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Effects of Yang-warming and Fluid Retention-resolving Method on the Expression of PI3K-Akt Pathway and Related Apoptotic Proteins in Chronic Heart Failure Rats

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Objective: Chronic heart failure（CHF）is one of the most common and also one of the highest mortality diseases, but there is no established effective treatment. The primary molecular mechanism of CHF is the apoptosis of myocardial cells through the PI3K-Akt signaling pathway. So, we investigated the possible mechanism of yang-warming and the fluid retention-resolving method in the treatment of CHF. ***Methods:*** The rat model of chronic heart failure was reproduced by intraperitoneal injection of adriamycin. Doppler echocardiography was used to determine rats' cardiac function. ELISA kits detected the levels of BNP and cTnI. H&E staining was used to observe the changes of myocardial pathological morphology in rats. The protein contents of p-PI3K, p-Akt were detected by Western Blot. The expression of Bcl-2 and Bax, and Caspase-8, and Caspase-3 were detected by immunohistochemistry. ***Results:*** We revealed that p-PI3K and p-Akt protein expression in the CHF group were decreased, the value of Bcl-2 and Bcl-2/Bax decreased, and the value of Bax, Caspase-8 and 3 increased; All of the treatment groups attenuated the levels of BNP, cTnI, Bax, Caspase-8 and Caspase-3. And the expressions of p-PI3K, p-Akt, Bcl-2, Bcl-2/Bax were increased. The yang-warming group ameliorated the CHF state from the amounts of the releases better than other treatment groups. ***Conclusions:*** These revealed that the yang-warming and fluid retention-resolving method might play a role in protecting the myocardium by regulating the PI3K-Akt signaling transduction pathway and subsequently inhibiting apoptosis.

Keywords: yang-warming and fluid retention-resolving, chronic heart failure, PI3K-Akt pathway

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INTRODUCTION

Chronic heart failure（CHF）is a complex clinical syndrome caused by myocarditis, coronary heart disease, myocardial infarction, hypertension, and other cardiovascular diseases. It is not only the ultimate destination of most cardiovascular diseases but also a global public health problem［1］. The number of patients with heart failure increases with age, but there is no established CHF therapy. So, CHF patients commonly have high morbidity and mortality and need to pay enormous medical bills to treat CHF. It was therefore considered necessary that we provide an effective, safe, and convenient treatment method.

Cardiomyocyte apoptosis in CHF's molecular mechanism is one of the main reasons for ventricular remodeling. It involves multiple signal transduction pathways such as Phosphatidylinositol-3-kinase（PI3K）-Akt, Bcl-2/Bcl-XL, and Caspase［2-3］. Phosphoinositide 3-kinase（PI3K）is composed of p85 and p110 subunits with modulating and catalytic functions. It is an enzyme with serine/threonine-protein kinase activity. Protein kinase B is also known as Akt. As a downstream target of PI3K, it has essential functions. It can regulate cell

apoptosis, transcription, proliferation, and growth and participate in protein synthesis and glucose metabolism and, other physiological processes [4]. PI3K-Akt signaling pathway also regulates vascular regeneration, regulating the proliferation, differentiation, apoptosis, and metabolism of cardiomyocytes [5]. These physiological processes and functions are closely related to CHF.

In the process of CHF, the primary way of myocardial cell loss is through apoptosis. It is the joint action of multiple apoptotic factors, and the PI3K-Akt signaling pathway plays a vital role in it. Akt can inhibit the activity of downstream target proteins such as pro-apoptotic molecules Bcl-2/Bcl-XL associated death promoter (Bcl-2/Bcl-XL associated death promoter, BAD) through phosphorylation. When BAD is inhibited, the Bcl-BAD complex can release the anti-apoptotic protein B-cell lymphoma/leukemia-2 (Bcl-2), thereby inhibiting apoptosis [6]. Akt can also phosphorylate the apoptosis-initiating factors of the Caspase family upstream of the cascade, such as Caspase-3, 8, etc., so that they lose the activity of proteolytic enzymes and inhibit apoptosis [7-8].

Yang-warming and fluid retention-resolving decoction is composed of Linggui Zhugan Tang and Tingli Dazao Xiefei decoction. In the prescription, Poria cocos (Fuling) was used as monarch medicine. Modern pharmacological studies have shown that the poriatin in Poria cocos has the effects of diuresis and swelling. Poria cocos polysaccharides have anti-inflammatory, anti-tumor, and immune-regulating impacts [9]. The minister of medicine is Cassia twig (Guizhi). Modern pharmacological studies have shown that one of the main components of cinnamon sticks, cinnamic aldehyde, is expanding blood vessels and enhancing coronary blood flow, so cinnamon sticks are widely used in the treatment of cardiovascular diseases [10]. The assistant medicine Atractylodes (Baizhu) and Licorice (Gancao) have found that they play an essential role in the body's gastrointestinal, urinary, cardiovascular, and other systems and can repair the stomach mucosal, anti-tumor, regulating water metabolism, protect the cardiovascular system [11]. In the Tingli Dazao Xiefei prescription, Semen lepidii (Tinglizi) inhibits myocardial hypertrophy and ventricular remodeling in cardiovascular aspects; the alcohol extract of Tinglizi also has the effect of anti-inflammatory and strong myocardial contractility [12]. Modern pharmacological research also found that the flavonoids and glycosides in Red jujube (Dazao) can relax the blood vessels and antagonize the platelet-activating factor, which can play a better protective effect on the cardiovascular system [13]. The two prescriptions are used to achieve the effect of Yang-warming and fluid retention-resolving and improve CHF symptoms.

The selected Traditional Chinese medicine (TCM) control drug in the experiment is Xuefu Zhuyu Capsule. In the prescription, Peach kernel (Taoren) and Safflower (Honghua) are the monarch medicine, which promotes blood circulation, removes blood stasis, and dredges the collaterals. The red paeony root (Chishao), Chinese angelica (Danggui), radix achyranthis bidentatae (Niuxi), adix rehmanniae (Shengdi), ligusticum wallichii (Chuanxiong) are all minister medicines. It can enhance the effect of promoting blood circulation and removing blood stasis with monarch medicine aid and has the effect of pain relief. All the drugs are combined to promote blood circulation, remove blood stasis, promote and improve heart function.

So, we established the Yang-warming and fluid retention-resolving decoction to look at this decoction on PI3K-Akt signaling pathways, and related apoptotic protein Bcl-2, Bax, Caspase8, and Caspase3 explored the action mechanism of the prevention and treatment of CHF.

MATERIALS AND METHODS

Animal

All animal-related experiments were conducted following the guidelines for the Care and Use of Laboratory Animals. Fifty male-specific pathogen-free (SPF) rats, 8–10 weeks old, weighing 180 ± 25 g, and in good health were selected. The animals were housed at three mice per cage in the SPF animal room of the Experimental Animal Center of Ningxia Medical University (License number: SCXK (Ning) 2015-0001) at room temperature (22 ± 2°C) and 60% relative humidity, under a 12 h light/dark cycle, and provided regular ad libitum access to food

and water. The experiment was conducted after allowing acclimatization for 1 week.

Drugs

Composition of Yang-warming and fluid retention-resolving decoction is Poria cocos (Fuling), Cassia twig (Guizhi), Atractylodes (Baizhu), Licorice (Gancao) (4:3:3:2), Semen lepidii (Tinglizi), Red jujube (Dazao) (6:5). The above medicinal materials were purchased from the outpatient department of the Fushu Huiyi Hospital of Ningxia Medical University. The pharmaceutical concentrate containing crude drug 2 g/ml was prepared with distilled water before application. The drugs we used were as follows; Adriamycin (ADR) (Zhejiang Haizheng pharmaceutical co., LTD., China), Xuefu Zhuyu (Tianjin Hongrentang pharmaceutical co., LTD., China), and Metoprolol (Astrazeneca pharmaceutical co., LTD., United Kingdom).

Reagents and instruments

Formaldehyde, Absolute ethyl alcohol, Xylene (sinopharmics group chemical reagent co., LTD., Shanghai China); Hematoxylin (Bioswamp, Wuhan, China); Protein G Magnetic Beads (CST, Boston, USA); SDS (Sigma-Aldrich, St. Louis, MO, USA); RIPA Lysis Buffer (Bioswamp); Enhanced BCA Protein Assay Kit (Bioswamp); anti-p-PI3K, anti-p-AKT, anti-GAPHD (Abcam, Cambridge, UK); Brain Natriuretic Peptide (BNP) and Cardiac Troponin I (cTnI), (eBioscience, San Diego, CA, USA). MaxVision TM HRP-Polymeranti-Mouse/Rabbit IHC Kit, PAB160022, (Bioswamp); anti-Caspase8, anti-Caspase3, anti-Bax, anti-Bcl-2 (Bioswamp); Automatic chemiluminescence analyzer (Tanon, Shanghai, China); Enzyme standard instrument MK3 (Thermo Finnpipette, Finland); DAB light microscope (Leica Microsystems Ltd., Wetzlar, Germany).

Modeling grouping and administration

After two weeks of adaptive feeding, rats were intraperitoneally injected with ADR [14]. 2 mg/kg of ADR was dissolved in 1mL normal saline once a week for a total of 6 injections to establish a CHF rat model. The left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular ejection fraction (LVEF), and left ventricular short-axis shortening rate (LVFS) were measured between the control group and the model group, determined by Doppler echocardiography, to evaluate the model building success. The rats were randomly divided into the CHF group, the yang-warming group, the xuefu group, and the metoprolol group after the modeling. Dosages according to a simple practice guide for dose conversion between animals and humans [15]. 9 g/(kg·d) in the yang-warming group, 0.25 g/(kg·d) in xuefu group, and 12 mg/(kg·d) in metoprolol group. Each group received oral administration once a day for four weeks. The control group and CHF group were given distilled water once a day, 3ml each time.

Rats symptoms and physical signs

Their mental status, diet, urine and excrement conditions, and activities of limbs were observed.

Detection of Cardiac function

Color Doppler echocardiography was performed for SD rats by ultrasound doctors. The rats were anesthetized by intraperitoneal injection of 0.03 ml/kg chloral 10% hydrated. The rats were fixed on the splint in the supine position, and their chest area was shaved; they were sterilized with 0.7% ethanol. Sternal high-frequency ultrasound was performed at 13 MHz with a 10 L probe, and two-dimensional images of the left long axis of the sternum were obtained with m-mode ultrasound sampling. LVFS, LVEF, LVEDD and, LVESD were calculated. The mean values of three consecutive cardiac cycles were taken from each group to evaluate the normal cardiac function of SD rats in each group; LVEF less than 45% was considered as a successful model.

Enzyme-Linked Immunosorbent Assay (ELISA)

The plasma samples for ELISA analysis were prepared following the instructions, and levels of Brain Natriuretic Peptide (BNP) and Cardiac Troponin I (cTnI) were detected by ELISA kits, according to the manufacturer's instructions. The optical density (OD) values from the samples were measured with a microplate reader in the 490 nm wavelength.

Hematoxylin Eosin (H&E) staining

To decalcify and fix the collected ventricular tissue, specimens were placed in a 12.5% ethylenediaminetetraacetic acid (EDTA) decalcification solution for 2 weeks. After dehydration and xylene-clearing treatment to render the tissue transparent, the specimens were paraffin-embedded, marked in groups, and stored at room temperature. Continuous 5 μm tissue sections were sliced, transferred onto glass slides, baked, and then subjected to hematoxylin and eosin (H&E) staining. Images of the stained sections were examined using a DAB light microscope, and representative images were captured.

Western blotting

Protein samples (20 μg) were separated using 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electro- phoresis and transferred to polyvinylidene fluoride (PVDF) membranes; the membranes were blocked by incubating in 5% skim milk in Tris-buffered saline (TBS)-Tween (TBS-T, 10 mM Tris-HCl, 50 mM sodium chloride (NaCl), 0.25% Tween 20) for 1 h. The membrane was incubated overnight at 4°C with primary antibodies (PI3K, p-PI3K, Akt, p-Akt). After three rinses with TBS-T, the membranes were incubated with horseradish peroxidase (HRP) anti-rabbit secondary antibody (1:10000) for 1 h. The signals were detected via exposure in a Bio-Rad imaging system. Gray levels corresponding to the indicated proteins were quantified and normalized relative to GAPDH using ImageJ software.

Immunohistochemical staining

The immunohistochemical method was used, and the specific steps have followed the instructions of the kit. Hematoxylin was restrained, sealed, and observed and photographed under the microscope. Samples were collected and analyzed by the Lecia Application Stiue image system, and the positive results of Bcl-2, Bax, and caspase-8 were semi-quantitatively analyzed under the x200 microscope. Six visual fields were observed randomly for each specimen. The antigen's relative content was represented by the area of the positive product/visual field, and its average value was taken.

Statistical analysis

Data processing and statistical analysis were performed using the statistical package for the social sciences (SPSS) 22.0 (IBM Corp., Armonk, NY, USA). Data are expressed as the mean ± standard error. One-way ANOVA was performed for statistical evaluation. Differences were considered statistically significant at $*p < 0.01$. All results were derived from at least three independent experiments.

RESULTS

The general situation

The control group rats gained weight rapidly and grew well. Responsive to external stimuli, the coat is supple and bright white, the tail thick but is long, the amount of food and urine is in line with average growth and development. After 6 weeks of modeling, the rats in each group had slow weight growth, slow movement, lethargy, and loss of appetite, and gradually saw listlessness and watery stool. After 4 weeks of a drug intervention, except for the CHF group, the weight of rats in each group gradually returned to increase, and the amount of food and water also increased.

Color doppler ultrasound in rat heart

Compared with the control group, LVEDD and LVESD of rats in the modeling group increased significantly, with statistically significant differences ($P < 0.01$), while LVFS and LVEF levels decreased,

Table 1. Results of cardiac ultrasound doppler examination in rats

Group	n	LVEDD (mm)	LVESD (mm)	LVFS%	LVEF%
Control group	10	5.31 ± 0.17	3.30 ± 0.25	40.79 ± 1.56	77.52 ± 3.92
CHF group	10	6.68 ± 0.50*	5.61 ± 0.50*	25.40 ± 1.93*	38.23 ± 2.42*

Results are expressed as Means±SD; *Statistically significant, $p < 0.01$

with statistically significant differences ($P < 0.01$) indicating successful modeling (Table. 1).

Effect of yang-warming therapy on serum BNP and cTnI in CHF rats

Cardiac functions were determined by BNP and cTnI ELISA assays, as revealed in (Fig. 1B and 1C). In contrast with the Control group, the CHF group extremely elevated the releases of BNP and cTnI levels in the plasma ($P < 0.01$). When compared with the CHF group, the yang-warming group, xuefu group, and metoprolol groups all attenuated BNP and cTnI ($P < 0.01$) from the amounts of the releases. We could conclude the yang-warming group ameliorated the CHF state better than xuefu and metoprolol groups.

Histological findings

The size of myocardial cells in the control group was regular and orderly, the cytoplasm was uniform, and the myocardial fibers were not ruptured. There were significant differences in myocardial cell hypertrophy in the CHF group, myocardial cell population, cell permutation disorder, cytoplasmic hyperchromasia, and myocardial fiber rupture. Compared with the CHF group, the treatment group's myocardial cells were significantly smaller, with a neat arrangement, uniform cytoplasm, and no cardiac fiber rupture. (Fig. 1A).

Expression of p-PI3K and p-Akt proteins in myocardial tissues of rats in each group

Compared with the control group, p-PI3K/PI3K and p-Akt/Akt expressions were decreased in the CHF

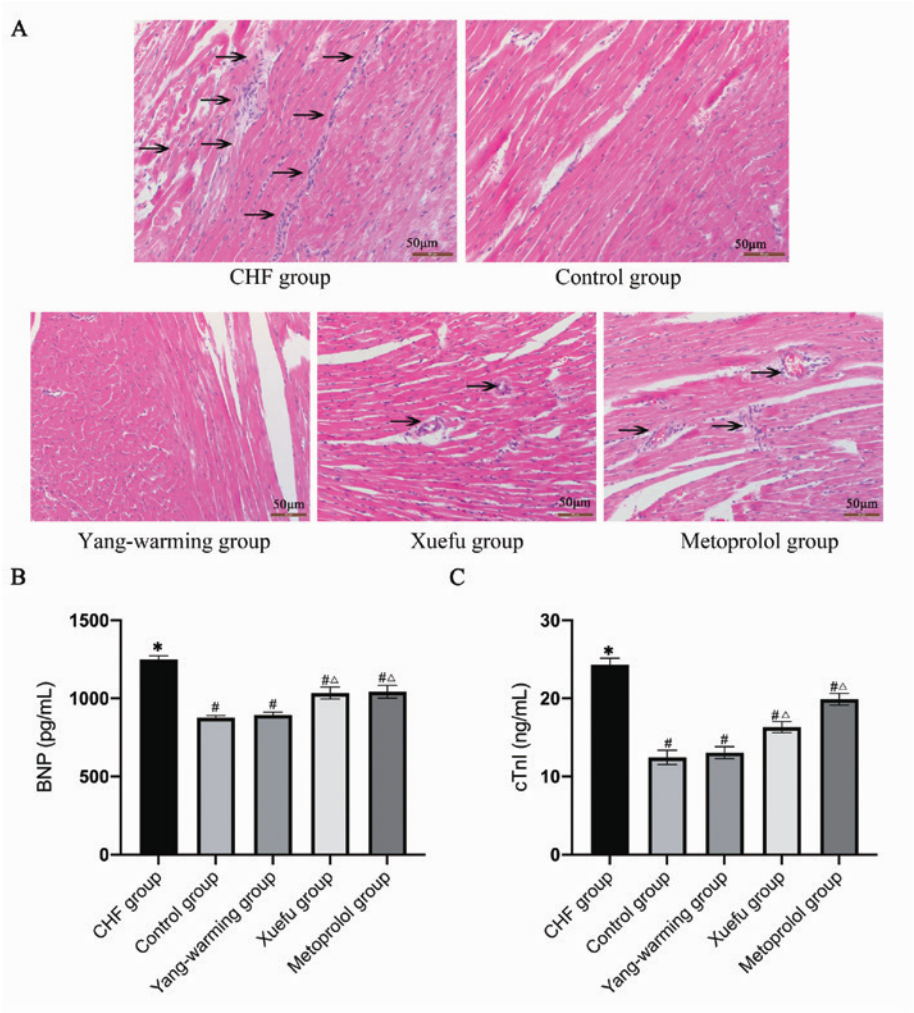

Fig. 1. H&E-stained images on light microscopy

(A) Arrow: Compared with the Control group, there were significant differences in myocardial cell hypertrophy, cell permutation disorder, cytoplasmic hyperchromasia, and myocardial fiber rupture. Scale bar, 50μm. (B) The levels of BNP in the plasma; (C) the levels of cTnI in the plasma. (*$p < 0.01$ compared with the Control group; #$p < 0.01$ compared with the CHF; $^{\triangle}p < 0.01$ compared with the Yang-warming group; n = 7 per group).

model group ($P < 0.01$) (Fig. 2). Compared with the CHF group, p-PI3K/PI3K and p-Akt/Akt expression were significantly increased in the yang-warming group, the xuefu group, and the metoprolol group ($P < 0.01$) (Fig. 2D, G). Compared with yang-warming group, the expression levels of p-PI3K, PI3K, p-Akt, and Akt in the xuefu group and metoprolol group were decreased ($P < 0.01$) (Fig. 2).

Expression of Bcl-2, Bax, Caspase-8, and Caspase-3 in myocardial tissue of rats

The positive expressions of Bcl-2, Bax, Caspase-8 and, Caspase-3 proteins in the myocardium of rats in each group were brown particles (Fig. 3A, B, C, D). Compared with the control group, the values of Bcl-2 and Bcl-2 /Bax in the myocardial tissue of the CHF group were significantly decreased ($P < 0.01$), and Bax was significantly increased ($P < 0.01$), Caspase-8 and Caspase-3 were significantly

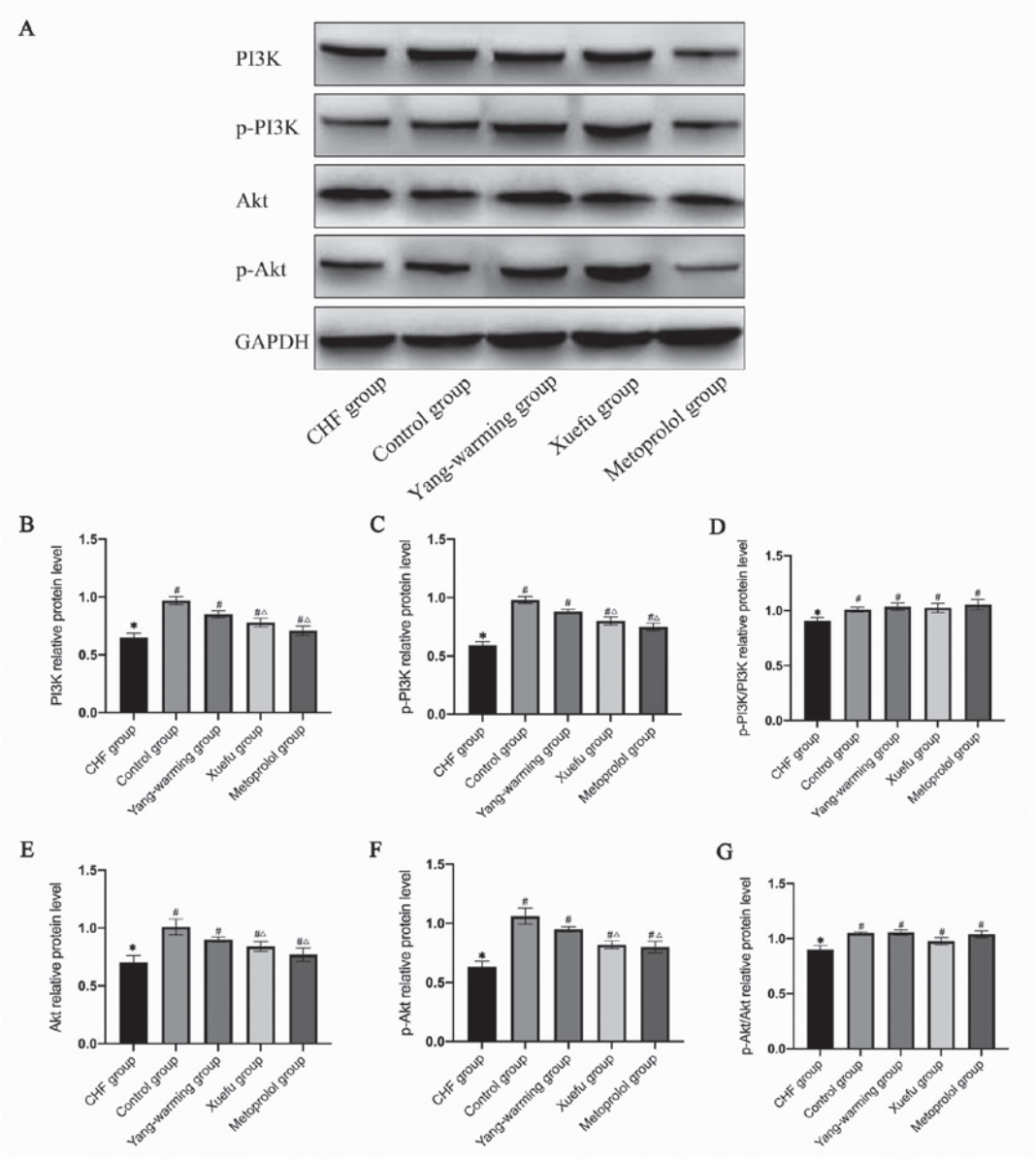

Fig. 2. Expression of p-PI3K and p-Akt

(A) Protein expression of the PI3K-Akt signaling pathway. (B, C, D, E, F, G) Gray band levels of the Phosphatidylinositol-3-kinase (PI3K) (B), p-PI3K (C), p-PI3K/PI3K (D), Protein kinase B (Akt) (E), p-Akt (F), and p-Akt/ Akt (G) were analyzed using Image J software. (*$p < 0.01$ compared with the Control group; #$p < 0.01$ compared with the CHF; △$p < 0.01$ compared with the Yang-warming group; n = 7 per group). Data are the means of at least three independent experiments with similar results.

increased ($P < 0.01$). Compared with the CHF group, Bcl-2 and Bcl-2/Bax values were significantly increased ($P < 0.01$) and Bax, Caspase-8 and, Caspase-3 values were significantly decreased ($P < 0.01$) in the yang-warming group, xuefu group and, metoprolol group (Fig. 3).

DISCUSSION

CHF model rats induced by ADR were used in the experiment. ADR has severe cardiotoxicity and can inhibit systolic myocardial function, leading to heart failure. It is an ideal CHF model in clinical practice

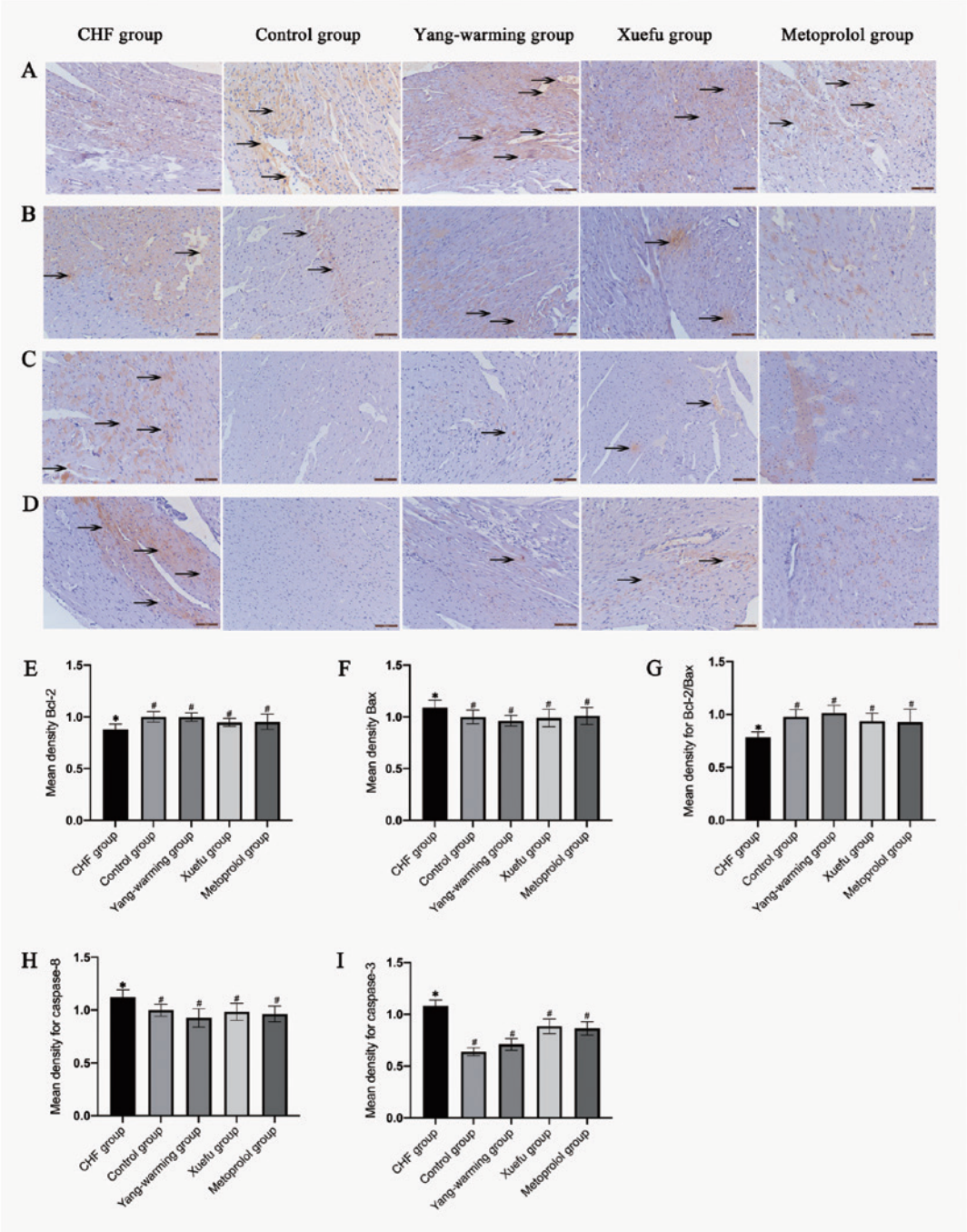

Fig. 3. Yang-warming treatment inhibits the expression of apoptotic factors.
(A) Immunohistochemical staining of Bcl-2. (B) Immunohistochemical staining of Bax. (C) Immunohistochemical staining of Caspases-8. (D) Immunohistochemical staining of Caspases-3. The mean density for Bcl-2, Bax, Caspases-8, and Caspases-3 were quantified. (E, F, G, H, I) (*p < 0.01 compared with the control group; #p < 0.01 compared with the CHF group; n = 7 per group). Black arrows indicate positive expression, respectively. Scale bar: 50 μm.

[16]. The results showed that after intraperitoneal injection of ADR, LVEDD and LVESD in the CHF model rats were significantly increased, while LVFS and LVEF levels were decreased, indicating that the systolic and diastolic functions of the heart of the rats in the CHF model group were impaired considerably and there was severe cardiac dysfunction. The results of H&E staining of the rats' myocardium were consistent with the pathological indexes of the heart failure rats [17], indicating that the model building was successful.

Other studies have demonstrated that the release of cTnI is associated with increased 1-year mortality and heart failure-related readmission. The cTnI level can act as an independent predictor and complete in CHF patients' prognostic utility [18]. The level of plasma BNP is a cost-effective method for evaluating the LV dysfunction. And some studies presented that BNP-guided therapies showed a decrease in death and hospital stay of CHF patients [19]. That means BNP is positively associated with all-cause mortality of CHF, and researchers also found that the standard of BNP was useful in estimating prognosis in stable CHF patients [20]. That is why in this research, the results of BNP and cTnI are consistent with these theories.

Studies have shown that extensive cardiomyocyte apoptosis exists in CHF, which plays a vital role in CHF's occurrence and development. The PI3K-Akt signaling pathway plays an essential role in regulating angiogenesis, cardiomyocyte apoptosis, and metabolism [3]. PI3K-Akt signaling pathway can directly or indirectly inhibit apoptosis factors and play a protective effect on the myocardium. As an upstream signaling molecule, PI3K cannot instantly activate downstream target molecule Akt. It can entrain Akt to the cell membrane and eventually activate Akt through phosphoinositide-dependent kinase, thereby initiating a series of signaling cascades. For example, Akt phosphorylation plays a crucial role in cell apoptosis, proliferation and transcription by regulating its downstream Bcl-2/Bcl-xl related death promoters, Bcl-2, Bax, Caspase3, and other target proteins to promote apoptosis [21]. Among them, pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 can regulate cell apoptosis by forming homologous dimer or heterologous dimer. When the proportion of homologous dimer or Bcl-2 protein is dominant, it will inhibit cell apoptosis. On the contrary, apoptosis will be induced, and its ratio determines whether the cells are stimulated to apoptosis or survival [22]. Both can also activate Caspase, the executor of apoptosis, to promote the completion of apoptosis, and caspase-8 and 3 is an essential molecule in the apoptotic death receptor pathway [23-24]. The results showed that when p-PI3K and p-Akt protein expressions were decreased in the model group, the ratio of anti-apoptotic proteins BCL-2 and BCL-2/Bax was also decreased, and the expressions of pro-apoptotic proteins Bax and caspase-8 were increased. After drug treatment in the three groups and p-PI3K and p-Akt protein expressions were significantly increased in the three groups; from the results, we could conclude the yang-warming group ameliorated the CHF state better than xuefu and metoprolol groups, apoptotic protein Bcl-2 and Bcl-2/Bax values were significantly increased, and apoptotic protein Bax, caspase-8 and caspase-3 values were significantly decreased.

It is suggested that Yang-warming and fluid retention-resolving decoction may better regulate the expression of the PI3K-Akt signaling pathway and affect the expression of related anti-apoptotic proteins and pro-apoptotic proteins by upregulation of PI3K-Akt signaling pathway to reduce apoptosis and improve cardiac function, thus playing a role in prevention and control of myocardial injury. In the next step, proteomics and metabolomics should be combined. The drug concentration gradient should be set up to explore the multi-target mechanism of Yang-warming and fluid retention-resolving decoction prevention and CHF treatment to provide a basis for CHF's clinical prevention and treatment with traditional Chinese medicine.

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Conflicts of interest

The authors report no conflicts of interest. The au-

thors alone are responsible for the content and writing of the paper.

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