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Original Research

Effects of Xinjierkang on Nrf2/HO-1 expression in viral myocarditis mice models

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Abstract: In this study, the changes of Nrf2/HO-1 and cytokines TNF-α, IL-6, IL-17 and IL-1β in cardiac muscle cells of Viral myocarditis (VMC) mice were detected in order to clarify the mechanism of action of Xinjierkang (XJEK). One hundred and fifty healthy male BALBC mice were randomly divided into the normal group, model group, low-, medium- and high-dose XJEK groups, with 30 in each group. Replication of the VMC model in mice inoculated with $CVB_{3m.}$ Serum inflammatory factors TNF-α, IL-6, IL-17 and IL-1β, Nrf2 and HO-1 protein levels in myocardial tissue were compared. The results showed that no apoptotic cells were found in the myocardium of normal mice. The percentage of cardiomyocyte apoptosis in the low, medium and high dose groups of XJEK was significantly lower than the model group ($P < 0.05$). At 3, 7, 14, 21, and 28 days after inoculation, compared with the normal group, the TNF-α, IL-6, IL-17 and IL-1β levels in the model group significantly increased ($p < 0.05$). After the administration of XJEK, compared with the model group, the TNF-α, IL-6, IL-17, and IL-1β levels in the low-, middle-, and high-dose XJEK groups significantly decreased ($p < 0.05$). At 28 days after inoculation, compared with the normal group, the expressions of Nrf2 and HO-1 proteins in the myocardial tissue of the model group were significantly down-regulated ($p < 0.05$); and compared with the model group, the expressions of Nrf2 and HO-1 proteins in the low-, medium-, and high-dose XJEK groups were significantly up-regulated ($p < 0.05$) in a concentration-dependent manner. In conclusion, XJEK can prevent myocardial injury in VMC mice, and its mechanism of action may be related to improving myocardial cell apoptosis, inhibiting inflammatory response, and up-regulating the expression of Nrf2 and HO-1 proteins in myocardial tissue.

Key words: Xinjierkang (XJEK); Viral myocarditis; Inflammatory response; Nrf2, HO-1.

Introduction

Inflammation of the heart muscle (myocardium) is called myocarditis. Inflammation of the heart muscle leads to the destruction or death of the heart muscle cells. Myocarditis (inflammation of the heart muscle) has many causes and a wide range of symptoms, from minor ailments, with mild and improving symptoms, to deadly diseases with high progression. Myocarditis should not be equated with pericarditis, because pericarditis is actually an inflammation of the sac that involves the heart and, like myocarditis, does not involve the heart muscle. However, many patients suffer from both complications. Myocarditis has many different types and different possible causes that lead to the disease. One of the most important factors in myocarditis is a virus called viral myocarditis (VMC). Viral myocarditis refers to non-specific inflammatory lesions of the myocardium caused by virus infection. Myocarditis occurs in about 5% of patients during the viral epidemic, and about 12.5% of acute VMC can progress to dilated cardiomyopathy (1-2). Enteroviruses, especially Group B coxsackievirus (CV), have traditionally been considered as a main viral cause (3). The pathogenesis of VMC is mainly divided into two stages. First, viral infection directly damages cardiac muscle (virus-mediated cardiomyocyte lysis); and second, the host immune response causes indirect damage by killing virally infected (antiviral immunity) and uninfected (autoimmune) cardiac muscle: some cases develop into dilated cardiomyopathy, and some may end in heart failure or sudden death. Regarding the etiology and pathogenesis of VMC, many believe that the lack of vital qi is the basis, and the invasion of the pathogenic toxin is the key. The heart qi and blood as well as yin and yang are disordered, so it produces phlegm, stasis, damp turbidity, and other pathological products. Traditional Chinese medicine has its unique advantages in treating VMC (4-5).

The drug Xinjierkang (XJEK) used in this study is composed of astragalus, ginseng, sophora flavescens, panax notoginseng, ophiopogon japonicus, aconite, and other drugs. It has the effects of boosting qi, nourishing yin, expelling evil, restoring heart, promoting blood circulation and restoring the pulse (6-7).

It has been reported (8) that viral infection can directly or indirectly induce apoptosis, which plays an important role in the occurrence and development of VMC and is regulated by many factors. Inflammatory cytokines play an important role in the progression and regulation of VMC. TNF-α, IL-6, IL-17, and IL-1β are the main inflammatory mediators and important components in the cytokine network (9-11). Studies have shown (12) that Nrf2 activation can prevent damage in VMC. Therefore, the Nrf2/HO-1 pathway may be

involved in the occurrence and development of VMC (13).

In this study, the changes of Nrf2/HO-1 and cytokines TNF-α, IL-6, IL-17, and IL-1β in cardiac muscle cells of VMC mice were detected in order to clarify the mechanism of action of XJEK.

Materials and Methods

Model establishment, grouping and administration

One hundred and fifty healthy BALBC mice (male, aged 4-5 weeks and weighed 13-17g) were randomly divided into the normal group, model group, low-, medium- and high-dose XJEK groups, with 30 in each group. Each mouse in the model group and the low-, medium-, and high-dose XJEK groups was intraperitoneally injected with 0.1 mL CVB3 virus solution containing 100 TCID50 for 3 consecutive days to establish VMC mice models. The normal group was injected intraperitoneally with an equal volume of normal saline. 2 hours after the first injection of the virus solution, XJEK was administered by gavage. The low-, medium-, and high-dose XJEK groups were administered with 5, 10, and 20 g/kg, respectively. The normal group and the model group were administered with an equal volume of distilled water. They were administered continuously until each observation time point, i.e., sampling at 3, 7, 14, 21, and 28 days.

Determination of serum inflammatory factors

At 3, 7, 14, 21, and 28 days after the inoculation of the above 5 groups, 6 mice were randomly selected for aseptic extraction of the eyeballs. Blood was centrifuged, and the serum was collected. Double antibody sandwich ELISA was used to detect serum inflammatory factors TNF-α, IL-6, IL-17, and IL-1β.

Detection of cardiomyocyte apoptosis by TUNEL

At 3, 7, 14, 21, and 28 days after the inoculation, 6 mice were randomly selected and sacrificed by spinal cord dissection, and their hearts were dissected to check for cardiomyocyte apoptosis. They were digested with proteinase K to expose the DNA. Under the mediation of terminal deoxynucleotidyl transferase, the DNA cleavage part was labeled with dUTP linked to alkaline phosphate. The color was then developed by immunohistochemistry, and the normal cardiac muscle tissue was treated with DNase as the positive control. The number of positive and total cells was counted under a 400x light microscope, and the percentage of cardiomyocyte apoptosis was calculated according to the formula: number of positive cells/total number of cells × 100%.

Detection of Nrf2 and HO-1 protein levels in myocardial tissue by Western Blot

At 28 days after the inoculation, 6 mice were sacrificed in each group and their hearts were dissected to detect Nrf2 and HO-1 protein levels in the myocardial tissue. New lysis containing protease inhibitor was added and lysed on ice to extract the total protein in the sample; an appropriate amount of loading buffer was mixed to denaturation at 100 °C for 5 min; 50-70 μg protein was taken for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); after the protein was completely separated, it was tank transferred to polyvinylidene fluoride (PVDF) membrane; the blocking solution containing 5% skim milk powder was used to incubate at room temperature for 1 h; the corresponding protein primary antibody was added to incubate overnight at 4 °C; then the secondary antibody labeled with horseradish peroxidase was added the next day to incubate for 1 h at 37 °C, and the electroluminescence (ECL) solution was added, and a gel imager was used to display protein bands, with GADPH as an internal reference to calculate the relative expression of Nrf2 and HO-1 proteins.

Statistical analysis

SPSS 25 software was used for data statistical analysis, and measurement data were expressed in $\bar{x} \pm s$. One factor analysis of variance was used for measurement data, the LSD method was used for intergroup comparison, and the X^2 test was used for counting data. $p < 0.05$ was statistically significant.

Results

Detection of myocardial cell apoptosis by TUNEL

Under light microscopy, apoptotic nuclei were brown-yellow and non-apoptotic nuclei were green. No apoptotic cells were seen in the myocardial tissue of the normal group, and apoptotic cardiomyocytes were all seen in the model group and the low-, medium-, and high-dose XJEK groups. The percentage of myocardial apoptosis was significantly reduced in the low-, medium-, and high-dose XJEK groups compared with that in the model group ($P < 0.05$), as shown in Table 1.

Comparison of serum TNF-α in each group

At 3, 7, 14, 21, and 28 days after inoculation, compared with the normal group, the TNF-α level in the model group significantly increased ($P < 0.05$). After the administration of XJEK, compared with the model group, the

Table 1. Detection of myocardial cell apoptosis by TUNEL.

Group	n	Positive number / tested number					Total detection rate	
		3d	7d	14d	21d	28d	n	%
Normal	30	0/6	0/6	0/6	0/6	0/6	0	0
Model	30	5/6	5/6	6/6	5/6	3/6	24	80
Low-dose XJEK	30	2/6	3/6	2/6	1/6	0/6	8	26.67*
Medium-dose XJEK	30	2/6	2/6	2/6	0/6	0/6	6	20*
High-dose XJEK	30	2/6	1/6	0/6	0/6	0/6	3	10*

*P<0.05, compared with model group.

TNF-α levels in the low-, middle-, and high-dose XJEK groups significantly decreased (P < 0.05), as shown in Table 2.

Comparison of serum IL-6 in each group

At 3, 7, 14, 21, and 28 days after inoculation, compared with the normal group, the IL-6 level in the model group significantly increased (P < 0.05). After the administration of XJEK, compared with the model group, the IL-6 levels in the low-, middle-, and high-dose XJEK groups significantly decreased (P < 0.05), as shown in Table 3.

Comparison of serum IL-17 in each group

At 3, 7, 14, 21, and 28 days after inoculation, compared with the normal group, the IL-17 level in the model group significantly increased (P < 0.05). After the administration of XJEK, compared with the model group, the IL-17 levels in the low-, middle-, and high-dose XJEK groups significantly decreased (P < 0.05), as shown in Table 4.

Comparison of serum IL-1β in each group

At 3, 7, 14, 21, and 28 days after inoculation, compared with the normal group, the IL-1β level in the model group significantly increased (P < 0.05). After the administration of XJEK, compared with the model group, the IL-1β levels in the low-, middle-, and high-dose XJEK groups significantly decreased (P < 0.05), as shown in Table 5.

Comparison of Nrf2 and HO-1 protein expression levels in each group

At 28 days after inoculation, compared with the normal group, the expressions of Nrf2 and HO-1 proteins in the myocardial tissue of the model group were significantly down-regulated (P < 0.05); and compared with the model group, the expressions of Nrf2 and HO-1 proteins in the low-, medium-, and high-dose XJEK groups were significantly up-regulated (P < 0.05) in a concentration-dependent manner, as shown in Figures 1 and 2.

Table 2. Comparison of serum TNF-α in each group (ng/L, $\bar{x} \pm s$).

Group	n	3 d	7 d	14 d	21d	28 d
Normal	6	14.96±3.32	15.41±3.45	15.03±3.86	15.03±4.15	15.11±4.03
Model	6	90.37±8.12#	127.58±12.06#	60.27±8.44#	40.47±6.33#	25.67±3.96#
Low-dose XJEK	6	58.51±7.43*	72.13±5.29*	30.74±6.15*	25.55±5.52*	21.09±4.18*
Medium-dose XJEK	6	27.45±5.16*	36.28±6.04*	25.02±7.43*	20.12±4.36*	17.54±3.07*
High-dose XJEK	6	20.66±4.21*	21.78±5.74*	18.18±3.92*	17.45±3.05*	16.09±3.91*

#P<0.05, compared with normal group, *P<0.05, compared with the model group.

Table 3. Comparison of serum IL-6 in each group (ng/L, $\bar{x} \pm s$).

Group	n	3d	7d	14d	21d	28d
Normal	6	25.45±3.11	25.06±4.14	25.53±3.85	25.31±4.22	25.28±4.03
Model	6	182.62±13.33#	314.83±15.22#	165.42±12.09#	137.56±13.45#	121.86±11.01#
Low-dose XJEK	6	87.73±6.29*	132.78±7.01*	79.56±9.32*	42.42±8.67*	33.57±7.49*
Medium-dose XJEK	6	55.01±7.94*	59.44±9.75*	38.41±7.03*	31.63±5.91*	29.04±3.63*
High-dose XJEK	6	40.45±6.72*	41.31±7.06*	31.17±5.51*	29.67±4.02*	27.13±3.88*

#P<0.05, compared with normal group, *P<0.05, compared with the model group.

Table 4. Comparison of serum IL-17 in each group (pg/mL, $\bar{x} \pm s$).

Group	n	3d	7d	14d	21d	28d
Normal	6	32.54±6.31	32.68±5.03	33.49±5.35	33.51±4.92	33.37±4.64
Model	6	136.41±17.85#	167.55±19.43#	158.02±15.98#	125.17±10.03#	115.56±9.09#
Low-dose XJEK	6	75.02±9.16*	80.31±8.45*	75.48±6.13*	39.96±5.04*	37.04±6.15*
Medium-dose XJEK	6	60.01±8.11*	64.92±6.32*	56.35±5.22*	37.63±4.67*	35.35±4.34*
High-dose XJEK	6	43.89±5.94*	47.67±8.15*	40.28±6.04*	35.91±5.33*	33.89±4.12*

#P<0.05, compared with normal group, *P<0.05, compared with the model group.

Table 5. Comparison of serum IL-1β in each group (pg/mL, $\bar{x} \pm s$).

Group	n	3d	7d	14d	21d	28d
Normal	6	28.36±4.23	28.45±4.91	28.51±5.03	28.77±4.67	28.41±5.05
Model	6	116.58±14.65#	123.96±16.28#	109.16±13.52#	94.35±9.12#	80.35±8.17#
Low-dose XJEK	6	60.66±7.43*	65.19±7.12*	60.85±5.04*	55.78±6.13*	49.16±7.04*
Medium-dose XJEK	6	50.43±6.51*	56.87±5.44*	50.68±3.17*	34.09±5.48*	32.47±3.56*
High-dose XJEK	6	40.65±7.17*	43.01±6.32*	38.14±5.22*	32.05±4.39*	29.15±3.83*

#P<0.05, compared with normal group; *P<0.05, compared with the model group.

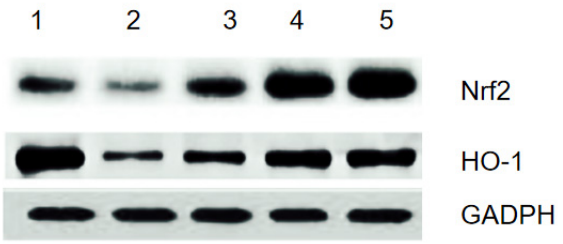

Figure 1. Detection of Nrf2 and HO-1 protein expressions in each group by Western Blot (n = 6).

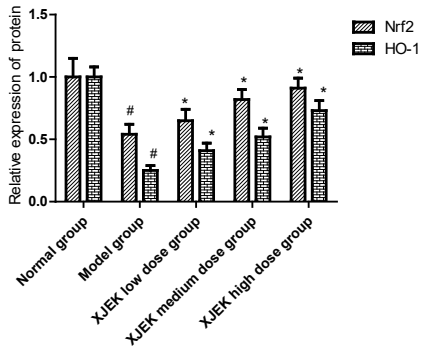

Figure 2. Semi-quantitative analysis of Nrf2 and HO-1 protein expressions in each group (n = 6) compared with the normal group, $^{\#}P < 0.05$; and compared with the model group, $^{*}P < 0.05$.

Discussion

After the virus invades the human body, it interacts with specific receptors and enters the cardiomyocytes to start replication, hindering the synthesis of cardiomyocytes' proteins and causing damage and necrosis of the cardiomyocytes. Meanwhile, after the human immune system recognizes the invasion of CVB3, it will release a large number of inflammatory factors to clear the virus, but the persistent presence of inflammatory mediators will cause inflammatory damage to the heart muscle as well (14). In this experiment, a double antibody sandwich ELISA method was used to detect the changes of serum TNF-α and IL-6 levels in BALBC mice at different stages after CVB_{3m} infection. The results showed that the levels of serum TNF-α and IL-6 in mice showed a dynamic change from increase before decrease. It starts to increase on the 3rd day after infection, increases significantly on the 7th day, and then gradually decreases. The levels of TNF-α, IL-6, IL-17 and IL-1β in each XJEK group were significantly lower than those in the model group ($P < 0.05$). Therefore, XJEK may exert preventive and therapeutic effects by regulating these inflammatory factors.

The location of VMC is the heart. Insufficient heart qi and yin, blood stasis block, poor heart pulse, and heart deficiency are the main pathogenesis of this disease. It was found in this study that XJEK could inhibit myocardial cell apoptosis and prevent myocardial damage in VMC mice. The reason may be that XJEK emphasizes supporting vital qi. It chooses astragalus and ginseng as main medicine, especially astragalus, which enters the spleen and lung channels, supplements the spleen-qi and secures the exterior, with a sweet taste and slight warm property; and pharmacological studies show that astragalus has the functions of enhancing humoral, cellular and non-specific immunities, regulating immunity and inducing interferon, and plays a role in anti-bacteria and anti-virus (15-16). Ginseng, with a slightly bitter taste and slightly warm property, nourishes vitality, promotes the production of body fluid to quench thirst, and relieves uneasiness of mind and body; and pharmacological studies show that ginseng has the functions of anti-cell peroxidation, scavenging free radicals and regulating the whole body. It can resist myocardial ischemia, heart rhythm disorders, and myocardial hypoxia, with effects of immune regulation, anti-inflammation, anti-bacteria, and anti-virus (17-18). Ophiopogon japonicus can increase cardiac coronary flow, enhance myocardial contractility, and regulate the body's immune function. Sophora flavescens, bitter and cold, is a product of pure yin. It can clear heat and dampness, and adjust the heart rhythm; and pharmacological studies show that sophora flavescens, with an antiarrhythmic effect, is used to treat various arrhythmias, and can increase coronary blood flow and prevent myocardial ischemia (19-20). All the medicines have the functions of benefiting qi, nourishing yin, promoting blood circulation, removing blood stasis, and clearing heat. Comprehensive pharmacological effects of XJEK include anti-virus, anti-myocardial ischemia & hypoxia, anti-arrhythmia, and regulation of the body's immunity.

Nrf2 is a nuclear transcription factor. Under normal physiological conditions, Nrf2 is localized in the cytoplasm, and it interacts with cytoplasmic protein chaperone molecules in an inactive state. When the cell is injured, external stimuli will activate the Nrf2 phase into the nucleus, where it combines with antioxidant response sequence elements, induces the expression of multiple antioxidant proteins (SOD, GSH, etc.) and phase II detoxifying enzymes (HO-1, etc.), reduces the production of reactive oxygen and peroxide end products MDA, and counteracts oxidative stress (21-22). Finding natural compounds that have anti-oxidative effects to neutralized oxidative stress is on demand (23). As oxidative stress plays an important contribution in variety diseases such as heart diseases (24). It was found in this study that XJEK up-regulated the expression of Nrf2 and HO-1 proteins in VMC mice, suggesting that XJEK may protect the myocardial tissue of model mice by increasing the expression of Nrf2 and HO-1 proteins, enhancing the level of anti-oxidation, and reducing oxidative stress injury. New technologies, such as gene editing, can play an important role in overcoming viral diseases (25).

In summary, XJEK can prevent myocardial injury in VMC mice, and its mechanism of action may be related to improving myocardial cell apoptosis, inhibiting inflammatory response, and up-regulating the expression of Nrf2 and HO-1 proteins in myocardial tissue.

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