

Impacts of Yangxin decoction on the expressions of MMP-9, CaN, NFAT3 and GATA4 in myocardial tissue of rats with chronic heart failure

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Abstract: To investigate the impacts of Yangxin decoction on the expressions of matrix metalloproteinase 9 (MMP-9), calcineurin (CaN), T cell activated nuclear factor 3 (NFAT3) and zinc finger transcription factor 4 (GATA4) in myocardial tissue of rats with chronic heart failure (CHF). 50 healthy SD rats were randomly divided into the normal control group ($n = 10$) and the operation group ($n = 40$). After successful modeling, the rats were randomly divided into 4 groups. And they were treated with Yangxin decoctions of low concentration (1.5 g/kg), medium concentration (2.5 g/kg), high concentration (3.5 g/kg) and distilled water (for 4 weeks). The LVSP, SAP, DAP and LVEDP in Yangxin decoction treatment groups were significantly superior to the model group. The LVEF, LVIDd and LVIDs in Yangxin decoction treatment groups were significantly superior to the model group. The activity of CaN in each group treated with Yangxin decoction was significantly lower than that in the model group. The expression levels of MMP-9, NFAT3, GATA4 protein in each group treated with Yangxin decoction were significantly lower than that in the model group. Yangxin decoction can significantly improve the cardiac function, reduce CaN activity, decrease the expression levels of MMP-9, NFAT3 and GATA4, inhibit CaN/NFAT3 signaling pathway, increase myocardial remodeling and protect myocardial tissue in rats with CHF.

Keywords: Yangxin decoction, chronic heart failure, MMP-9, CaN, NFAT3, GATA4.

INTRODUCTION

Chronic heart failure (CHF) is the advanced stage of cardiovascular disease, with the myocardial structure reconstructed and the myocardial function weakened. In recent years, the incidence of CHF has gradually increased. At present, the poor prognosis of western medicine treatment is the main cause of death in patients with cardiovascular disease, which seriously impacts the life and health of patients (Skrzypek *et al.*, 2018). CHF belongs to the category of “palpitation”, “asthma syndrome”, “heart arthralgia” and “edema” in traditional Chinese medicine, and its inducement is related to factors such as physical weakness, chronic illness, invasion of exogenous pathogens, improper diet and labor, injury caused by seven emotions and imbalance of viscera (Bihua *et al.*, 2018). Chronic failure mostly occurs from the heart arteries, which is related to the lack of blood in spleen, deficiency of kidney yin, deficiency of lung qi and dysfunction of stagnation of liver qi. It is originated in the asthenia, so there is asthenia in origin and asthenia in superficiality. The asthenia transforms in origin and superficiality. The deficiency refers to the deficiency of heart-qi yang or heart-blood yin, the losses of yin and yang, and mind and spirit. Superficiality refers to blood stasis, pulse block, phlegm, stopping drinking water and edema. CHF is mainly divided into qi deficiency and blood stasis syndrome, qi and yin deficiency and yang

deficiency syndrome, etc. Meanwhile, the treatment is mainly to invigorate qi and promote blood circulation and warm yang and nourish yin (Juan *et al.*, 2019). The main efficacy of Yangxin decoction is to benefit the temperature and yang, nourish yin and promote blood circulation. It has been reported that it has achieved good results in the treatment of cardiovascular diseases such as coronary heart disease, angina pectoris and heart failure, but its mechanism is less researched (Yongping and Ruyi, 2018). In this research, the mechanism of CaN/NFAT3 signaling pathway of Yangxin decoction in rats with CHF was researched. It is reported as follows.

MATERIALS AND METHODS

Main reagents and drugs

There were matrix metalloproteinase-9 (MMP-9) antibody, T cell activated nuclear factor 3 (NFAT3) antibody, zinc finger transcription factor 4 (GATA4) antibody, β -actin (American Cell Signaling Technology Co., Ltd.), calcineurin (CaN) activity detection kit (Beijing Biolab Technology Co., Ltd.), goat anti-rabbit IgG (H&L) unconjugated (Wuhan Boster Biological Technology Co., Ltd.), western blot reagent (Beijing Solarbio Technology Co., Ltd.), Yangxin decoction (formula: Korean ginseng 12g, corni fructus 12g, trichosanthes kirilowii 12g, cooked aconite tablets 6g, allium macrostemon 6g, safflower 6g, ophiopogon japonicus 18g, angelica sinensis 18g, pinellia ternata 9g, coptis chinensis 3g, Chinese rhubarb 9g), chloral hydrate and penicillin.

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Main instruments

There were Vevo®2100 small animal high resolution ultrasound imaging system (Fuji VisualSonics Co., Ltd., Canada), R415 small animal ventilator (Shenzhen RWD Life Science and Technology Co., Ltd.), PowerLab multi-channel physiological recorder (Australian AD Instrument Co., Ltd.), LS-20ECG animal ECG recording analyzer (American iWorx Co., Ltd.), electrophoretic instrument (American BIO-RAD Co., Ltd.), Bole ChemiDoc XRS + gel imaging system (American BIO-RAD Co., Ltd.).

Groups and treatment of experimental animals

50 clean SD rats, weighing 200 - 230 g, 50% male and 50% female, were provided by the animal experimental center of our university. 40 SD rats were randomly selected to establish the CHF model by coronary artery ligation (Shan *et al.*, 2018). The specific methods were as follows. 0.3ml/100g of 10% chloral hydrate was injected intraperitoneally for anesthesia and fixed on the operating table to connect the small animal ventilator. The pericardium was opened to make the heart by incision at the third rib of the left anterior chest was exposed. The anterior descending branch of the left coronary artery was ligated from the lower edge of the left atrial appendage to the pulmonary cone. Wound disinfection was sutured, it was shown by ECG that the ST segment dropped by more than 50% or the towering T segment recovered as a successful operation. 20IU penicillin was injected intramuscularly for 3 days to prevent infection. After normal feeding for one week, it was adjusted to feed with high fat. The sleep time of rats was reduced, and after 4 weeks, it was shown by the test the cardiac function that the heart index $\leq 180 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ (Chaplin, 2019), and the diagnostic criteria of qi deficiency and blood stasis syndrome of traditional Chinese medicine (2014) met as the model was successful. 30 SD rats were randomly divided into three groups, which were given Yangxin decoction with different concentrations. The low concentration group was given Yangxin decoction of 1.5g/kg, the medium concentration group was given Yangxin decoction of 2.5g/kg, the high concentration group was given Yangxin decoction of 3.5g/kg, and the model group and the blank control group were given distilled water with the same dose once a day for 4 weeks.

Detection indexes and methods

Cardiac function detection

The left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), arterial systolic pressure (SAP) and arterial diastolic pressure (DAP) were detected by multi-channel physiological recorder through noninvasive impedance method.

Ultrasonic detection of heart

The left ventricular ejection fraction (LVEF), left ventricular end diastolic diameter (LVIDd), left

ventricular end systolic diameter (LVIDs) and other indexes of rats in vivo were detected for real time by using the small animal high resolution ultrasound imaging system.

CaN activity detection

A proper amount of rat myocardial tissue was taken, and the CaN activity detection kit was used to detect the CaN activity in the tissue. The operation process was strictly in accordance with the kit instructions.

The expressions of MMP-9, NFAT3 and GATA4 detected by Western Blot

(1) Protein extraction and quantification: A proper amount of rat heart tissue was taken, liquid nitrogen was added, it was fully grinded and transferred to a centrifuge tube. And a proper amount of RIPA lysate and PMSF was added, and it was made stand at room temperature for 30 min to fully lyse. The supernatant was collected by centrifugation at 8000 g/min for 15 min, and the protein concentration was detected by BCA protein quantitative method. (2) SDS-PAGE electrophoresis: Separation gel and concentration gel were prepared, protein Marker and sample were added into gel hole respectively, and electrophoresis was performed at 80 V for 50 min, and then 120 V electrophoresis for 2 h was switched when protein marker reaches separation gel. (3) Mold transfer: After electrophoresis, the separation gel was taken out, it was put into the membrane transfer system with PVDF membrane, and wet transfer at low temperature of 100 V for 60 min. (4) Membrane washing: After the mold transfer, the PVDF membrane was taken out, it was washed with TBST for 3 times, 5min each time, and it was put into the antibody incubation box after the membrane washing. (5) Incubation of primary antibody: The prepared diluent of primary antibody was added, it was put into a horizontal shaker at 4°C overnight, TBST clean was performed for 3 times after incubation, and it was put into an incubation box. (6) Incubation of secondary antibody: The prepared secondary antibody diluent was added, it was put into a horizontal shaker at 4°C overnight, and TBST clean was performed for 3 times after incubation. (7) Color development: ECL chemiluminescence method was adopted, and the luminescent liquid was fully contacted with PVDF membrane and then placed in a cassette. (8) Development and imaging: In a dark room, developer was added for exposure for 5min, then fixer for fixation was added for 5 - 10 min, and the gray value on PVDF film was calculated by using BIO-RAD gel imaging system. The ratio of gray value / β -actin in the blank control group was taken as 1, and the values in each sample were calculated for statistics.

Ethical approval

Ethical approval was taken from Kunming Medical University, Kunming, China with reference No.KMU1H2018467.

STATISTICAL ANALYSIS

All the data obtained in this research were analyzed by SPSS22.0. The expression form of measurement data and independent sample *t* test were conducted. Univariate analysis of variance was used for comparison among groups, and LSD-*t* method was used for pairwise comparison among groups. The difference was statistically significant was recorded as $P < 0.05$.

RESULTS

Comparison of LVSP, LVEDP, SAP, DAP and other cardiac function indexes of rats in each group

Compared with the normal control group, LVSP, SAP, DAP in the model group were significantly lower, while LVEDP was significantly higher ($P < 0.05$). Compared with the model group, LVSP, SAP, DAP in Yangxin decoction treatment groups were significantly higher, while LVEDP was significantly lower ($P < 0.05$). Compared with the low concentration group, LVSP, SAP and DAP in the medium concentration group and high concentration group were significantly higher, while LVEDP was significantly lower ($P < 0.05$). There were no statistically significant differences in LVSP, LVEDP, SAP and DAP between the medium concentration group and high concentration group ($P > 0.05$). Table 1.

Comparison of echocardiographic indexes such as LVEF, LVIDd and LVIDs in each group

Compared with the normal control group, LVEF of the model group was significantly lower, LVIDd and LVIDs were significantly higher ($P < 0.05$). Compared with the model group, LVEF was significantly higher, LVIDd and LVIDs were significantly lower in each group treated with Yangxin decoction ($P < 0.05$). Compared with the low concentration group, the medium concentration group and high concentration group were significantly higher, and LVIDd and LVIDs were significantly lower ($P < 0.05$). There were no statistically significant differences in LVEF, LVIDd and LVIDs between the medium concentration group and high concentration group ($P > 0.05$). Table 2.

Comparison of CaN activity and MMP-9, NFAT3, GATA4 protein expressions in myocardium of rats in each group

Compared with the normal control group, CaN activity in the model group was significantly higher ($P < 0.05$). Compared with the model group, the activity of CaN in each group treated with Yangxin decoction was significantly lower ($P < 0.05$). Compared with the low concentration group, the CaN activity in the medium concentration group and high concentration group was significantly lower ($P < 0.05$), while there was no statistically significant difference between the medium concentration group and high concentration group ($P > 0.05$). The expression levels of MMP-9, NFAT3 and

GATA4 in the model group were significantly higher than those in the normal control group ($P < 0.05$), while the expression levels of MMP-9, NFAT3 and GATA4 in Yangxin decoction treatment groups were significantly lower than those in the model group ($P < 0.05$). The expression levels of MMP-9, NFAT3 and GATA4 in the medium concentration group and high concentration group were significantly lower than those in the low concentration group ($P < 0.05$), while there was no statistically significant difference between the medium concentration group and high concentration group ($P > 0.05$). Table 3.

DISCUSSION

Cardiovascular disease has always been a difficult disease in modern medicine, with rising incidence, poor therapeutic efficacy and prognosis, and high mortality. CHF is a common type of cardiovascular disease, and myocardial remodeling is considered as the key process of CHF (Jiabin *et al.*, 2019). However, Chinese medicine believes that CHF belongs to the syndrome of deficiency of essence, excess of superficiality and deficiency of heart-qi, which is explained in ancient books such as *Plain Questions*, *Treatise on Febrile Diseases*, *Synopsis of Golden Chamber*, *Recipes for Saving Lives* and *Medical Biography*. The disease is mainly in the heart, followed by liver, spleen, lung and kidney, showing “deficiency”, “stasis” and “stagnation”. The deficiency of Qi is the main factor for the development of heart failure, and blood stasis is the main factor of heart failure. Both of them jointly determine the development and evolution of heart failure (Lingjuan *et al.*, 2018; Ting *et al.*, 2018). In treating CHF, TCM advocates treating both the symptoms and root causes, warming yang and nourishing yin, promoting blood circulation and benefiting qi, and assisting with resolving phlegm and promoting diuresis.

In Yangxin decoction, Korean white ginseng belongs to lung, spleen and heart meridians, which can tonify spleen and lung, invigorate vitality, revive pulse, promote salivation and calm nerves. *ShenNong's Herbal*: “The main supplement is the five internal organs, the spirit is safe, the shock is stopped, and the evil spirits are removed.” *Cornus officinalis* belongs to the liver and kidney meridians, which astringent essence, tonify liver and kidney. *Pharmaceutical Theory*: “To treat brain and bone pains, stabilize the menstruation, and invigorate the kidney; And to stimulate the penis and nourish the essence. “*Trichosanthes kirilowii* belongs to the lung, stomach and bowel meridians, clearing away heat and phlegm, widening chest and resolving stagnation, moistening dryness and smoothing intestines. *Description of Materia Medica*: “*Trichosanthes kirilowii* is solid, thick and fat, so the phlegm of heat and dryness is the agent to be treated.” Cooked aconite tablets belong to heart, kidney and spleen meridians, which can restore yang to

Table 1: Comparison of cardiac function indexes of rats in each group

Groups	Cases	LVSP (mmHg)	LVEDP (mmHg)	SAP (mmHg)	DAP (mmHg)
The low concentration group	10	111.99±6.5 ^{ab}	8.67±2.15 ^{ab}	120.47±9.61 ^{ab}	95.55±6.66 ^{ab}
The medium concentration group	10	126.7±7.5 ^{abc}	6.67±1.49 ^{abc}	152.36±11.43 ^{abc}	103.43±7.45 ^{abc}
The high concentration group	10	128.6±7.3 ^{abc}	6.37±2.44 ^{abc}	156.54±11.87 ^{abc}	104.53±7.89 ^{abc}
The model group	9	92.7±5.6 ^a	12.11±3.94 ^a	98.52±8.15 ^a	86.54±5.62 ^a
The normal control group	10	145.8±8.5	4.58±1.77	169.63±13.55	115.68±8.52
<i>F</i> value		46.077	6.949	48.141	9.741
<i>P</i> value		<0.001	<0.001	<0.001	<0.001

Table 2: Comparison of cardiac ultrasound indexes of rats in each group

Groups	Cases	LVEF (%)	LVIDd (mm)	LVIDs (mm)
The low concentration group	10	54.03±5.14 ^{ab}	5.66±0.69 ^{ab}	3.15±0.46 ^{ab}
The medium concentration group	10	61.76±5.49 ^{abc}	4.63±0.63 ^{abc}	2.55±0.33 ^{abc}
The high concentration group	10	62.66±5.56 ^{abc}	4.57±0.64 ^{abc}	2.52±0.32 ^{abc}
The model group	9	40.73±4.66 ^a	6.07±0.75 ^a	3.78±0.58 ^a
The normal control group	10	68.87±5.97	4.26±0.64	1.96±0.35
<i>F</i> value		28.317	8.552	13.101
<i>P</i> value		0	0	0

Table 3: Comparison of CaN activity and MMP-9, NFAT3, GATA4 protein expressions in myocardial tissue of rats in each group

Groups	Cases	CaN (U/mg)	MMP-9	NFAT3	GATA4
The low concentration group	10	9.03±0.91 ^{ab}	2.31±0.38 ^{ab}	2.15±0.16 ^{ab}	2.57±0.23 ^{ab}
The medium concentration group	10	7.04±0.72 ^{abc}	1.56±0.26 ^{abc}	1.54±0.11 ^{abc}	1.68±0.14 ^{abc}
The high concentration group	10	6.97±0.76 ^{abc}	1.51±0.23 ^{abc}	1.52±0.10 ^{abc}	1.65±0.12 ^{abc}
The model group	9	11.12±1.07 ^a	3.24±0.43 ^a	2.63±0.22 ^a	3.35±0.31 ^a
The normal control group	10	5.86±0.59	1.00±0.00	1.00±0.00	1.00±0.00
<i>F</i> value		36.995	40.716	84.215	102.559
<i>P</i> value		0.000	0.000	0.000	0.000

Note: A was compared with the normal control group, *P*<0.05; B was compared with the model group, *P*<0.05; C was compared with the low concentration group, *P*<0.05

save adverse effects, dispel cold and relieve pain, and tonify hot and nourish yang. *Secret Essentials for Attending Diseases*: “To remove the internal cold. It can help increase yang qi and remove deficiency to warm spleen and stomach”.

Allium macrostemon Bunge belongs to lung, stomach and large intestine meridians, which dredging yang and dissipating mass, promoting qi and resolving stagnation. *Notes on Materia Medica*: “To benefit the orifices and treating asthma in lung.” Safflower belongs to the heart and liver meridians. It can activate blood circulation and dredge meridians, disperse blood stasis and relieve pain.

Compendium of Materia Medica: “To promote blood circulation, moisten dryness, relieve pain and disperse swelling, and dredge meridians.” Radix ophiopogonis belongs to lung, stomach and heart meridians and nourish yin and moisten lung, clear heart hot and remove vexation,

benefit stomach and promote fluid production. *Theory of Drug Properties*: “To treat heat toxin and stop polydipsia, and the limbs of the main wet face edema. It can cure flaccidity and pus discharge of the lung and govern the drainage of essence. “*Angelica sinensis* belongs to liver, heart and spleen meridians, regulates menstruation, relieves pain, invigorates blood circulation, moistens intestines and defecation. *Bie Lu*: “To warm the internal body can relieve pain, remove congestion in the blood, apoplexy, spasm, inability to sweat, damp impediment, mild aversion to the internal body and deficiency cold, which can supplement the five zang and generate muscle. “*Pinellia tuber* belongs to the meridians of spleen, stomach and lung, and has the efficacies of drying dampness, eliminating phlegm, removing mass and resolving hard lump, lowering adverse effects and stopping vomiting. *ShenNong's Herbal*: “It mainly cures cold and heat caused by typhoid fever, as well as the hardness in the lower heart, the lower qi, sore throat,

dizzy head and chest distension, cough reversal, borborygmi and antiperspiration.” The whole prescription altogether plays the role of benefiting yang, nourishing yin and activating blood. Relevant researches have proved that (Honglei *et al.*, 2019; Xiaoya *et al.*, 2019; Yingfei *et al.*, 2020) Yangxin decoction, an effective component of Chinese herbal medicines, has the efficacies of anti-inflammation, bacteriostasis, treatment of cardiovascular diseases, improvement of arrhythmia, anti-platelet aggregation, lowering blood glucose, reducing blood lipid, anti-thrombosis, anti-neurasthenia, and improving immune function.

MMP-9 is a kind of enzyme that can degrade collagen in the main components of extracellular matrix and basement membrane. Some scholars have found that MMP-9 is activated in the process of CHF, which destroys the extracellular matrix and basement membrane structure of myocardium, thus causing myocardial remodeling (Li *et al.*, 2018). CaN is a protein phosphatase which depends on calcium ion. It is a heterodimer composed of two subunits. It can directly or indirectly regulate multiple signaling pathways through calcium ion. Under normal circumstances, CaN is in an inactivated state. When the concentration of calcium ion is high, calcium ion and its subunits form a complex to change the structure of CaN, and it is activated to regulate a series of downstream signaling pathways (Schulz and Yutzey, 2004). NFAT is secreted by T-cell nuclear factor. In cardiac tissue, NFAT can interact with transcription factors such as GATA4 in myocardial cell nucleus, activate the expression of BNP, ANF and other downstream genes, and make myocardial cells have pathological features such as myocardial hypertrophy and decreased myocardial contractility, which leads to myocardial remodeling and may result in heart failure in the continuous process. Its secretion is regulated by CaN (Qi *et al.*, 2018). The results of this research indicated that the decline of cardiac function and abnormal cardiac ultrasound indexes in the CHF model group showed that myocardial remodeling occurred in rat myocardium during the process of CHF. After a period of treatment with Yangxin decoction, the cardiac function and new ultrasound indexes recovered obviously. Increasing the concentration of Yangxin decoction is helpful to accelerate the recovery of cardiac function and improve myocardial remodeling. Simultaneously, the expression of MMP-9 was increased in the CHF model, but was decreased after Yangxin decoction treatment, suggesting that Yangxin decoction can inhibit the expression of MMP-9 and protect myocardial structure. The activity of CaN in myocardial tissue of rats with CHF was significantly enhanced, and the expressions of NFAT3 and GATA4 in its downstream were significantly up-regulated. After treatment with Yangxin decoction, the activity of CaN and the expressions of NFAT3 and GATA4 in its downstream were significantly down-regulated,

suggesting that Yangxin decoction can inhibit CaN activity, thereby inhibiting CaN/NFAT3 signaling pathway and improving myocardial remodeling.

CONCLUSION

Yangxin decoction can down-regulate MMP-9 expression, inhibit CaN activity, inhibit CaN/NFAT3 signaling pathway, protect myocardial cells, restore cardiac function and improve myocardial remodeling. Meanwhile, Yangxin decoction with higher concentration has better efficacy.

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