Drug-containing serum of Xinfeng capsules protect against H9C2 from death by enhancing miRNA-21 and inhibiting toll-like receptor 4/phosphorylated p-38 (p-p38)/p-p65 signaling pathway and proinflammatory cytokines expression

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Abstract

OBJECTIVE: To investigate effect of drug-containing serum of Xinfeng capsules on myocardial cell growth.

METHODS: Drug-containing serum of Xinfeng capsules rat models were established by intragastrically administrated Xinfeng capsules. MTT assay was used to evaluated H9C2 cells viability. H9C2 cells were divided into normal control group, triptolide group, lipopolysaccharide (LPS) group, drug-containing serum group and miRNA-21 inhibitor group. microRNA-21 (miRNA-21) inhibitor was structured and transfected into H9C2 cells. Western blot and immunofluorescence assay were applied to examine toll-like receptor 4 (TLR4), phosphorylated p-38 (p-p38) and p-p65 expression. Quantitative real-time PCR (qRT-PCR) was used to evaluated mRNA levels of miRNA-21. Enzyme linked immunosorbent (ELISA) was used to measure tumor necrosis factor α (TNF-α), IL-6 and IL-17 levels.

RESULTS: Drug-containing serum treatment significantly increased cell viability compared to LPS treated group. qRT-PCR results indicated that miRNA-21 levels were significantly decreased in drug-containing serum group compared to LPS group. Early and late apoptosis in drug-containing serum group were significantly decreased compared to LPS group. Western blot and immunofluorescence assay results showed that TLR4, p-p38 and p-p65 levels in drug-containing serum group were significantly decreased compared to LPS group. ELISA findings indicated that drug-containing serum significantly decreased inflammatory cytokine levels of TNF-α, IL-6 and IL-17.

CONCLUSION: Drug-containing serum of Xinfeng capsules protect against lipopolysaccharide instructed H9C2 cells from death by enhancing miRNA-21 and inhibiting TLR4/p-p38/p-p65 signaling pathway and proinflammatory cytokines expression.
INTRODUCTION

The ischemic heart diseases, such as the ischemia-reperfusion (IR) injury, myocardial infarction, are the leading reason for the death in the whole world.\(^1\) When the myocardial blood supply to the ischemic regions, and the subsequent reperfusion does not ensure achievement of complete myocardial reperfusion and recovery of the normal internal environment, the ischemia/reperfusion injury may be occurred.\(^1,4\) The dys-regulation of the molecules in cardiac cells following the IR injury always be associated with some of the risk factors, such as the inflammatory response, pathological signaling pathways. Therefore, we discussed the myocardial cells related signalling pathways undergoing the treatment of Xinfeng capsules, which is a Traditional Chinese Medicine that could replenish the Qi and strengthen the spleen, and resolves Dampness and freeing channel.\(^1,6\)

The mechanisms for the cardiac injury in IR have been concluded be mediated by the activation of nuclear kappa B (NF-kB)/tumor necrosis factor α (TNF-α) pathway.\(^2\) toll-like receptor 4 (TLR4)/NF-kB pathway.\(^3\) Li et al.\(^5\) reported that the transforming growth factor β-activated kinase 1 signaling pathway critically regulates myocardial survival and remodelling. Hu et al.\(^6\) also proved that the PI3K/Akt/GSK-3β pathway involves in the myocardial cells injury. Therefore, investigating the signaling pathways participating in the myocardial cell injury may provide the novel methods for clinical therapy of the IR injury.

MicroRNAs (miRNAs) are a series of the non-coding small eukaryotic RNAs with the length about 19 to 25 nucleotides, which are highly conserved and play the endogenous regulatory functions.\(^1,1\) Especially, at the post-transcriptional level, the miRNAs could regulate the cognate target genes expression, and further cause the transcript degradation and the translational repression.\(^1,2\) The miRNAs have proved to be correlation with a series types of the heart disorders and the cardiovascular diseases.\(^1,3\) The miRNA-21 is the first miRNA that was confirmed to be transcribed by RNA polymerase II, and illustrates strong evolutionary conservation in avian, fish clades and mammalian.\(^5,6\) miRNA-21 participates in many biological functions, such as inflammation, cell proliferation and growth, apoptosis, and participates in some diseases, such as cardiovascular diseases.\(^3\) Therefore, the inhibition or up-regulation for the miRNA-21 may exert a obvious effects on the myocardial cell growth or apoptosis.

In this study, we evaluated the effects of drug-contain- ing serum of Xinfeng capsules on the myocardial cell growth and the mechanism or signaling pathways of Xinfeng capsules in reducing the cell apoptosis and enhancing the cell growth.

MATERIALS AND METHODS

Animals

Twenty specific pathogen free (SPF) Sprague-Dawley (SD, weight from 250 to 300 g, and 6-8 weeks old) were used in the present study, which were purchased from Western Biotech., Chongqing, China. The SD rats were maintained with the cycle of 12 h light/12 h dark at the temperature of (25 ± 2) °C. The SD rats were fed with the standard commercial diet and water freely, which were purchased from CLEA Japan Inc. (Shizuoka, Japan). This study was performed as the guidance of Care and Use of Laboratory Animals of HNI, and was approved by Institutional Animal Care and Use Committee of China.

Preparation of drug-containing serum

Twenty SD rats were divided into two groups, including normal serum group and drug-containing serum group. For the drug-containing serum group, the Xinfeng capsules (a Chinese drug for strengthening spleen, removing dampness and clearing collaterals, produced by the drug-making center of the first Affiliated Hospital of Anhui University of Traditional Chinese Medicine with the batch number 2016O0B. Xinfeng capsules consists of Astragali, Coix Seed, Hook and Centsipes. Each capsule contains extract 0.5 g of crude drugs) were intragastrically administrated to the SD rats in a dose of 0.3 g/100 g body weight once daily at 9:00 am for 3 d. For the normal serum group, the normal saline was intragastrically administrated to the rats in the control group (1 mL/100 g body weight) once daily at 9:00 am for 3 d. Any food intake was restricted 12 h before the intragastrically administration of Xinfeng capsules, but freely access to the water. Then, the SD rats were anesthetized by injecting intraperitoneally with the 7% chloral hydrate (0.5 mL/100 g). The abdominal cavity of rats were cut opened, and the blood samples were drawn from the abdominal aorta and kept still for 2 h. In order to induce blood coagulation, the blood samples were kept in the refrigerator at 4 °C for 4 h, and then were centrifugated at 3000 r/min for 15 min. The obtained supernants were inactivated at 56 °C for 30 min, and were sterilized by using the millipore filters (0.2 μm). Finally, the serum isolated from both normal serum group and drug-containing serum group were stored at –70 °C.

H9C2 cell culture and trial grouping

The myocardial cell line of rat, H9C2, was purchased from the Shanghai Chinese Academy of Science cell Bank, Shanghai, China. The H9C2 cells were cultured
by using DMEM (Gibco, Waltham, MA, USA) containing the 100 mg/mL streptomycin (Beyotime Biotech., Shanghai, China), 100 units/mL penicillin (Beyotime Biotech.) and 10% fetal bovine serum (FBS, Gibco, Waltham, MA, USA). H9C2 cells were cultured at 37 °C and in a humidified atmosphere supplementing with 5% CO₂.

**Triptolide treatment and MTT assay**

The triptolide (10 mg) was dissolved into the DMSO (5 mL) to prepare the mother liquid at the concentration of 2 mg/mL, and stored at 4 °C. In order to evaluate the cytotoxicity of the triptolide, the H9C2 cells were divided into 1 ng/mL triptolide treatment group, 10 ng/mL triptolide treatment group and 20 ng/mL triptolide treatment group. Meanwhile, the H9C2 cells without triptolide treatment were assigned as the normal control group.

The cell cytotoxicity caused by triptolide was evaluated by using the MTT assay according to the previous published study. Briefly, H9C2 cells were seeded into the 96-well plates (Corning-Costar, NY, USA) and incubated with 5% CO₂ at 37 °C. Then, the MTT solution (Sigma-Aldrich, St. Louis, Missouri, USA) was added into the 96-well plates at the concentration of 5 mg/mL, and incubated at 37 °C for 4 h. Finally, 150 μL DMSO (Amresco Inc., Solon, OH, USA) was added into the 96-well plates to dissolve the crystal and incubated for 15 min. The optical density of the obtained solution were examined by using the enzyme-linked immunosorbent assay (ELISA) reader (Thermo Fisher Scientific, Hudson, NH, USA) at the wavelength of 490 nm.

**Trial grouping**

The H9C2 cells were divided into 5 groups, including normal control group (incubating with normal serum), triptolide group (incubating with normal serum + 10 mg/mL triptolide), LPS group (incubating with control serum + 40 mg/L LPS), drug-containing serum group (incubating with drug-containing serum + 40 mg/L LPS) and miRNA-21 inhibitor group (transfected with miRNA-21 siRNA and incubating with drug-containing serum + 40 mg/L LPS).

**miRNA-21 inhibitor (siRNA) sequence and transfection**

The miRNA-21 inhibitor and the negative sham oligonucleotides for the miRNA-21 (miRNA-21 control) were constructed and synthesized by GenePhama Co., Ltd., (Suzhou, China). The oligonucleotide sequence for miRNA-21 inhibitor was listed as followsings: 5’-TCAACATCAGTCTGATAAGCTA-3’. The oligonucleotide sequence for miRNA-21 control was listed as followings: 5’-CAGTACTTTTGTGTAAGCTA-3’. Both of the miRNA-21 inhibitor and miRNA-21 control siRNAs were transfected into the H9C2 cells by using the Lipofectamine 2000 kit (Invitrogen/Life Technologies (Carlsbad, CA, USA) according to the manufacturer’s instruction. For the key process, the H9C2 cells were transfected with miRNA-21 inhibitor and miRNA-21 control siRNAs, and cultured in the Opti-MEM at 37 °C for 6 h. Then, the Opti-MEM was discarded and the cells were cultured by using complete DMEM 24 h culture. Finally, the miRNA-21 siRNAs transfected H9C2 cells were prepared for the following tests.

**Western blot assay**

The cells in every group were extracted by using RIPA lysis buffer (Beyotime Biotech., Shanghai, China). The extracted protein were separated by using 15% SDS-PAGE (Amersham Biosciences (Little Chalfont, Buckinghamshire, England), and electrotransferred onto the polyvinylidene fluoride (PVDF, Dupont, USA). The PVEF membranes were blocked by using the 5% bovine serum albumin in phosphate buffered saline (PBS, supplementing with 0.05% Tween-20 solution and adjusting pH 7.5). Then, the membranes were incubated by using mouse anti-rat TLR4 monoclonal antibody (1: 3000; Catalogue No. ab30667, Abcam Biotech., Cambridge, Massachusetts, USA), mouse anti-rat p-p38 monoclonal antibody (1: 3000; Catalogue No. ab45381, Abcam Biotech.) and rabbit anti-rat p-p65 polyclonal antibody (1: 2000; Catalogue No. ab86299, Abcam Biotech.) at room temperature for 2 h. The membranes were washed by using PBST and incubated by using the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Catalogue No. A0545, Sigma-Aldrich, St. Louis, MO, USA) and HRP-conjugated goat anti-mouse IgG (Catalogue No. AP127P, Sigma-Aldrich, St. Louis, Missouri, USA). The reactive protein signals were visualized by employing the enhanced chemiluminescence kit (ECL, Thermo Scientific Pierce, Rockford, IL, USA). Finally, the gel images were scanned and analyzed under a GDS8000 image scanning system (UVP, Sacramento, CA, USA).

**Immunofluorescence assay**

The TLR4, p-p38 and p-p65 in H9C2 were stained by utilizing the direct immuno-fluorescence assay in this study. The H9C2 cells were cultured using 6-well plates (Corning-Costar, NY, USA), and fixed by using 10% formalin, washed with PBS solution. Then, the H9C2 cells were treated by using 3% H₂O₂ to block the endogenous peroxidase activity. The 6-well plates were incubated by using 100 μL 10% FBS in a humidified chamber at room temperature. Then, the cells in 6-well plates were incubated by using mouse anti-rat TLR4 monoclonal antibody (1: 3000; Catalogue No. ab30667, Abcam Biotech., Cambridge, Massachusetts, USA), mouse anti-rat p-p38 monoclonal antibody (1: 3000; Catalogue No. ab45381, Abcam Biotech.) and rabbit anti-rat p-p65 polyclonal antibody (1: 2000; Catalogue No. ab86299, Abcam Biotech.) at 4 °C over-
night. Then, the TLR4 and p-p65 expression were observed utilizing the Alex Fluor 647 red conjugated goat anti-mouse IgG (Catalogue No. ab150115; 1: 500; Abcam, Cambridge, UK), and the p-p38 expression was observed by employing Alex Fluor 647 red conjugated goat anti-rabbit IgG (Catalogue No. ab150097; 1: 500; Abcam, Cambridge, UK). The laser confocal scanning microscopy (Leica Inc., Ltd., Germany) was employed to capture the immunofluorescence images.

ELISA
The cell TNF-α, IL-6 and IL-17 levels were measured by using commercial enzyme linked immunosorbent (ELISA) kits (Lkcx Tech., Beijing, China) according to the instruction of manufacturer. All of the measurements were performed in duplicate and expressed as ng/L. The levels of TNF-α, IL-6 and IL-17 were evaluated and measured by employing a ELISA plate reader (Bio-Tek Inc., Winooski, VT, USA).

Flow cytometry assay
The H9C2 cell apoptosis was evaluated by using flow cytometry that monitors the Annexin V-PE binding and 7-AAD (BD Biosciences, San Jose, CA, USA) uptake simultaneously. In brief, the H9C2 cells were harvested from the 6-well plates, and resuspended in Annexin V-PE binding buffer (BD Biosciences,) and incubated by using the Annexin V-PE and 7-ADD for 15 min at room temperature in dark. The cells were analyzed by using a FACS Vantage SE flow cytometry (BD Biosciences, San Jose, CA, USA). The fluorescence was measured by using a 530/578 band filter to monitor the Annexin V-PE binding and using a 546/647 band filter to monitor the 7-ADD uptake.

Statistical analysis
The data were analyzed using SPSS 19.0 (IBM Corp., Released 2010. IBM SPSS Statistics for Windows, Version 19.0, Armonk, NY, USA), and described as mean ± standard deviation (±). The One-way analysis of variance and Student’s t test were used test the differences between groups. A P value less than 0.05 was considered statistical significant.

RESULTS

H9C2 growth
The results indicated that there were not significant differences for the cell viability of H9C2 cells between the normal control group and the 10 ng/mL (or the 1 ng/mL) triptolide group (P > 0.05). However, the viability of H9C2 cells was significantly decreased in 20 ng/mL triptolide group compared to the 10 ng/mL triptolide group (P < 0.05). Therefore, in the following experiments, we selected the concentration of 10 ng/mL to discuss the protective role of triptolide for myocardial cells. And the results also showed that there were no obvious differences between the 10 ng/mL triptolide group (Figure 1). However, a plenty of death cells appeared in the cells of LPS treatment group, and the drug-containing serum treatment and miRNA-21 inhibitor treatment obviously suppressed the LPS caused cells death (Figure 1).

Drug-containing serum inhibits miRNA-21 expression
The results indicated that the miRNA-21 in triptolide group was significantly decreased compared to normal control group (P < 0.05). The miRNA-21 levels in LPS group was significantly increased compared to the normal control group (P < 0.01). However, the levels of miRNA-21 in drug-containing serum group and miRNA-21 inhibitor group were significantly decreased compared to the LPS group (P < 0.01).

Drug-containing serum enhances the cell viability
The results showed that cell viability in LPS group was significantly decreased compared to normal control group (P < 0.05) at 24, 48 and 72 h, respectively. However, the drug-containing serum treatment could significantly increase the cell viability compared to the LPS treated group (P < 0.05) at 24, 48 and 72 h, respectively.

Drug-containing serum decreases the early apoptosis and late apoptosis
The flow cytometry findings (Figure 2) showed that the both of the early apoptosis and late apoptosis rate

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**Table 1.** The results are expressed as mean ± standard deviation (±) and were analyzed by using the One-way ANOVA test and Bonferroni post-hoc test for multiple comparisons. The significant differences were indicated by *P* < 0.05.

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**Figure 1.** H9C2 growth in different groups
A: normal control group; B: triptolide group; C: LPS group; D: drug-containing serum group; E: miRNA-21 inhibitor group. LPS: lipopolysaccharide.
in LPS group were significantly increased compared to the normal control group \( P < 0.01 \). Meanwhile, the early apoptosis and late apoptosis in drug-containing serum group were significantly decreased compared to LPS group \( P < 0.05 \). However, the late apoptosis in drug-serum combining miRNA-21 inhibitor group was significantly increased compared to drug-containing serum group \( P < 0.05 \).

**Drug-containing serum decreases levels of TLR4, p-p38 and p-p65**

The results illustrated that the levels of TLR4, p-p38 and p-p65 in tripotide group were significantly decreased compared to the normal control group \( P < 0.05 \), and in LPS group were significantly increased compared normal control group \( P < 0.01 \). Moreover, the TLR4, p-p38 and p-p65 levels in drug-containing serum group were significantly decreased compared to LPS group \( P < 0.05 \). The miRNA-21 combining drug-containing serum treatment also significantly decreased TLR4, p-p38 and p-p65 levels compare LPS group \( P < 0.05 \), however, increased compared to drug-containing serum group \( P > 0.05 \).

Meanwhile, the immunofluorescence assay also indicated that the drug-containing serum treatment significantly inhibited the LPS caused enhancing levels of TLR4, p-p38 and p-p65. The miRNA-21 inhibitor also obviously suppressed the LPS induced enhancing levels of TLR4, p-p38 and p-p65, the effects of which were slightly weakened compared to drug-containing serum group (Figure 3).

**Drug-containing serum decreases inflammatory cytokine levels**

The cell TNF-\( \alpha \), IL-6 and IL-17 levels in LPS group were significantly increased compared to the normal control group \( P < 0.01 \). However, the TNF-\( \alpha \), IL-6 and IL-17 levels were significantly decreased in Drug-containing serum group compared to the LPS group \( P < 0.01 \). However, the drug-serum combining miRNA-21 inhibitor treatment (miRNA-21 inhibitor group) enhanced levels of TNF-\( \alpha \), IL-6 and IL-17 levels compared to drug-containing serum group \( P < 0.05 \).

**DISCUSSION**

In this study we investigated the effect of drug-containing serum of Xinfeng capsules on myocardial cell growth. Meanwhile, the mechanism or signaling pathways undergoing the Xinfeng capsules’ effects in reducing the cell apoptosis and enhancing the cell growth were also evaluated. Our findings demonstrated that the LPS deduced the H9C2 cell growth and caused the apoptosis, and firstly investigated the role of drug-containing serum of Xinfeng capsules in cell growth, apoptosis by regulating the levels of inflammatory cytokine, miRNA-21 and the other death-associated molecules. Generally, the endogenously expressed miRNAs are always changed in response to the pathological and physiological stimuli, tissue or cell injury, and the milieu in interior diseases. The previous study also reported that the I/R injury diseases could induce the miRNA-21 expression in the heart tissues, and cause the myocardial cell injury. According to the quantitative RT-PCR analysis, we discovered that miRNA-21 levels in LPS group was significantly increased compared to the normal control group, however, the levels of miRNA-21 in Drug-containing serum group and miRNA-21 inhibitor group were significantly decreased compared to the LPS group. This result suggests that the drug-containing serum could inhibit the expression of the miRNA-21 expression, which was discovered for the first time due to the previous documents. Meanwhile, the combining of the miRNA-21 inhibitor with drug-containing serum strengthened the inhibitive effects of drug-containing serum on the miRNA-21 expression. The previous study reported that the miRNA-21 could induce the damage or death of the myocardial cells in myocardial ischemia in clinical. Therefore, we examined the H9C2 cell viability by using the MTT assay. The results showed that the drug-containing serum could significantly increased the cell viability compared to the LPS group, which suggests that the drug-containing serum could inhibit the LPS induced cell death and enhance the cell viability.

Apoptosis is a kind of cell death that is caused by the genetic programs, which could lead to the cell death directly. Therefore, we detected the cell apoptosis in every group. The results indicated that the early apoptosis and late apoptosis in drug-containing serum group were significantly decreased compared to LPS group, which hints that drug-containing serum could inhibit the cell apoptosis. However, the late apoptosis in drug-serum combining miRNA-21 inhibitor group was significantly increased compared to drug-containing serum group, which also suggests that miRNA-21 inhibitor may induce the cell apoptosis.

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**Figure 2 Evaluation for the early apoptosis and late apoptosis by using flow cytometry assay**

A: normal control group; B: triptolide group; C: LPS group; D: drug-containing serum group; E: miRNA-21 inhibitor group. LPS: lipo-polysaccharide.
The TLR family is described as a group of critical regulators that involves in the immune response and the inflammatory responses. The TLR family proteins could activate some common signaling pathways, such as the mitogen-activated protein kinase (MAPKs), nuclear factor-kB (NF-kB, p65) cascades, and then regulate the inflammatory genes transcription, such as the TNF-α and the interleukin family proteins. In this study, we examined the levels of TLR4, p-p38 and p-p65 protein levels in every group. Both of the western blot assay and immunofluorescence assay results indicated that the LPS triggered high levels of TLR4, p-p38 and p-p65. However, the TLR4, p-p38 and p-p65 levels in drug-containing serum group were significantly decreased compared to LPS group, which suggests that drug-containing serum protects the H9C2 growth by activating the TLR4/p-p38/p-p65 signaling pathway. Moreover, the levels of TLR4, p-p38 and p-p65 in miRNA-21 inhibitor group (treatment with both miRNA-21 inhibitor and drug-containing serum) were obviously increased compared to drug-containing serum group, which suggest that miRNA-21 inhibitor increased the TLR4, p-p38 and p-p65 levels, however, the mechanism has not been fully investigated till now.

The previous studies reported that LPS-induced cell death was associated with the proinflammatory cytokines, such as the TNF-α, IL-6 and IL-17, therefore, we examined levels of these cytokines of cells undergoing drug-containing serum treatment. The results in this study showed that the TNF-α, IL-6 and IL-17 levels were significantly decreased in Drug-containing serum group compared to the LPS group. However, the drug-serum combining miRNA-21 inhibitor treatment (miRNA-21 inhibitor group) enhanced levels of TNF-α, IL-6 and IL-17 levels compared to Drug-containing serum group, the mechanism of which needs to be further investigated in the following study.

In conclusion, the drug-containing serum of Xinfeng capsules enhanced the H9C2 cell viability and inhibited the cell apoptosis. Drug-containing serum of Xinfeng capsules significantly decreased the TLR4, p-p38 and p-p65 protein expression and inhibited the proinflammatory cytokines, TNF-α, IL-6 and IL-17 levels in H9C2 cells. Therefore, drug-containing serum of Xinfeng capsules protect against the lipopolysaccharide instructed H9C2 cells from death by enhancing miRNA-21 expression and inhibiting TLR4/p-p38/p-p65 signaling pathway and proinflammatory cytokines expression.

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