplasmic protein. To carry out immunoblotting, proteins were separated by SDS-polyacrylamide gel electrophoresis

(PAGE), followed by transfer onto the polyvinylidene difluoride (PVDF) membranes. Then, the PVDF membranes were blocked with 5% defatted milk for 2 h at room temperature, and incubated with primary antibodies diluted by TBST overnight at 4 °C. The membranes were incubated with the following primary antibodies: SIRT3 (1:1000, Proteintech Group, Inc, # 10099-1-AP), PGC-1α (1:1000, Proteintech Group, Inc, #66369-1-Ig), NRF1 (1:500, BOSTER, #AO1129-2), TFAM (1:500, BOSTER, #PB0413), Cleaved Caspase-3 (1:1000, Affinity, # AF7022), Cyt C (1:800, Proteintech Group, Inc, # 10993-1-AP), GAPDH (1:1000, Cell Signaling Technology, #5174). After washing with TBST for 3 times, the membranes were incubated with peroxidase (HRP)-conjugated secondary antibody, and visualized using the luminol reagent (Engreen Biosystem Co, Ltd., Beijing, China).

### Statistical analysis

All experimental data were expressed as means±S.D. The SPSS 19.0 (Lead Technologies, Chicago, Illinois) was utilized for all statistical analyses. Significance was determined by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. A difference of $P < 0.05$ was considered as statistically significant.

## Results

### TMYXP mitigates cardiac dysfunction in rats induced by ISO

Sixty rats were given subcutaneous injection of ISO for 2 weeks, and then the corresponding drugs were given intragastrically for 6 weeks (Figure 1A). With this treatment, rats began to die on day 10; 10 rats survived after the last injection. We found that the hearts of the 10 mice that died had varying degrees of enlargement. After the injection of ISO, the rats experienced weight loss. According to Figure 1AB, ISO stimulation markedly slowed down the weight gain in rats, while TMYXP 1.0 g/kg, 2.0 g/kg and BIS treatment suppressed such changes. In addition, the changes in HWI suggested that hearts were remarkably expanded in ISO group, while TMYXP 1.0 g/kg, 2.0 g/kg and BIS treatment reduced HWI ($P<0.01$) (Figure 1C). Moreover, the serum biochemical index levels suggested that ISO evidently induced BNP in rats. TMYXP 1.0 g/kg, 2.0 g/kg and BIS intervention restrained the secretion of BNP in rats (Figure 1D). Moreover, the variations in heart function, shape and size were evaluated by echocardiography. In comparison with control group, animals treated with ISO displayed ventricular remodeling at the $2^{nd}$ week, along with cardiac function decline, as evidenced by the increased LVESD while reduced LVEF and LVFS. The LVESD and LVEDD levels in ISO group were evidently increased upon the end of the $8^{th}$ week compared with those at the $2^{nd}$ week. Additionally, LVFS and LVEF were apparently decreased compared with those at the $2^{nd}$ week, indicating further deterioration of rat heart function, while TMYXP 1.0 g/kg, 2.0 g/kg and BIS could prevent such changes (Figure 1E, F).

### Effect of Tongmai Yangxin Pill (TMYXP) on Changes of Histopathological changes in ventricular myocardial tissues

Results of H&E staining demonstrated that, the ISO group was associated with inflammatory cell infiltration, disordered cardiac muscular fibers, and cardiac hypertrophy relative to those in control group. Nonetheless, such impacts were reduced in TMYXP 1.0 g/kg, 2.0 g/kg and BIS treatment group (Figure 2A). Besides, compared with control group, results of Masson's trichrome staining revealed that, the ISO group was linked with disorderly arranged myocardial tissues, along with large area of congestion, and irregular collagen fibers. Additionally, fibrotic connective tissues were dramatically reduced in TMYXP 1.0 g/kg, 2.0 g/kg and BIS group, which conformed to the prior H&E staining results (Figure 2B).

### TMYXP mitigates OS and enhances mitochondrial function in myocardial tissues of ISO-induced rats

The activities of antioxidant enzymes and ROS contents were examined in the myocardial tissues of rats. Relative to control group, ISO stimulation markedly increased myocardial OS, as verified by the elevated MDA and ROS levels, together with the reduced GSH-Px and Mn-SOD activities ($P<0.01$). TMYXP 1.0g/kg, 2.0 g/kg and BIS intervention evidently prevented ROS accumulation and enhanced antioxidant function (Figure 3A, B and C). These results suggest that TMYXP may function by attenuating the mitochondrial stress caused by ISO. To further assess this possibility, we evaluated the effects of each group of drugs on ATP levels. ISO significantly decreased the mitochondrial ATP content, and PER attenuated this decrease ($P < 0.01$; Figure 3D). To verify that these effects on mitochondrial ATP content were mediated at the level of ATP synthesis, we evaluated the activities of the mitochondrial respiratory chain complexes I, II, III, and IV. The activities of each of these complexes were significantly reduced in comparison with those of the control group; however, TMYXP 1.0 g/kg, 2.0 g/kg and BIS statistically reversed the decreases ($P<0.01$) (Figure 2E). These results demonstrated that TMYXP intervention protected the function of electron transport chain in rat myocardial cells, thus decreasing ROS production and elevating ATP synthesis.

### TMYXP decreased ROS-derived myocardial apoptosis in cardiomyopathy rats induced by ISO

To further assess the functional outcome of PER-mediated attenuation of OS, we evaluated the effects of TMYXP on ISO-inducted myocardial apoptosis. We assessed the effects of ISO, TMYXP and BIS on the mitochondrial membrane potential (MMP). The myocardial MMP of the ISO group was notably lower than that of control group ($P<0.01$), while the MMP of TMYXP 0.5 g/kg, 1.0 g/kg, 2.0 g/kg and BIS group was higher than that of ISO group ($P<0.01$) (Figure 4A, B). Furthermore, TUNEL staining results had verified the remarkably aggravated myocardial apoptosis in ISO group ($P<0.01$), but that in TMYXP 1.0 g/kg, 2.0 g/kg and BIS group was markedly decreased compared with that in ISO group ($P<0.01$) (Figure 4C, D). ISO stimulation resulted in the changed contents of cleaved caspase-3, mitochondrial cytochrome C (Mito Cyt C). These results demonstrated that the pro-apoptotic factor Cyt C had leaked from mitochondria to cytoplasm of myocardial cells in ISO group, which activated caspase-3 and initiated apoptosis. Nevertheless, TMYXP 1.0 g/kg, 2.0 g/kg and BIS suppressed Cyto Cyt C leakage and caspase-3 activation (Figure 4E, F). These results suggest that TMYXP reduces the level of myocardial apoptosis in the ISO-induced cardiomyopathy model.

### TMYXP modulates PGC-1α, SIRT3, TFAM, and NRF1 protein and mRNA levels in myocardial tissues from ISO-induced rats

Influence of TMYXP on the protein levels of PGC-1α, SIRT3, TFAM, and NRF1 in myocardial tissues from cardiomyopathy rats induced by ISO. To determine whether TMYXP might ameliorate cardiomyocyte dysfunction by improving mitochondrial biosynthesis, we performed Western blotting of proteins that are known to have a role in mitochondrial biosynthesis. Relative to control group, ISO treatment markedly down-regulated the protein expression of PGC-1α, SIRT3, mitochondrial transcription factor A (TFAM), and nuclear respiratory factor 1 (NRF1) ($P<0.01$). Besides, the expression of PGC-1α, SIRT3, TFAM, and NRF1 were increased following TMYXP 1.0 g/kg, 2.0 g/kg and BIS intervention ($P < 0.01$) (Figure 4A, B). These findings suggested that TMYXP enhanced mitochondrial biosynthesis via activating the SIRT3/PGC-1α signal transduction pathway.

# Discussion

Our research had concentrated on the efficacy of TMYXP in treating ISO-induced myocardial damage in rats. This study aimed to investigate the mechanism of action of TMYXP and to provide new therapeutic targets for the clinical HF prevention and treatment. Our experimental results demonstrated that PER attenuated myocardial remodeling in rats with ISO-induced cardiomyopathy, and prevented from cardiac function decline, which might be related to the TMYXP-induced up-regulation of SIRT3 and PGC-1α.

Subcutaneous injection of  ISO led to the accumulation of catecholamine in animals, which would thereby induce cardiomyocyte hypertrophy, necrosis, and apoptosis, resulting in myocardial remodeling and finally HF (15). In this experiment, a rat cardiomyopathy model was established by subcutaneous injection of ISO at 10.0 mg/kg/d for 2 weeks. Changes in the volume and shape of the left ventricle are often referred to as cardiac remodeling in clinical practice (16). At the end of the second week of modeling, the results of echocardiography showed that ISO stimulation caused left ventricular dilatation and left ventricular systolic function decreased in rats, suggesting that the cardiac structure of rats changed and cardiac remodeling occurred. At the end of the eighth week, the echocardiographic indicators of the rats in the ISO group suggested that the ventricular dilatation in rats increased, and the left ventricular systolic function decreased further. Rat weight, total heart mass index, and serum BNP also demonstrated that the rats in the ISO group had slower growth, enlarged hearts, and decreased heart function. Pathological examination found that ISO can induce myocardial necrosis, hypertrophy and fibrosis in rats. The above results suggest that ISO intervention can aggravate rat heart remodeling and promote the development of heart failure. TMYXP 1.0 g/mg, TMYXP 2.0 g/mg and bisoprolol intervention can reduce serum BNP levels, reduce myocardial fibrosis, inhibit the tendency of heart cavity enlargement, improve the pump function of the heart and increase the output of the heart. It shows that TMYXP can protect myocardial cells, inhibit cardiac remodeling, and delay the development of heart failure.

Related articles (13) found that Tongmai Yangxin Pills have a protective effect on cardiomyocytes, and its mechanism may be related to antioxidant effects. Recent studies have shown (17,18) that a large

amount of ROS produced by OS induces apoptosis, eventually changes the heart structure, causes abnormal heart function, and promotes the evolution of heart failure. Our data also show that administration of TMYXP 1.0 g/mg, TMYXP 2.0 g/mg and bisoprolol to ISO-induced rats can significantly reduce ROS content, increase the activity of Mn-SOD and GSH-Px, and MDA content in myocardial tissue. SODs is an antioxidant enzyme that can eliminate superoxide anion free radicals and protect cells from oxidative damage (19) while Mn-SOD located in mitochondria mainly acts to remove ROS in mitochondria (20). In addition, GSH-Px has the function of eliminating lipid peroxides and forms an antioxidant defense system in the body with other oxidases (21). On the other hand, MDA is a product of lipid peroxide, and its content reflects the degree of tissue oxidative damage. Although the relative role of upstream regulators in this process has yet to be fully elucidated, these findings are related to the cardioprotective effects of TMYXP and OS remission.

Electron transport chain (ETC) is the main place for mitochondrial ROS production. When electron leakage occurs during electron transfer, a large number of superoxide anions (02-) can be formed. Among them, complexes I and III are the main sites for electron leakage and mitochondrial ROS production (22). Superoxide anions form hydrogen peroxide and hydroxyl radicals through further conversion, causing oxidative damage to cells (23,24). In addition to its role as a source of ROS, mitochondria may themselves be damaged by ROS. Under the condition of OS, the increase of ROS production in mitochondria may lead to abnormal mitochondrial respiratory chain function, leading to reduced ATP synthesis and activation of endogenous apoptotic pathways. In addition, insufficient energy production and cardiomyocyte apoptosis contribute to ventricular remodeling and ultimately lead to HF (25). The results of this study indicate that the production of ROS in the myocardial tissue of ISO-induced cardiomyopathy rats is accompanied by a decrease in mitochondrial respiratory chain I, II, III, IV activity and ATP production. However, TMYXP 1.0 g/mg, TMYXP 2.0 g/mg and BIS can improve respiratory chain function and increase myocardial ATP content. Therefore, we speculate that protecting mitochondrial function is a key link in TMYXP-mediated cardiac protection.

Myocardial apoptosis causes ventricular remodeling, which ultimately leads to HF (26,27). Previous studies (28) have suggested that excessive production of ROS in mitochondria can cause damage to lipids and proteins of mitochondrial inner membrane, induce intimal fluidity and Permeability changes, permeability transition pores (mPTP) open. The opening of mPTP can rapidly destroy the mitochondrial membrane potential and change the osmotic pressure in mitochondria, causing membrane rupture, promoting Mito Cyt C outflow, activating caspase-3 and eventually lead to apoptosis (29). In this experiment, TMYXP 1.0 g/mg, TMYXP 2.0 g/mg and BIS treatment increased MMP, inhibited the release of the proapoptotic factor Cyt C from the mitochondria into the cytosol and prevented caspase-3 activation, reducing cardiomyocyte apoptosis. Combined with the results of previous experimental data, it is fully demonstrated that TMYXP reduces the activation of endogenous apoptotic pathways by reducing ROS production in mitochondria.

Mitochondrial biosynthesis can be defined as the process of mitochondrial proliferation and mitochondrial system synthesis and individual synthesis. It is an important mechanism for cells to

achieve self-renewal and regulation. Mitochondrial biosynthesis up-regulates both mitochondrial respiratory chain enzymes and mitochondrial antioxidant factors (such as Mn-SOD and GSH-Px) (30,31). Mitochondrial biosynthesis depends on the expression of mitochondrial genome (mtDNA) and nuclear genome (nDNA), and mtDNA is the main target of ROS-mediated damage (32). PGC-1α plays a central role in the regulation of mitochondrial production. PGC-1α activates the downstream nuclear respiratory factors NFR-1 and Tfam, which activates the transcription of oxidative phosphorylated proteins encoded by mt DNA, thereby promoting mitochondrial biosynthesis (22). Decreased PGC-1α expression can be observed in various HF models with OS and mitochondrial dysfunction (10,11). SIRT3 acts on a wide range of substrates and is closely related to cardiovascular disease. SIRT3 is involved in almost every major aspect of mitochondrial biology, such as fatty acid oxidation, tricarboxylic acid cycle, antioxidant defense, mitochondrial protein synthesis and translation, and mitochondrial dynamics (33). Previous studies have shown that SIRT3 regulates mitochondrial biosynthesis by activating PGC-1α. Studies (12) have shown that myocardial protective active substances in TMYXP, the identified compounds are mainly derived from licorice, Schisandra chinensis, Ophiopogon. Glycyrrhizin, the main active compound of TMYXP, can activate the AMPK/SIRT1/PGC-1α signaling pathway in the kidneys of db/db mice with diabetic nephropathy in rats, and play a role in inhibiting ROS activation and apoptosis (14). Schisandrol is extracted from Schisandra chinensis, the main component of TMYXP, and can significantly increase SIRT1 and PGC-1α in the hippocampus of mice, thereby increasing the activity of SOD and GSH-px, and reducing the level of MDA (34). SIRT3 is a deacetylase that is homologous to SIRT1 in myocardial tissue, and is similar to SIRT1 in the way of activation. This study suggests that the protein expression of SIRT3, PGC-1α, NRF-1 and TFAM is reduced in the myocardial tissue of the ISO group rats. However, the intervention of TMYXP 1.0 g/mg, TMYXP 2.0 g/mg and BIS can increase the expression of SIRT3, PGC-1α, NRF-1 and TFAM, suggesting that Tongmai Yangxin Pill may improve mitochondria by activating SIRT3/PGC-1α Biosynthesis.

In summary, our results indicate that cardiac remodeling and decreased cardiac function in ISO-induced cardiomyopathy model rats may be related to the down-regulation of SIRT3 and PGC-1α expression. TMYXP can improve mitochondrial biosynthesis and attenuate mitochondrial OS and apoptosis by restoring SIRT3 and PGC-1α expression, thereby improving cardiac function. These results have provided a deeper understanding of the mechanism of TMYXP in preventing and treating HF in clinical practice.

# Abbreviations

HF, heart failure; TMYXP, Tongmai Yangxin Pill; ISO, isoproterenol; ROS, reactive oxidative species; SIRT3, Sirtuin 3; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; NRF1, nuclear respiratory factor 1; TFAM, mitochondrial transcription factor A; NADH/NAD, nicotinamide adenine dinucleotide; AngII, Angiotensin II; OS, oxidative stress; SD, Sprague-Dawley; MMP, mitochondrial membrane potential; TUNEL, transferase dUTP nick end labeling; HWI, Heart weight index; BNP, B-type natriuretic peptide; MDA, malondialdehyde; GSH-Px, glutathione peroxidase; Mn-SOD, manganese superoxide dismutase.

# Declarations

**Ethics approval:** The procedures of the study were approved by the Animal Care and Use Committee of Tianjin Union Medical Center.

**Consent for publication:** This manuscript has not been published and is not under consideration for publication elsewhere in whole or in part. No conflicts of interest exist in the submission of this manuscript, and the manuscript has been approved for publication by all listed authors.

**Availability of data and material:** The data used to support the findings of this study are available from the corresponding author upon request.

**Competing interests:** None of the authors has any financial and personal relationships with other people or organizations that could potentially and inappropriately influence this work and its conclusions. Authors declared no competing interest on publishing this paper.

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**Authors' contributions**: ZZ conceived and designed the study, collected, analyzed and interpreted the data, and wrote the manuscript. HL conceived and designed the study, and collection and presented the data. WC analyzed and interpreted the data. YC was involved in the animal experiments. AH assessed western blotting analysis. XQ conceived and designed the study, wrote the manuscript, and gave final approval of the manuscript.

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# Figures

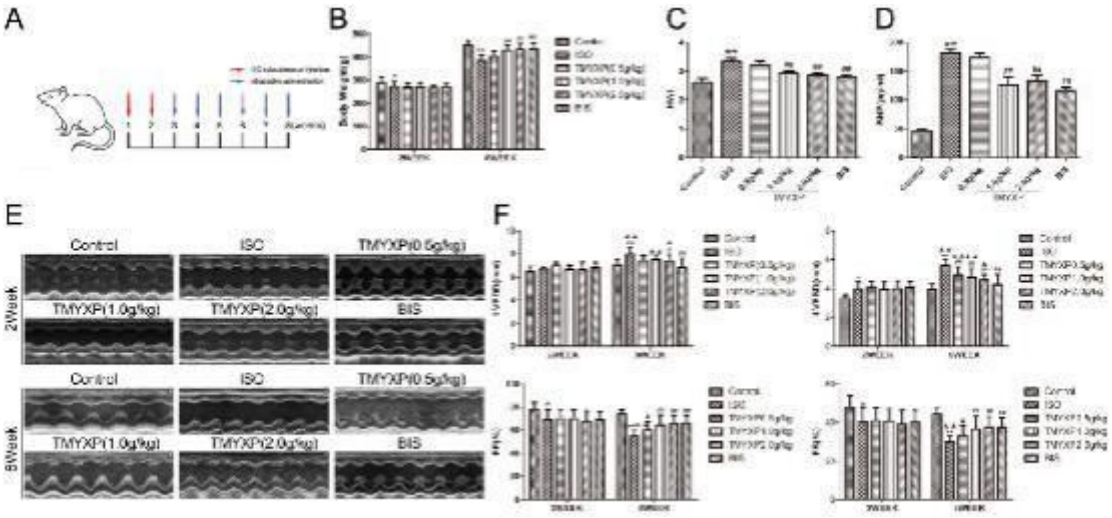
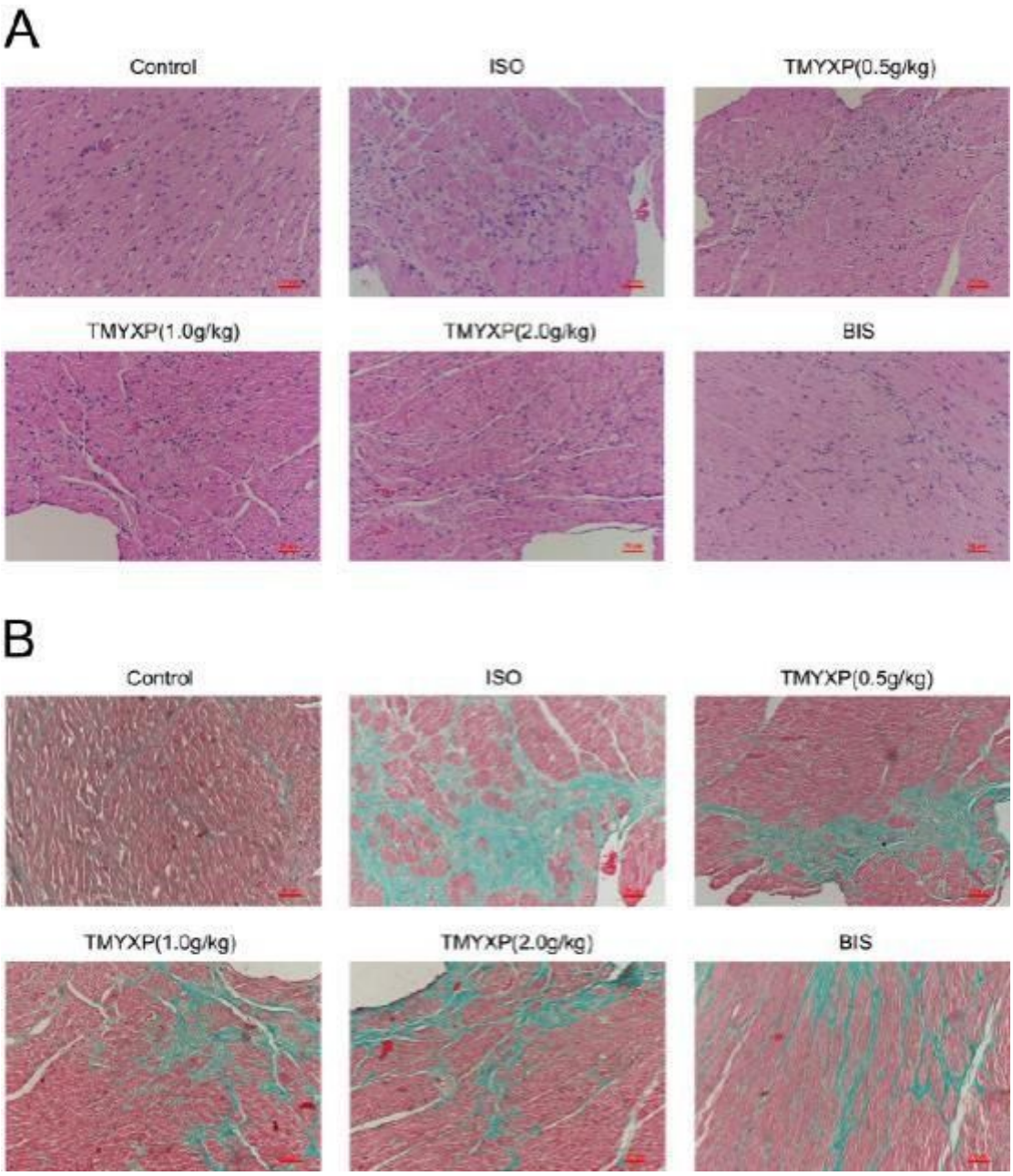


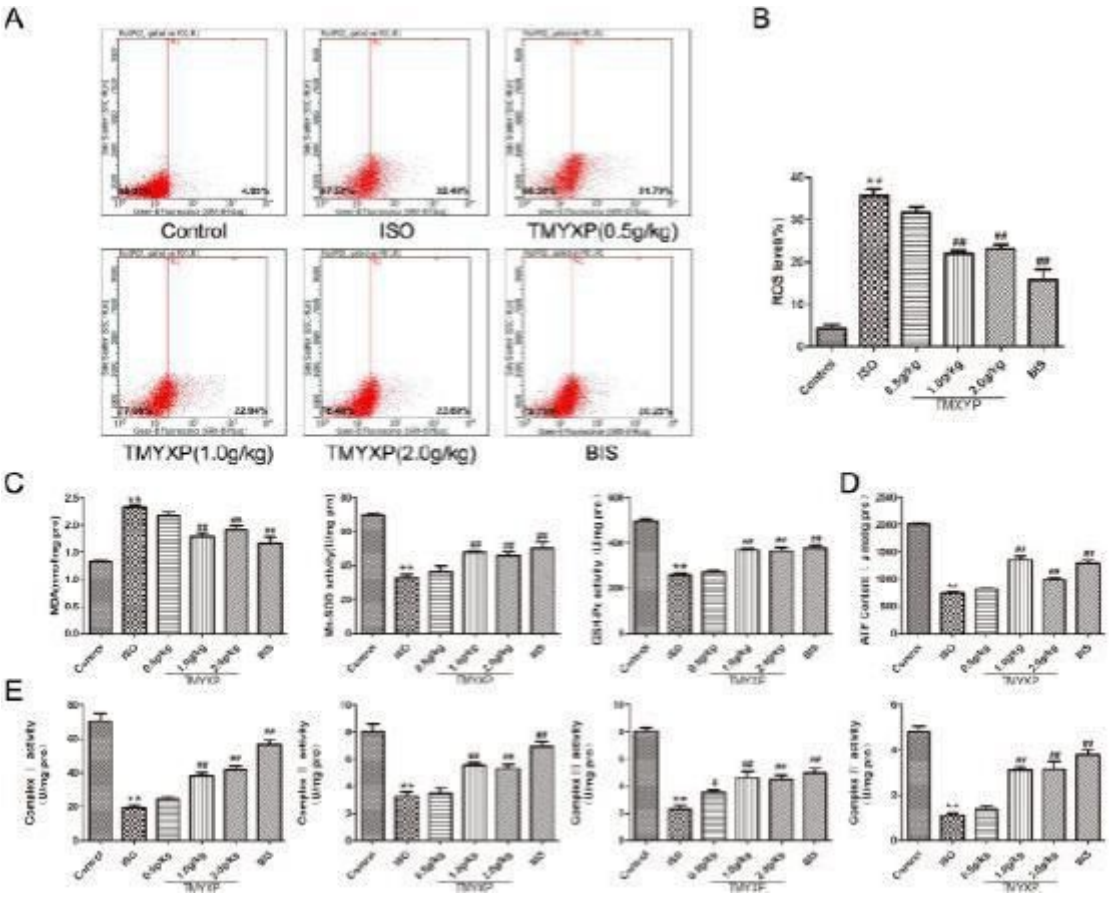

**Figure 1**

Tongmai Yangxin Pill (TMYXP) enhances heart function in the isoproterenol (ISO)-induced cardiomyopathy model rats. (A) Schematic diagram of the experimental flow (B) Changes in body weights for control rats and ISO-induced cardiomyopathy rats treated with out without PER. (C) Changes in rat heart weight index (HWI) for different groups. (D) B-type natriuretic peptide (BNP) levels in sera were measured by ELISA assay. (E) Representative M-mode echocardiograms for ISO, TMYXP (0.5 g/kg), TMYXP (1.0 g/kg), TMYXP (2.0 g/kg), BISand control groups. (F) Bar charts showing LVESD, LVEDD, LVFS% and LVEF%. Data were represented as mean±SD, #P<0.05, ##P<0.01 compared with ISO group, **P<0.01 compared with control group, ▲P<0.05, ▲▲P<0.01 compared with 2Week, LVESD: Left ventricular end diastolic diameter; LVEDD: Left ventricular end diastolic diameter; LVFS%: Left ventricular fractional shortening; LVEF%: Left ventricular ejection fraction.



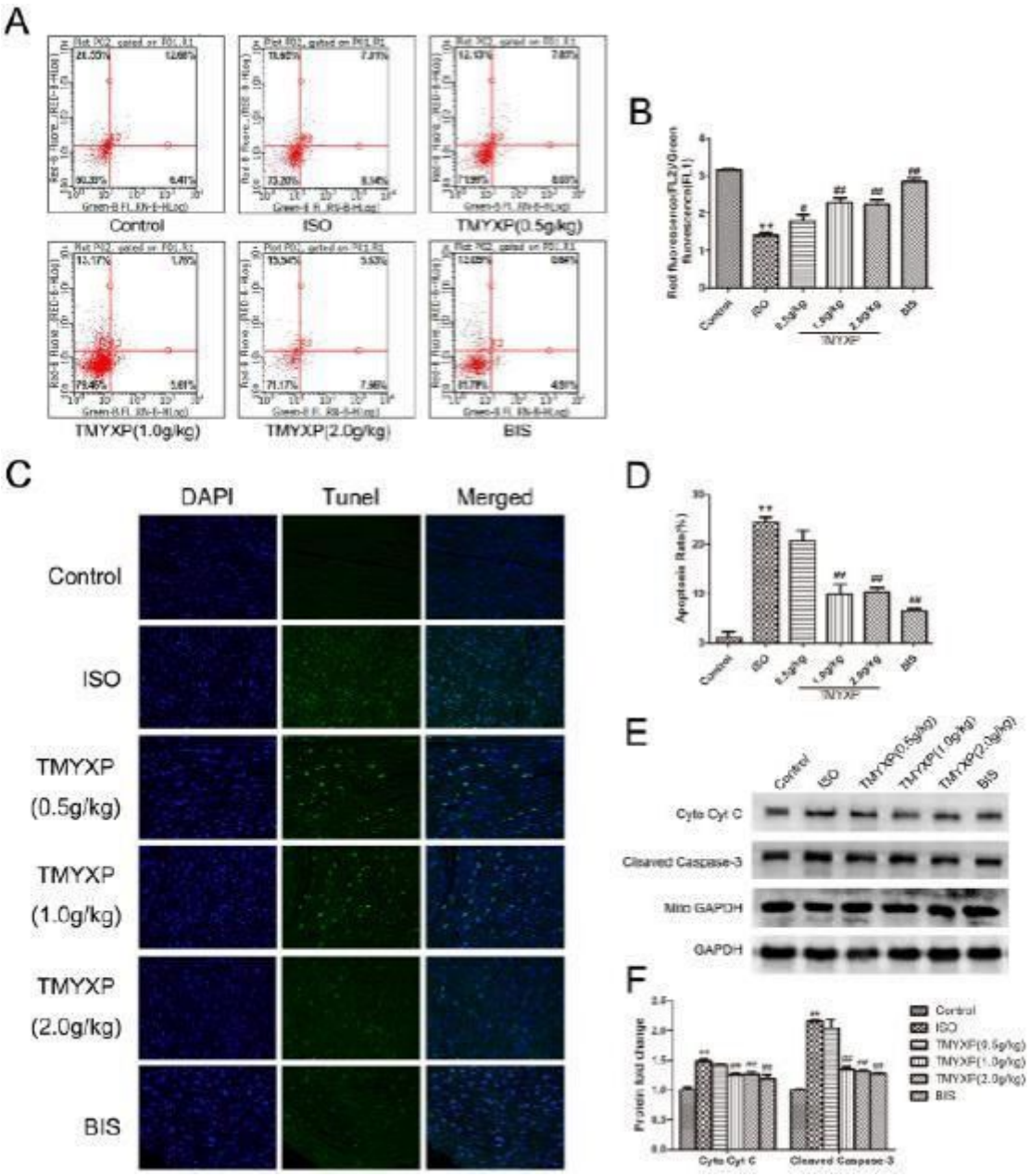

## Figure 2

Effect of Tongmai Yangxin Pill (TMYXP) on Changes of Histopathological changes in ventricular myocardial tissues (A) Histopathological changes in ventricular myocardial tissues observed by H&E staining (×200 magnification). (B) Masson's Trichrome Staining of cardiac tissues (×200 magnification). Blue color represents fibrosis.



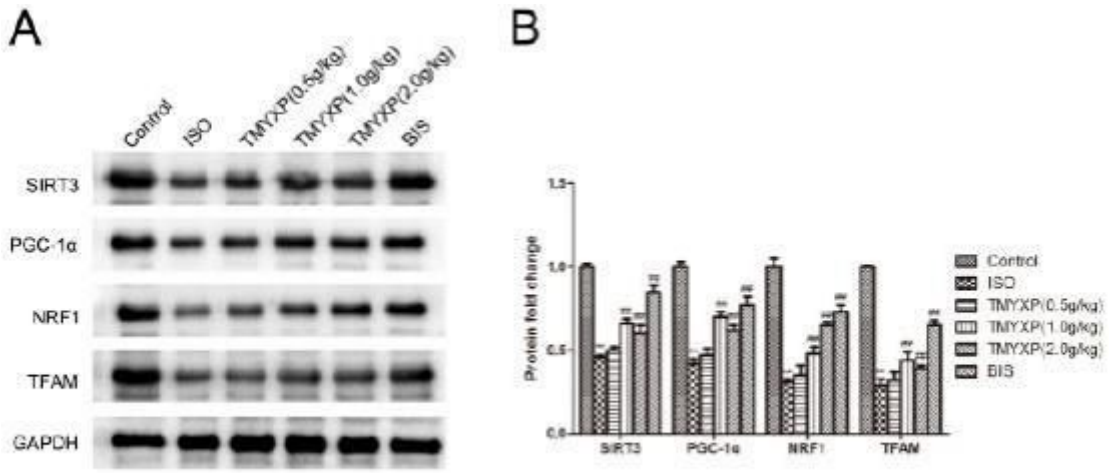

## Figure 3

Tongmai Yangxin Pill (TMYXP) attenuates myocardial mitochondrial function and oxidative stress in isoproterenol (ISO)-induced cardiomyopathy rats. (A) Flow cytometry was performed to analyze the total reactive oxygen species (ROS) levels in podocytes based on DCHF-DA staining. (B) Quantitative analysis of ROS. (C) Myocardial tissues were homogenized, and then malondialdehyde (MDA), Mn-SOD, and GSH-px levels were assessed. (D) Mitochondria were isolated from myocardial tissues for detection of Complex I, II, III, IV concentrations. Data are represented as means±SD, ##P < 0.01 compared with the ISO group, and **P < 0.01 compared with the control group.




## Figure 4

Tongmai Yangxin Pill (TMYXP) attenuates myocardial apoptosis in isoproterenol (ISO)-induced cardiomyopathy rats. (A) Flow cytometry was employed to analyze the mitochondrial membrane potential (MMP) of myocardial cells based on JC-1 staining. (B) The MMP was quantified as the red-to-green fluorescence ratio. (C) Representative DNA fragmentation assays measured by DAPI and TUNEL staining. (D) The apoptosis rate was computed as the positive cell percentage. Statistics were calculated from 3 views from every group. (E) Western blotting of Cyto Cyt C in cytoplasmic lysates and Cleaved Caspase-3 in total cell lysates. (F) Quantification of Cyto Cyt C and Cleaved Caspase-3 protein levels was performed using densitometry. Data are represented as means±SD, ##P<0.01 compared with the ISO group, **P<0.01 compared with the control group.




## Figure 5

Tongmai Yangxin Pill (TMYXP) restores the protein levels of Sirtuin 3 (SIRT3), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (TFAM) in myocardial tissues from isoproterenol (ISO)-induced cardiomyopathy rats. (A) Western blotting of SIRT3, PGC1α, NRF1 and TFAM within myocardial tissues. (F) Quantification of SIRT3, PGC1α, NRF1 and TFAM protein levels was performed using densitometry. Data are represented as means±SD, #P<0.05 compared with the ISO group, ##P<0.01 compared with the ISO group, **P<0.01 compared with the control group.