

Original Article

Effects of Wenyangzhenshuai Granule on ERK1/2 and ERK5 activity in the myocardial tissue in a rabbit model of adriamycin-induced chronic heart failure

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Abstract: Objective: To elucidate the effects of Wenyangzhenshuai granule on expression of extracellular signal-regulated kinase 1/2 (ERK1/2) and 5 (ERK5) in the myocardial tissue using a rabbit model of adriamycin-induced chronic heart failure. Materials and methods: Rabbits were divided into heart failure positive control, adriamycin injection, and adriamycin injection with Wenyangzhenshuai treatment (low, medium and high dose) groups. Cardiac function and cardiac hypertrophy were measured in all groups. Besides, myocardial expression of ERK1/2 and ERK5 phosphorylation were evaluated by Western blotting and ERK1/2 and ERK5 mRNA levels by RT-PCR. The cardiac structure and cardiac function were also compared using histology staining and electron microscope. Results: Adriamycin injection led to cardiac failure reflected by decreased left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), E/A ratio, and increased cardiac hypertrophy, both of which have been improved by Wenyangzhenshuai granule treatment (all $P < 0.05$). Mechanistically, increased p-ERK1/2 and decreased p-ERK5 levels were observed in myocardial tissues of mice treated with Adriamycin for 8 weeks. However, such signaling change could be partially corrected by Wenyangzhenshuai treatment. In addition, no significant difference was detected in the expression of ERK1/2 and ERK5 mRNA levels between adriamycin injection groups and Wenyangzhenshuai treatment groups ($P > 0.05$), indicating an alteration in the activity/phosphorylation levels of these proteins instead of the transcription levels. Conclusion: we found a beneficial effect of Wenyangzhenshuai treatment in partially decelerating the progression of CHF. Such effect was probably through the role of Wenyangzhenshuai in diminishing p-ERK1/2 and raising p-ERK5 level in myocardial tissue.

Keywords: Chronic heart failure, Wenyangzhenshuai granule, adriamycin, ERK1/2, ERK5, myocardial tissue

Introduction

Chronic heart failure (CHF) is a group of syndromes due to the impaired ventricular filling and/or ejection function as a result of structural or functional cardiac diseases. Ventricular remodeling is one of its basic pathological changes in which several signal transduction abnormalities of myocardial cells are involved [1, 2]. Mitogen activated protein kinase (MAPK) cell signaling pathway is one of the most important signaling pathways in the pathogenesis of ventricular remodeling and CHF, with two major mediators extracellular signal-regulated kinase 1/2 (ERK1/2) and 5 (ERK5) signaling pathways having been reported [3]. MAPK is a group of protein kinase which distributes in cytoplasm and can transmit signals from the cytosol to the

nucleus after phosphorylation by its upstream primers. MAPK is also the common pathway of extracellular signal caused by nuclear reactions [4-6].

Wenyangzhenshuai granule is a complex of several traditional Chinese medicines (TCMs) which have been widely used in patients suffered from CHF in clinical practice in China. Wenyangzhenshuai granule is composed of ingredients of TCMs such as dried ginger, red ginseng and licorice root, etc. Earlier studies by others have shown that Wenyangzhenshuai granules play a vital role in improving cardiac function and inhibiting inflammatory [7]. However, the precise mechanisms need further investigation. In this study, we tested our hypothesis that Wenyangzhenshuai granules

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prevent the progression of CHF through regulating ERK1/2 and ERK5 signaling pathways in a rabbit model of CHF.

Materials and methods

Animals

42 normal, healthy male New Zealand rabbits, weighing 1.87 ± 0.12 kg, were provided by Laboratory Animal Center of Hunan University of Traditional Chinese Medicine. Rabbits were fed for a week under conditions of free diet, a light/dark cycle of 12 h/12 h (illumination time: 6:00-18:00), a background noise of (40 ± 10) db at $20 \pm 3^\circ\text{C}$.

Reagents

Adriamycin hydrochloride was purchased from Shenzhen Main Luck Pharmaceutical Inc. Wenyangzhenshuai granules were obtained from The First Affiliated Hospital of Hunan University of Traditional Chinese Medicine. Enalapril tablets was purchased from Shandong Cisen Pharmaceutical Ltd. Antibodies used for western blot including ERK1/2, p-ERK1/2, ERK5 and p-Erk5 were obtained from Santa Cruz Biotechnology, Inc., USA.

Grouping and CHF model

37 rabbits were administered with doxorubicin hydrochloride (Adriamycin) injection through marginal ear vein at a dose of 1.0 ml/kg each time, twice a week for a total of 4 weeks to induce chronic heart failure as previously reported [8]. At the end of the fourth week of doxorubicin hydrochloride injection, a total of 35 rabbits survived and then were randomized into groups as follows: (1) 4 weeks adriamycin injection group (sacrificed immediately after 4 weeks of Adriamycin injection), (2) 8 weeks adriamycin injection group who continued to receive another 4 weeks of adriamycin injection, (3) positive control group (oral administration of enalapril tablets with a dosage of 5 mg/kg/d) to induce chronic heart failure as reported previously [9], (4) Wenyangzhenshuai low dose group (0.4 g/kg/d orally) for 4 weeks, (5) Wenyangzhenshuai medium dose group (0.8 g/kg/d orally) for 4 weeks and (6) Wenyangzhenshuai high dose group (1.6 g/kg/d orally) for 4 weeks. All wenyangzhenshuai groups were treated with a total of 8 weeks of adriamycin injection. Another 5 rabbits did not

undergo Adriamycin injection, serving as a normal control group. Experimental scheme was shown in **Figure 1**.

Echocardiography assessment of heart function

Echocardiography assessment of heart function was performed in all rabbits. Animals were anesthetized with 25% urethane at a dose of 4 ml/kg. Ultrasound probe was placed in the left sternal border and the long axis of left ventricle, and the maximum diameter was obtained. Portable software automatically calculated left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), peak E and peak A ratio (E/A), left ventricular end diastolic diameter (LVDd), left ventricular end systolic diameter (LVDs), interventricular septal thickness (IVS) and left ventricular posterior wall thickness (LVPW), and the averages of a total of 5 heart-beat cycles were reported in this study.

Western blot of myocardial tissues

Rabbits in all groups were sacrificed and left ventricular myocardial tissues were quickly collected after a thorough cold PBS perfusion. Tissues were placed in liquid nitrogen for cryopreservation. The frozen myocardial tissues were then homogenized in tissue lysis buffer at a 1:8 tissue mass to lysis buffer volume ratio. After letting the lysis buffer with tissue stand on ice for 1 hour, the homogenate was centrifuged at 18,000 g for 30 min at 4°C , and the supernatant was collected. Protein concentration was determined by Bradford method, and the supernatant was then packaged and stored at -70°C for later experiments. Before the western blot, 50 μg of myocardial total proteins were added to 2 \times SDS sample buffer (0.1 mol/L Tris pH 6.8, 0.2 mol/L DTT, 4% SDS, 20% glycerol and 0.02% bromophenol blue), and then were denatured at 100°C for 8 min before run on Tris-glycin SDS gel. Proteins on the gel were transferred into a nitrocellulose membrane using standard protocol. Primary antibodies including anti-p-ERK1/2 and anti-p-ERK5 antibody were incubated with nitrocellulose membrane at 4°C overnight. The according HRP conjugate secondary antibodies were used and blots were developed with ECL reagents. Films were scanned and images were analyzed using ImageJ.

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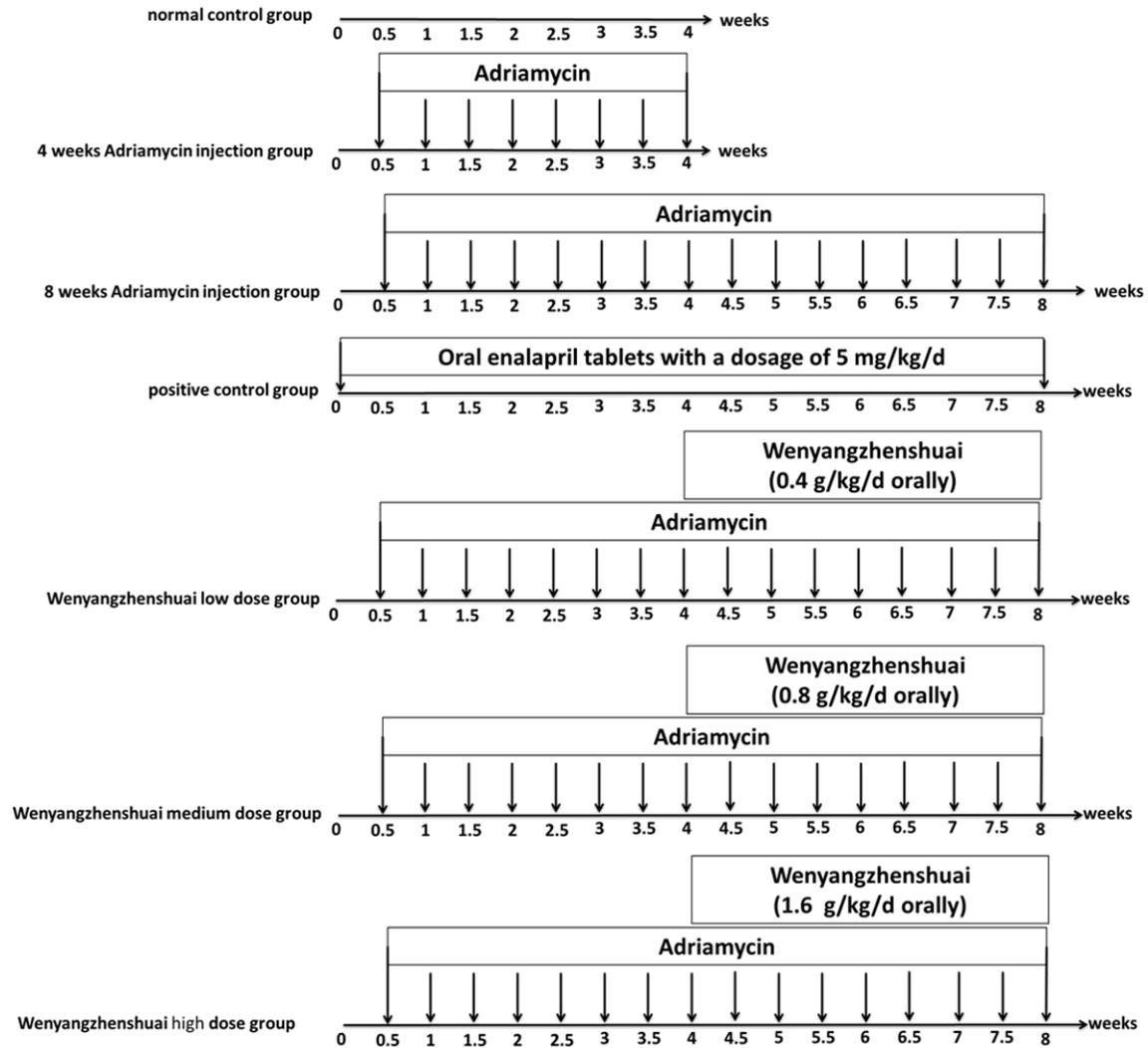


Figure 1. Experimental scheme.

RT-PCR detecting myocardial ERK1/2 and ERK5 mRNA levels

Total RNAs from myocardial tissues were extracted by using the Trizol one-step method. Standard method of transverse reverse reaction to synthesize cDNA and semi-quantify PCR were used in the analysis of gene expressions. The PCR products were run on an agarose gel, pictures were taken and images were analyzed by ImageJ. Primers for target genes are listed as follow: ERK1/2 (XM_008257945) forward primer 5'-cctggaagccatgagggatgtctac-3', reverse primer 5'-gcagatgtggtcgttcttagttgc-3'; ERK5 (XM_008250339) forward primer 5'-cccc-tcccccttctacatcagagtc-3', reverse primer 5'-gtcgaccacacccatgtcaaaagac-3'; GAPDH (NM_001082253) forward primer 5'-ttcaacagtgc-caccactcctcta-3', reverse primer 5'-ccctgttgcttagcctcaaatcgtt-3'.

H&E staining and transmission electron microscopy of pathological change of cardiac tissues

The myocardium of experimental rabbits was fixed in 4% paraformaldehyde and 2.5% glutaraldehyde respectively. Standard H&E staining were performed and pictures were taken using light microscope. On the other hand, transmission electron microscope was used to observe the detailed pathological change of myocardial tissues.

Statistical methods

All measurement data were expressed as mean \pm SD. All data were tested for normality and processed by the SPSS17.0 statistical analysis software package (SPSS, Chicago, IL, USA). The differences of the means among groups were

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Table 1. Comparison of cardiac function between experimental groups (mean \pm SD)

Group	N	LVEF (%)	LVFS (%)	E/A
Normal control group	5	73.52 \pm 7.34	45.61 \pm 4.29	1.38 \pm 0.19
4 weeks Adriamycin injection group	5	54.43 \pm 5.23 ^a	33.12 \pm 2.57 ^a	1.13 \pm 0.12 ^a
8 weeks Adriamycin injection group	5	40.20 \pm 4.32 ^{a,b}	22.53 \pm 2.60 ^{a,b}	0.61 \pm 0.05 ^{a,b}
Wenyang low dose group	6	46.49 \pm 4.02 ^{a,b}	26.49 \pm 2.23 ^{a,b}	0.74 \pm 0.06 ^{a,b}
Wenyang medium dose group	5	54.71 \pm 4.68 ^{a,c,d}	28.84 \pm 2.31 ^{a,b,d}	0.89 \pm 0.08 ^{a,b,c,d}
Wenyang high dose group	6	55.35 \pm 5.18 ^{a,c,d}	28.16 \pm 2.25 ^{a,b,d}	0.91 \pm 0.08 ^{a,b,c,d}
Positive control group	5	47.39 \pm 3.79 ^{a,b}	26.46 \pm 2.28 ^{a,b}	0.74 \pm 0.05 ^{a,b}

^aP<0.05 compared with normal control group; ^bP<0.05, compared with 4 weeks Adriamycin injection group; ^cP<0.05 compared with wenyang low dose group; compared with wenyang high dose group. ^dP<0.05 compared with positive control group. Abbreviation: LVEF: left ventricular ejection fraction; SD: standard deviation; LVFS: left ventricular fractional shortening.

Table 2. Comparison of cardiac structure between experimental groups (mean \pm SD)

Group	N	LVDd (mm)	LVDs (mm)	IVS (mm)	LVPW (mm)
Normal control group	5	11.39 \pm 1.12	8.12 \pm 1.06	2.12 \pm 0.24	2.10 \pm 0.18
4 weeks Adriamycin injection group	5	12.21 \pm 1.18	8.14 \pm 1.10	2.54 \pm 0.28 ^a	2.39 \pm 0.26 ^a
8 weeks Adriamycin injection group	5	13.83 \pm 1.14 ^a	9.49 \pm 1.08	2.96 \pm 0.26 ^{a,b}	2.87 \pm 0.27 ^{a,b}
Wenyang low dose group	6	12.37 \pm 1.15	8.89 \pm 1.09	2.75 \pm 0.25 ^{a,b}	2.69 \pm 0.25 ^a
Wenyang medium dose group	5	12.28 \pm 1.12	8.64 \pm 1.07	2.74 \pm 0.27 ^{a,b}	2.56 \pm 0.27 ^a
Wenyang high dose group	6	12.25 \pm 1.09	8.72 \pm 1.05	2.74 \pm 0.22 ^{a,b}	2.54 \pm 0.28 ^a
Positive control group	5	12.39 \pm 1.12	8.81 \pm 1.07	2.76 \pm 0.28 ^{a,b}	2.71 \pm 0.29 ^a

^aP<0.05, compared with normal control group; ^bP<0.05 compared with 4 weeks Adriamycin injection group; compared with wenyang low dose group. Abbreviation: LVDd: left ventricular end diastolic diameter; LVDs: left ventricular end systolic diameter; IVS: interventricular septal thickness; SD: standard deviation; LVPW: left ventricular posterior wall thickness.

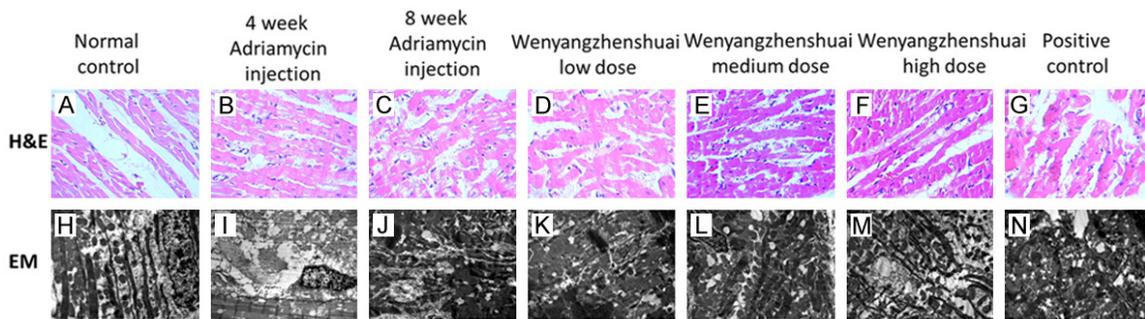


Figure 2. Pathological change of cardiac tissue in each group using H&E staining and transmission electron microscopy (EM).

analyzed by one-way analysis of variance. The least significant difference test (LSD) was used when equal variances were assumed and Tamhane's T2 test when they were not. The significance level was defined as a P<0.05.

Results

Survival rate of rabbits in each group

Among the total of 37 New Zealand rabbits, 2 rabbits died at 3 and 4 weeks after Adriamycin injection respectively. Before the end of the 8th

week of this study, one rabbit in the Adriamycin injection control group, one in enalapril positive control group and one in Wenyangzhenshuai medium dose group died. All animals in the normal control group survived. Experimental scheme was shown in **Figure 1**.

Wenyangzhenshuai granules prevent the cardiac dysfunction in rabbits with CHF

The cardiac function of each group was summarized in **Table 1**. LVEF, LVFS and E/A of both 4 weeks and 8 weeks Adriamycin injection

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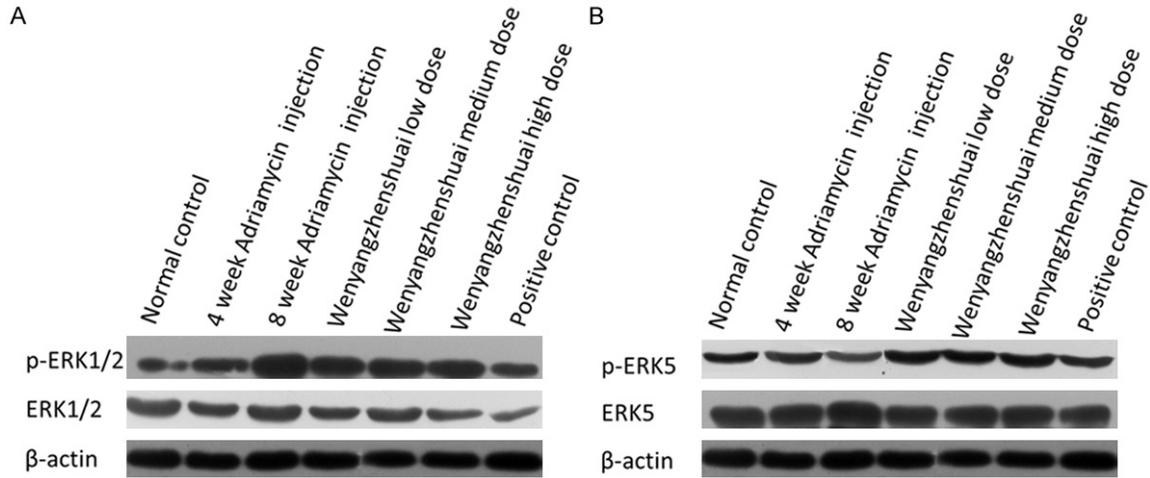


Figure 3. Myocardial ERK1/2 and ERK5 phosphorylation in rabbits of Adriamycin-induced chronic heart failure in each group.

Table 3. Comparison of myocardial ERK1/2 expressions between experimental groups (mean ± SD)

Group	N	P-ERK1/2/Actin (protein from western blot)	ERK1/2mRNA/GAPDH (mRNA from q-PCR)
Normal control group	5	0.46±0.07	1.19±0.17
4 weeks Adriamycin injection group	5	0.82±0.08 ^a	1.18±0.19
8 weeks Adriamycin injection group	5	1.29±0.09 ^a	1.21±0.19
Wenyang low dose group	6	1.16±0.07 ^{a,b,c,d}	1.15±0.18
Wenyang medium dose group	5	1.15±0.08 ^{a,b,c,d}	1.17±0.16
Wenyang high dose group	6	1.05±0.06 ^{a,b}	1.16±0.18
Positive control group	5	1.04±0.06 ^{a,b}	1.19±0.18

^aP<0.05 compared with normal control group; ^bP<0.05 compared with 8 weeks Adriamycin injection group; compared with wenyang medium dose group; ^cP<0.05 compared with positive control group. ^dP<0.05 compared with positive control group. Abbreviation: SD: standard deviation; ERK5: p44/p22 Mitogen-activated protein kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; SD: standard deviation; RNA Ribonucleic acid.

Table 4. Comparison of myocardial ERK5 expressions between experimental groups (mean ± SD)

Group	N	p-ERK5/Actin (protein from western blot)	ERK5mRNA/GAPDH (mRNA from q-PCR)
Normal control group	5	0.79±0.02	0.43±0.01
4 weeks Adriamycin injection group	5	0.67±0.02 ^a	0.44±0.01
8 weeks Adriamycin injection group	5	0.41±0.01 ^a	0.45±0.02
Wenyang low dose group	6	0.47±0.01 ^{a,b,c,d}	0.43±0.01
Wenyang medium dose group	5	0.48±0.01 ^{a,b,c,d}	0.43±0.01
Wenyang high dose group	6	0.56±0.02 ^{a,b}	0.45±0.02
Positive control group	5	0.55±0.02 ^{a,b}	0.45±0.02

^aP<0.05 compared with normal control group; ^bP<0.05 compared with 8 weeks Adriamycin injection group; compared with wenyang medium dose group; ^cP<0.05 compared with positive control group. ^dP<0.05 compared with positive control group. Abbreviation: SD: standard deviation; ERK5: p44/p22 Mitogen-activated protein kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

groups were significantly reduced compared with those of the normal control group (all P<0.05), with 8 weeks Adriamycin injection

having a further deteriorated cardiac function. These data suggested a successful modeling of CHF. Treating animals suffered from CHF

with Wenyangzhenshuai granules, including low, medium and high dosage, led to a better LVEF, LVFS and E/A compared with those of the animals in 4 week Adriamycin injection group (all $P < 0.05$), indicating a beneficial effect of Wenyangzhenshuai treatment in preventing cardiac dysfunction in this chronic heart failure model. Furthermore, such beneficial effect of Wenyangzhenshuai treatment had a dose-response-effect on preserving cardiac function reflected by the fact that LVEF and E/A of rabbits in Wenyangzhenshuai medium dose and high dose groups had a significantly higher LVEF and E/A compared with those of the rabbits in Wenyangzhenshuai low dose group (both $P < 0.05$).

Wenyangzhenshuai granule partially slows down the progression of cardiac hypertrophy in rabbits with CHF

Rabbits in 4 weeks Adriamycin injection group developed cardiac hypertrophy reflecting by an increased LVDd, LVDs, IVS and LVPW (all $P < 0.05$ compared with normal control groups). However, only LVPW and IVS had a further increase after 8 weeks of Adriamycin injection compared with 4 weeks of injection (both $P < 0.05$), rather than another two parameters including LVDd and LVDs (both $P < 0.05$) (**Table 2**). Although Wenyangzhenshuai treatment groups, including low, medium and high dose groups, had a trend towards a less degree of cardiac hypertrophy compared with 4 weeks Adriamycin injection group without treatment, beneficial effect of Wenyangzhenshuai treatment on cardiac hypertrophy did not reach a statistical significance in most of the cardiac hypertrophy parameters including LVDd, LVDs and LVPW (all $P > 0.05$ compared with 4 weeks Adriamycin injection group) (**Table 2**). However, we did find a significantly decreased IVS in Wenyangzhenshuai medium and high dose groups compared with 4 weeks Adriamycin injection group (both $P < 0.05$) (**Table 2**), suggesting a partially slowed down progression of cardiac hypertrophy after Wenyangzhenshuai treatment.

High-dose of Wenyangzhenshuai granule yields less pathological changes in cardiac tissues

Using H&E staining to show the general morphology of cardiac tissue, we found that myo-

cardial cell of control group had normal size and morphology, muscle fibers arranged in neat, the cytoplasm and nuclear stained uniform with no cell body edema and inflammatory cell infiltration (**Figure 2A**). The myocardial cells of 4 week Adriamycin injection group showed irregular shape, myocardial fibers disarrangement, muscle fiber fracture, myocardial cell edema and inflammatory cell infiltration (**Figure 2B**). The myocardial fibers of 8 weeks Adriamycin injection group had a further disarranged myocardial fiber, muscle fiber fracture, with severe myocardial cell edema and vacuolar degeneration, partially visible myocardial necrosis accompanied by a significant inflammatory cell infiltration (**Figure 2C**). Wenyangzhenshuai high-dose group showed a less degree of myocardial cells edema and necrosis, muscle fiber disarrangement compared with the Adriamycin injection group and other dose of Wenyangzhenshuai groups (**Figure 2D-F**).

By using transmission electron microscopy to further study the detailed cellular structures, we found that sarcomere of control group was clear, the number and structure of mitochondrial were normal with no inflammatory cell infiltration in the interstitial tissues (**Figure 2H**). The muscle fiber of model 4 weeks Adriamycin injection group showed necrosis or atrophy, mitochondrial edema and vacuolization, with unclear structure of myofilament and visible inflammatory cell infiltration (**Figure 2I**). Such phenotype deteriorated after another 4 week of Adriamycin injection (**Figure 2J**). The mitochondria and sarcoplasmic reticulum of 8 weeks Adriamycin injection group showed extensive edema and vacuolization, muscle fiber irregular contraction or necrosis accompanied by sarcomere fault (**Figure 2J**). Wenyangzhenshuai high-dose group showed a less degree of mitochondrial edema and vacuolization and muscle bundle became thinner and arranged disorderly compared with the 4 weeks and 8 weeks Adriamycin injection groups and other Wenyangzhenshuai treatment groups (**Figure 2K-M**).

Wenyangzhenshuai granule treatment down-regulates ERK1/2 phosphorylation and up-regulates ERK5 phosphorylation

Myocardial p-ERK1/2 levels in both Adriamycin injection groups at 4 weeks were significantly

higher than that of the normal control group with representative western blot shown in **Figure 3A** and statistical analysis shown in **Table 3** column ERK1/2/actin ($P < 0.05$). ERK1/2 phosphorylation level had a further increase at 8 weeks Adriamycin injection group compared with that of the 4 weeks Adriamycin injection group ($P < 0.05$), suggesting a possible role of ERK1/2 activity increase as cardiac dysfunction progressed. At the end of 8th week, the myocardial p-ERK1/2 level in all Wenyangzhenshuai treatment groups were significantly lower than that in 8 weeks Adriamycin injection groups (all $P < 0.05$), indicating a protective role of Wenyangzhenshuai treatment possibly through down-regulating ERK1/2 signaling pathway. Such effect existed in all Wenyangzhenshuai treatment groups with three different doses (all $P < 0.05$) (**Table 3** and **Figure 3A**).

In contrast, we found a significantly decreased phosphorylation level of ERK5 in cardiac tissues of 8 weeks Adriamycin injection groups compared with that of the normal control group, with representative western blot shown in **Figure 3B** and statistical analysis shown in **Table 4** column ERK5/actin (both $P < 0.05$), indicating a deactivated ERK5 signaling in the progression of CHF. Wenyangzhenshuai treatment partially corrected the decreased phosphorylation levels of ERK5 compared with that of the 8 week Adriamycin injection group and such effect also existed in all Wenyangzhenshuai treatment groups with three different doses (all $P < 0.05$) (**Table 4** and **Figure 3B**).

To further check the change of gene expression levels of ERK1/2 and ERK 5 in the myocardial tissue of rabbits in all groups, we performed the RT-PCR on cardiac tissues in each group (**Table 3** column ERK1/2 mRNA/GAPDH and **Table 4** column ERK5 mRNA/GAPDH, respectively). We did not find a significant change of ERK1/2 and ERK5 mRNA levels in either Adriamycin injection or Wenyangzhenshuai treatment group compared with normal control group. There was also no difference among all treatment groups indicating that the ERK1/2 and ERK5 activity regulation was at the protein activity levels, rather than gene expression levels in this rabbit model of chronic heart failure.

Discussion

CHF is a clinical syndrome with progressive cardiac remodeling and myocardial apoptosis as

the major pathological manifestations. Recent studies have shown that MAPKs signal transduction pathway abnormalities are important in the pathogenesis of CHF. In this study, we used a rabbit model of CHF to test our hypothesis that Wenyangzhenshuai, a broadly used traditional Chinese medicine, had a beneficial effect on protesting cardiac dysfunction through regulating MAPK signaling pathways.

First of all, we found that Adriamycin injection leads to cardiac hypertrophy and dysfunction in rabbits which was similar to the traditional enalapril feeding-induced CHF model, indicating a success of CHF rabbit model establishment. Treating CHF rabbits with Wenyangzhenshuai granules at different dosages lead to a partial decelerated cardiac dysfunction and cardiac hypertrophy compared with CHF animals without Wenyangzhenshuai treatment, suggesting a protecting role of this traditional Chinese medicine in the progression of CHF.

Wenyangzhenshuai granule is well known for its ability to warm yang and reinforce qi as well as induce diuresis for removing edema in traditional Chinese medicine theory. The main ingredients are dried ginger, red ginseng and liquorice. Dried ginger warms the spleen and stomach for dispelling cold, and restores yang to resolve stagnation. As it is stated in *Bencao Qizhen*, dried ginger has great heat but no toxicity. It has lasting, local effects but no extensive activities in the whole body. It brings the immediate effect of restoring yang for patients with weakness and coldness in the stomach. Red ginseng is prepared by steaming and drying the ginseng. With its mild nature and sweet flavor, the red ginseng reinforces the vital energy and invigorates the spleen to benefit the lung. Liquorice also has a sweet flavor and mild nature, with the effect of invigorating the spleen and replenishing qi.

A recent study showed that Wenyangzhenshuai granules could be used effectively in treating chronic heart failure, because it significantly improved heart function, reduced the serum levels of tumor necrosis factor- α and endothelin-1, and up-regulated the level of interleukin-10 [10]. Wenyangzhenshuai granules can also reduce the plasma levels of angiotensin-II and atrial natriuretic peptide [11]. In the aspect of myocardial apoptosis, Wenyangzhenshuai granules could reduce the density ratio of myocardial apoptosis and had a protective effect

on myocardial cells [12]. In the current study, we have further discovered that Wenyangzhenshuai granules decreased the phosphorylation of ERK1/2 and increased the phosphorylation of ERK5 in the myocardium of a rabbit suffered from chronic heart failure. Activation of ERK1/2 is a typical characteristic in the development and the progression of chronic heart failure ERK includes ERK1 and ERK2, which are activated primarily by following two pathways: (1) Activation of receptor tyrosine kinase/Ras pathway (2) activation of the ERK pathway by G protein coupled receptor. Once activated, it can phosphorylate different target proteins including cytoplasmic proteins, membrane proteins and a variety of transcription factors [13]. ERK1/2 could initiate the transcription and translation of primary and secondary response genes, such as proto-oncogene like c-fos, c-jun and c-myc, and also mediate the effect of external stimuli on cell proliferation [14]. One study has shown that the role of ERK1/2 on cardiac hypertrophy was precisely regulated at the phosphorylation level of the protein, rather than being regulated on the ERK mRNA levels and ERK1/2 phosphorylation degree is positively correlated with the degree of left ventricular hypertrophy [15]. Further, blocking ERK1/2 phosphorylation may be beneficial to delay or even block the development of left ventricular hypertrophy. In *in vitro* experiments, ERK1/2 agonist can significantly enhance myocardial cell hypertrophy and hyperplasia, which shares the similar phenotype when treating myocardial cells with different hypertrophic-related stimuli [16]. Similar studies also suggested that change in the activity of ERK1/2 is closely related to the degree of CHF, and ERK1/2 activity in the left ventricular myocardia of cardiomyopathy patients with compensatory myocardial hypertrophy are not changed significantly [17]. All these data suggested an important role of ERK1/2 activity in CHF. The finding of current study added another evidence of ERK1/2 activity in CHF and mechanistically provided a potential pathway through which a traditional Chinese medicine could have played a role in preventing CHF progression.

As part of the non-typical MAPK family pathway, ERK5 is localized in the cytoplasm, where it receives extracellular signals. It is activated by an upstream kinase and transposed into the

nucleus, where it exhibits transcriptional regulatory activity by phosphorylating the myocyte enhancer factor-2 (MEF2). Like the typical MAPK pathways, the signals are transmitted via the extracellular stimulation of the MAPKKK-MAPKK (MAPKK is the only specific upstream kinase for ERK5)-ERK5 activation. Early research showed that cytokines such as epidermal growth factor and nerve growth factor may induce the activation of ERK5, which in turn promotes the phosphorylation of the cAMP response element-binding protein (CREB), a member of the MEF2 family. These transcriptional factors are closely related to cell survival. ERK5 enters the nucleus and regulates the expression of other transcriptional factors. For example, c-Jun participates in the regulation of the cell cycle, while c-Fos mediates the anti-apoptotic effect of ERK5. ERK5 was shown to be associated with blood vessel development and proliferation [18]. Since Zhou et al firstly cloned ERK5 in 1995, a series of functions originally believed to be performed by ERK1/2 were finally attributed to ERK5 [19]. It is now believed that ERK5 plays important roles in sustaining blood vessel integrity and cell growth under oxidative stress, hypoxia, stimulation of reactive oxygen species, and a variety of mitogens. Regan et al indicated that ERK5-deficient mice experienced delayed development of several extraembryonic blood vessels and the cardiovascular system during the embryonic phase [20]. Hayashi et al [21] also found that ERK5-knockout adult mice died within 2 to 4 weeks. Therefore, ERK5 fulfills important functions during cardiovascular development. Our finding unraveled a novel role of ERK5 activity in the pathogenesis of CHF development.

Last but not least, although we found a changed activity of ERK1/2 and ERK5 (phosphorylation) in our rabbit model treated with Wenyangzhenshuai granule, no difference as observed on the mRNA levels of ERK1/2 and ERK5, This strongly suggested that among all treatment groups indicating that the ERK1/2 and ERK5 activity regulation was at the protein activity levels, rather than gene expression levels in this rabbit model of chronic heart failure.

Conclusion

In this rabbit model of CHF, we found a beneficial effect of Wenyangzhenshuai treatment in

partially decelerating the progression of CHF. Such effect was probably through the role of Wenyangzhenchuan in diminishing p-ERK1/2 and raising p-ERK5 level in myocardial tissue. Blocking the activation of ERK1/2 and ERK5 signal transduction pathway and illustrating its role in decelerating the progression of cardiac remodeling need to be further investigated.

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Disclosure of conflict of interest

None.

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