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# A Novel Chinese Medicine, Xinfeng Capsule, Modulates Proinflammatory Cytokines via Regulating the Toll-Like Receptor 4 (TLR4)/ Mitogen-Activated Protein Kinase (MAPK)/ Nuclear Kappa B (NF-κB) Signaling Pathway in an Adjuvant Arthritis Rat Model

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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**Background:** Rheumatoid arthritis (RA) is a chronic autoimmune disease targeting joints. This research aimed to explore the effects of Xinfeng capsules (XFC) on cardiac injury in adjuvant arthritis (AA) model rats and assessed the associated mechanism.

**Material/Methods:** An adjuvant arthritis (AA) rat model was established by intracutaneously injection with Freund's complete adjuvant (FCA). Model rats were divided into 4 groups: an AA model group, an astragalus polysaccharides (APS) group, a methotrexate (MTX) group, and an XFC and triptolide (TPT) group. Hematoxylin-eosin (HE) staining was used to observe histopathologic changes. TUNEL assay was utilized to evaluate the apoptosis of cardiomyocytes. ELISA was utilized to evaluate levels of tumor necrosis factor α (TNF-α), interleukin 17 (IL-17), and interleukin 6 (IL-6) in myocardial tissues. Quantitative RT-PCR (qRT-PCR) was used to detect microRNA-21 (miRNA21) levels. Mitogen-activated protein kinase (MAPK)/p38, Toll-like receptor 4 (TLR4), and nuclear kappa B (NF-κB)/p65 levels were evaluated using Western blot.

**Results:** XFC significantly improved proinflammatory response compared to the AA model group ($p<0.05$). XFC treatment significantly decreased the number of cells staining TUNEL-positive compared with the model group ($p<0.05$). XFC treatment significantly reduced TNF-α, IL-17, and IL-6 levels in myocardial tissues compared to the model group ($p<0.05$). Levels of miRNA21 were significantly lower in the XFC group compared to the AA model group ($p<0.05$). TLR4, MAPK/p38, and NF-κB/p65 expression levels were significantly lower in the XFC group than in the model group ($p<0.05$).

**Conclusions:** Xinfeng capsule, a traditional Chinese medicine preparation, protects against cardiac injury in AA rats by modulating proinflammatory cytokines expression via the TLR4/MAPK/NF-κB signaling pathway.

**MeSH Keywords:** Cardiomyopathies • Hereditary Autoinflammatory Diseases • Myocardium • Toll-Like Receptor 4

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## Background

Ischemic heart disease is the leading cause of death worldwide [1,2]. In clinical practice, ischemia/reperfusion usually occurs when myocardial blood reaches ischemic regions and later reperfusion cannot completely achieve myocardial reperfusion [3,4]. High levels of dysregulated molecules distributed in cardiac cells after heat-induced IR injury are usually correlated with certain risk factors, including the pathological response and the inflammatory activations. Thus, in this study we investigated the signaling pathways that participate in IR injury of myocardial cells treated with a Traditional Chinese medicine, Xinfeng capsule (XFC). According to Chinese Medicine, XFC obviously strengthens the spleen, effectively replenishes the *Qi*, and alleviates the symptoms of freeing channel and dampness [5].

A previous study [6] reported that cardiac injury occurring in IR processes is triggered by activating tumor necrosis factor α (TNF-α) molecules or nuclear kappa B (NF-κB), both of which are key molecules of the NF-κB/TNF-α signaling pathway, and by activating the Toll-like receptor 4 (TLR4)/NF-κB signaling pathway [7]. A previous study [8] also demonstrated that myocardial survival and remodeling in IR are mainly modulated by transforming growth factor β-activated kinase 1 (TAK1) signaling pathway-associated molecules. Hu et al. [9] reported that the phosphoinositide 3-kinase/protein kinase B/glycogen synthase kinase-3β (PI3K/Akt/GSK-3β) signaling pathway participates in injury to myocardial cells. Thus, discovering the signaling pathways involved in IR injury caused myocardial cell damage help develop a new strategy for treatment of IR injury.

MicroRNAs (miRNAs) are a group of small non-coding RNAs 19–25 nucleotides in length [10]. Normally, miRNAs are highly conserved and endogenously regulate the biological functions of cells [10]. Interestingly, miRNAs play critical roles in regulating the cognate targeting genes, and subsequently induce transcriptional repression or transcriptional degradation [11]. A previous study [12] reported that miRNAs are clinically correlated with various heart diseases and cardiovascular disorders. Jazbutyte et al. [13] reported that miRNA-21 plays a series of biological roles, including cell growth, inflammatory response, and cell apoptosis, and is involved in the pathological processes of many disorders, such as heart diseases and cardiovascular disorders. Therefore, the modulation or suppression of miRNA-21 might have critical effects on the apoptosis or growth of myocardial cells.

Therefore, the present study aimed to evaluate the protective effects of Xinfeng capsule on an AA rat model caused cardiac injury and to explore the associated mechanisms of this protection.

## Material and Methods

### Rats

Thirty-six specific pathogen-free (SPF) Sprague-Dawley rats, age 6–8 weeks, and weight 250–300 g, were obtained from Western Biotech. Co. (Chongqing, China). The rats were housed in an environment with 23–27°C, and a 12 h/12 h dark/light cycle. Standard commercial feed (CLEA Japan Inc., Shizuoka, Japan) and water were freely available. This research was conducted according to the HNI Guide to the Care and Use of Laboratory Animals, and was approved by the Animal Care and Use Committee (Ethics Committee) of Nanjing University of Chinese Medicine (Nanjing, China).

### Adjuvant arthritis (AA) rat model establishment and grouping

Using a random number table, the rats were divided into 2 groups: an AA group (n=30) and a normal control group (NC group, n=6). Rats in the AA group received intracutaneously injection of 0.1 ml Freund's complete adjuvant (FCA, Sigma-Aldrich., St Louis, MO, USA) via the left vola pedis. Twelve days after the first FCA administration, 0.05 ml FCA was injected again to enhance the inflammation and intensify the immunization [14]. Nineteen days after the first FCA administration, rats in the AA model group were randomly divided into 5 groups – an AA model group, an astragalus polysaccharides (APS) group, a methotrexate (MTX) group, an XFC group, and a triptolide (TPT) group – with 6 rats in each group. Rats in the NC group and AA model groups were intragastrically administered normal saline (1 ml/100 g, Sigma-Aldrich., St Louis, MO, USA) once a day for 30 days. For the XFC group, XFC (0.12 g/100 g, Shanghia Fuhua Pharma Industry, Shanghai, China) was intragastrically administrated once a week for 4 weeks. The MTX group were intragastrically administered MTX (1 mg/100 g, Sine Pharma Shanghai Pharma, Shanghai, China) once a week for 4 weeks. In the APS group, the rats were intragastrically administered APS (1 mg/100 g, Sine Pharma Shanghai Pharma, Shanghai, China) once a week for 4 weeks.

### Cardiac tissues and blood sample collection

At the end of the study, normal rats and AA rats were anesthetized by thiopental sodium (at final a concentration of 50 mg/kg) and then killed. The cardiac tissues were isolated and stored at –80°C for experiments. Blood samples were collected from the heart using cardiac puncture, and centrifuged for 10 min at 1500 rpm. Then, the obtained serum samples were collected and stored at –80°C for experiments.

### Hematoxylin-eosin (HE) staining

For the inflammation and morphological evaluation of cardiac tissues, the tissues were isolated and immersed in 4%





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paraformaldehyde (Sigma-Aldrich., St Louis, MO, USA) to fix cardiac tissues. Then, cardiac tissues were dehydrated, permeabilized, embedded, and cut into 6-μm-thick sections for hematoxylin staining (Nanjing Jiancheng Pharma, Nanjing, China) and eosin staining (Beyotime Biotechnology, Shanghai, China) according to the method reported everywhere. Finally, the stained HE sections were examined using a microscope (Mode: CX31, Olympus, Tokyo, Japan).

### TUNEL assay

The apoptosis of the cardiac tissues was evaluated using TUNEL assay according to the instructions of the manufacturer (Roche Pharma., Basel, Switzerland). In brief, the cardiac tissues were cut into 15-μm sections and mounted onto glass slides. The sections were incubated using DNase-free proteinase K (Biyotime Biotech. Shanghai, China) and 3% $H_2O_2$ (Beyotime Biotech, Shanghai, China) to inactivate the activity of endogenous peroxidase. The Free 3'-OH termini were treated with the terminal deoxynucleotidyl transferase at 37°C for 60 min. Finally, the sections were cleared using xylene (Beyotime Biotech, Shanghai, China), mounted using Canada balsam (Beyotime Biotech, Shanghai, China), and covered using coverslips (Corning Costar, Acton, MA, USA).

### Enzyme-linked immunosorbent assay (ELISA)

The serum levels of TNF-α, IL-17, and IL-6 were detected using commercially obtained ELISA kits (Lkcx Tech., Beijing, China) following the manufacturers' instructions. All ELISA tests in this study were conducted and repeated at least 6 times. TNF-α, IL-17, and IL-6 levels were measured with an ELISA plate reader (Mode: EL-311, Bio-Tek, Inc., Winooski, VT, USA).

### Quantitative RT-PCR (qRT-PCR)

The cardiac tissues were lysed with a tissue homogenate machine (Scientz Bio. Tech., Inc., Ningbo, China) and protein lysis buffer (Biyotime Biotech. Shanghai, China) according to instructions of the manufacturer. Total RNAs were extracted with cell lysis buffer (Beyotime Biotech. Co., Shanghai, China). These RNAs were reversely transcribed with reverse transcription regents and kits (Western Biotech, Chongqing, China) to obtain complementary DNAs (cDNAs). PCR primers (Table 1) for miRNA21 and β-actin were synthesized by Western Biotech., Inc. (Chongqing, China). The SYBR Green I real-time PCR kit (Catalogue No. 218073, Western Biotech, Chongqing, China) was used to amplify the miRNA21 gene following the instructions of the manufacturer. Amplification conditions for quantitative RT-PCR (qRT-PCR) were: 94°C for 4 min, followed by 35 cycles of 20 s at 94°C, 30 s at 60°C, and 30 s at 72°C, and terminated for 10 min at 72°C. All experiments were conducted at least for 6 times. The $2^{-\Delta\Delta Ct}$ method was used to analyze the qRT-PCR results.

**Table 1.** Primer sequences for quantitative RT-PCR.

| Gene | | Sequences |
|---|---|---|
| miRNA21 | Forward | 5'-CGGTAGCTTATCAGACTG-3' |
| | Reverse | 5'-GAGCAGGCTGGAGAA-3' |
| β-actin | Forward | 5'-CCCATCTATGAGGGTTACGC-3' |
| | Reverse | 5'-TTTAATGTCACGCACGATTTC-3' |

### Western blot assay

Cardiac tissues were lysed with a tissue homogenate machine (Scientz Bio. Tech., Inc., Ningbo, China) and radioimmunoprecipitation assay (RIPA) regents (Sigma-Aldrich, St. Louis, MO, USA). The harvested protein was isolated and separated with 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime Biotech. Co., Shanghai, China), and electro-transferred onto the PVDF membrane (DuPont, Wilmington, DE, USA). We used 5% bovine serum albumin (Gibco BRL. Co. Ltd., Grand Island, New York, USA) dissolved into phosphate-buffered saline (PBS) to block the PVDF membranes. Then, the PVDF membranes were treated with mouse anti-rat p-p38 monoclonal antibody (1: 3000; Cat. No. ab45381), mouse anti-rat TLR4 monoclonal antibody (1: 3000; Cat. No. ab30667), and rabbit anti-rat p-p65 polyclonal antibody (1: 2000; Cat. No. ab86299) for 2 h at room temperature. All of the above antibodies were purchased from Abcam Biotech (Cambridge, MA, USA). PVDF membranes were continuously washed with PBST and treated with horse radish peroxidase (HRP)- conjugated goat anti-mouse IgG (Cat. No. AP127P, Sigma-Aldrich, St. Louis, MO, USA) and HRP-conjugated goat anti-rabbit IgG (Cat. No. A0545, Sigma-Aldrich) at 37°C for 2 h. The Western blot bands on the PVDF membranes were developed and imaged with the ECL Western Blot Substrate Kit (Cat. No. 32109, Thermo Scientific/Pierce, Rockford, IL, USA). The developed films were photographed and analyzed using a professional image-scanning system (Mode: GDS8000, UVP, Sacramento, CA, USA).

### Statistical analysis

Data were analyzed using SPSS software 19.0 (SPSS Inc., Chicago, IL, USA) and are expressed as mean ± standard deviation (SD). All experiments were performed at least 6 times. Multiple group comparisons were performed using ANOVA. Comparisons of differences between 2 groups were conducted using the *t* test. $P<0.05$ was set as the level of statistical significance.





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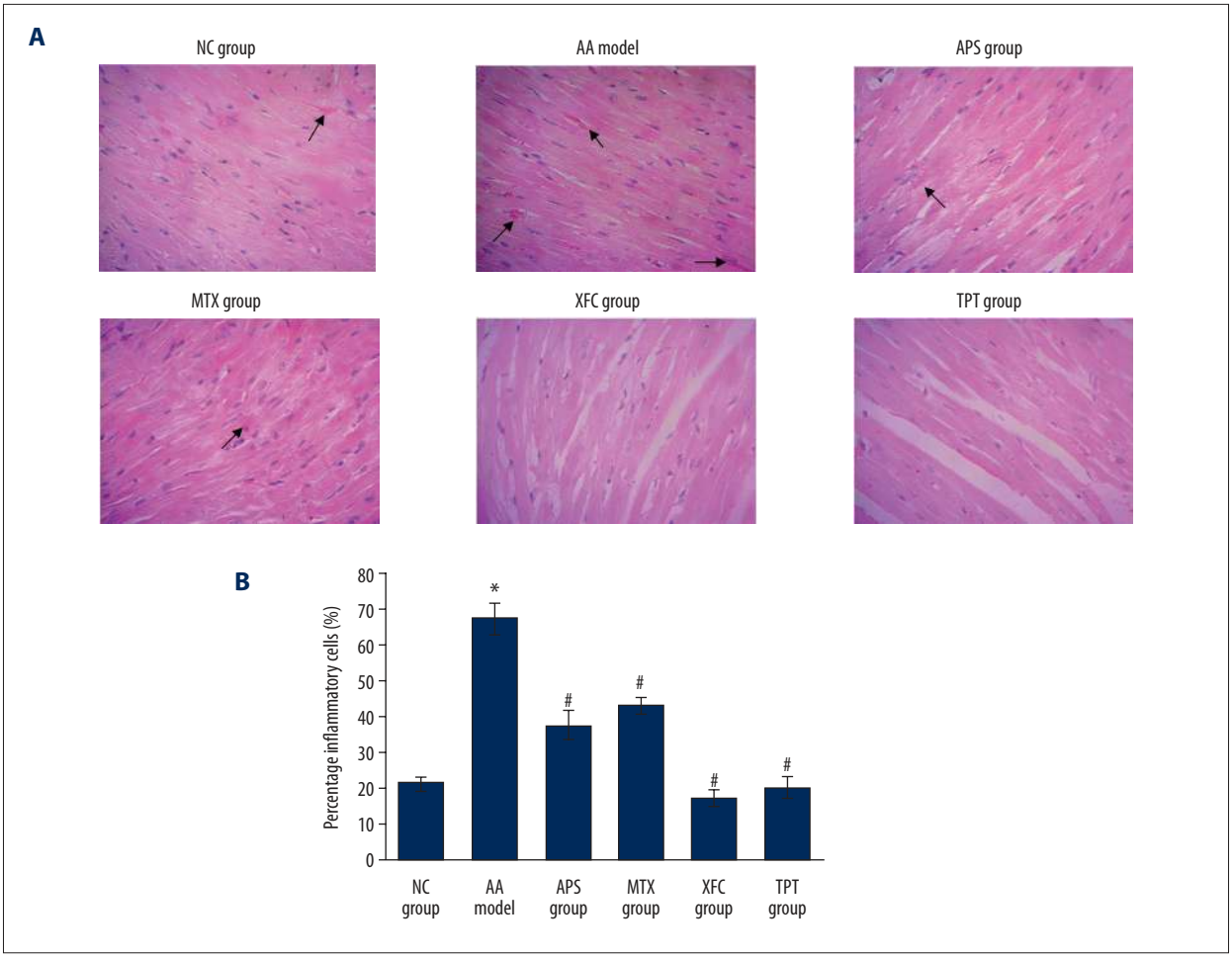


**Figure 1.** HE staining indicating inflammation of cardiac tissues of AA model rats in different groups. (**A**) HE staining images. (**B**) Statistical analysis of HE staining. The black arrows indicate inflammatory cells. * $p<0.05$ *vs.* NC group, $^{\#}p<0.05$ *vs.* AA model group.

## Results

### XFC reduced inflammatory response of AA rat model

HE staining was used to evaluate inflammation in the AA model rats (Figure 1A). The results showed that the inflammatory response in the AA model group was greater compared to that of the NC group (Figure 1B, $p<0.05$). All of the drugs (APS, MTX, XFC, and TPT) significantly reduced the inflammatory response compared to that of the AA model group (Figure 1B, $p<0.05$). Interestingly, XFC treatment had the best inflammation-inhibiting effects (Figure 1B).

### XFC treatment inhibited apoptosis of AA rat cardiac tissues

The TUNEL assay was used to evaluate apoptosis in cardiac tissues of AA model rats (Figure 2A). TUNEL-positive staining indicated apoptotic tissues. The results indicated significantly more TUNEL-positive cells (apoptosis) than in the NC group (Figure 2B, $p<0.05$). The percentage of cells staining TUNEL-positive (apoptosis) in the APS group, MTX group, XFC group, and TPT group was significantly lower compared to that of the AA model group (Figure 2B, $p<0.05$). XFC had the greatest inhibitive effect on apoptosis of cardiac tissues (Figure 2B).

### XFC decreased proinflammatory cytokine levels

Serum levels of TNF-α (Figure 3A), IL-6 (Figure 3B), and IL-17 (Figure 3C) in the AA model group were significantly higher than in the NC group (Figure 3, $p<0.01$), but the levels of TNF-α, IL-17, and IL-6 in the APS, MTX, XFC, and TPT groups were significantly lower than in the AA model group (Figure 3, $p<0.01$). XFC treatment was most effective in inhibiting pro-inflammation effects compared with other drug treatments (Figure 3).





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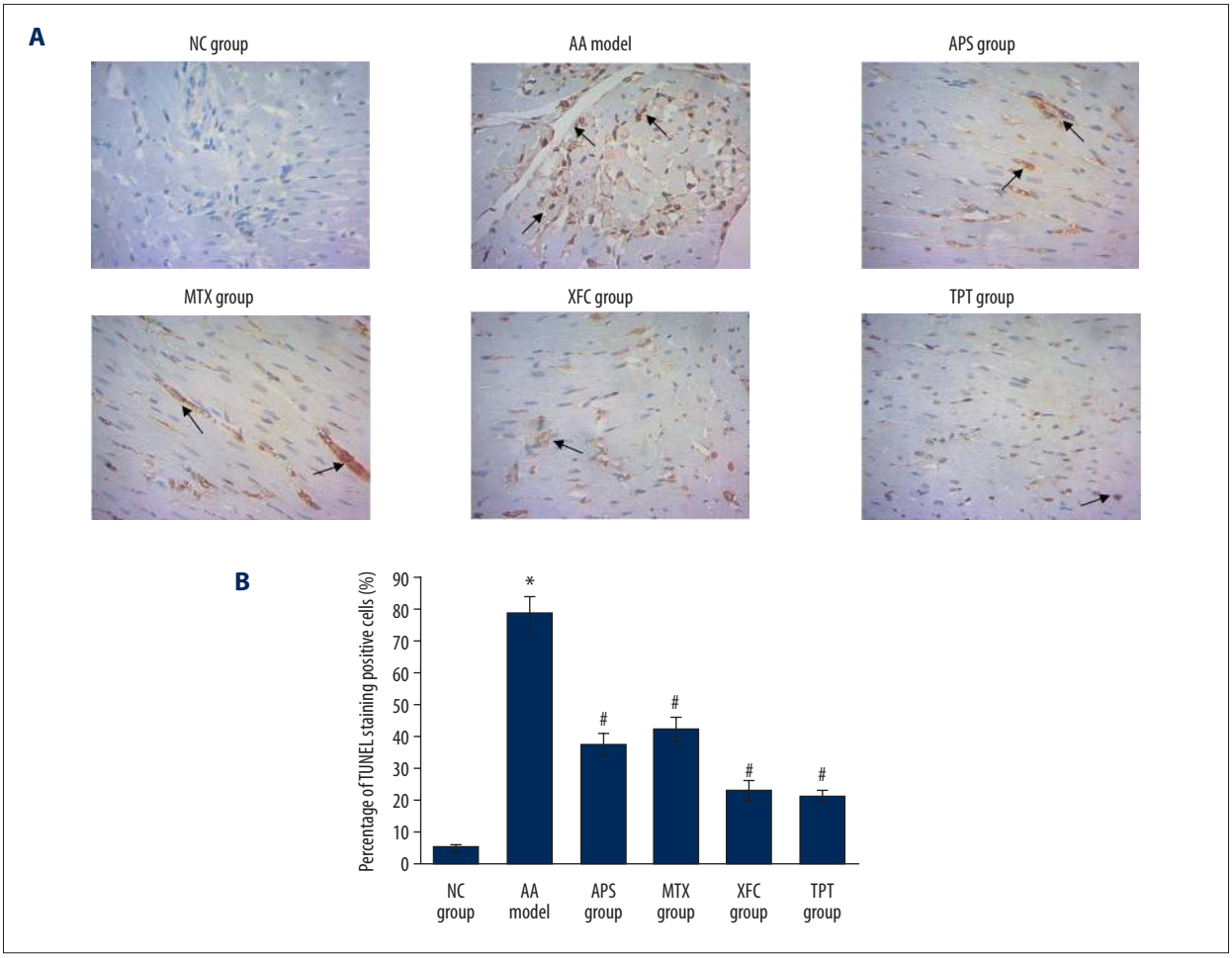


**Figure 2.** Evaluation of apoptosis by TUNEL staining. (**A**) TUNEL staining images for apoptosis. (**B**) Statistical analysis of TUNEL staining. The black arrows indicate staining TUNEL-positive. * $p<0.05$ *vs.* NC group, # $p<0.05$ *vs.* AA model group.

### XFC treatment inhibited miRNA-21 expression

To assess effects of XFC on miRNA-21 expression, the miRNA-21 levels were evaluated with qRT-PCR assay. The findings showed that levels in the miRNA-21 in AA model group were significantly higher than in the NC group (Figure 4, $p<0.05$), while the levels of miRNA-21 in the APS, MTX, XFC, and TPT groups were significantly lower than in the AA model group (Figure 4, $p<0.01$). Moreover, miRNA-21 levels in the XFC group were even obviously lower compared to the other drug treatment groups (Figure 4).

### XFC treatment decreased the p-p38, p-p65, and TLR4 levels

In this experiment, the cell death-associated molecules p-p38, p-p65, and TLR4 were examined with Western blot assay (Figure 5A). The findings showed that the TLR4 levels (Figure 5B), p-p38 levels (Figure 5C), and p-p65 levels (Figure 5D) in the AA model group were significantly higher than that of the NC group ($p<0.05$). Levels of p-p38, p-p65, and TLR4 in the APS group, MTX group, XFC group, and TPT group were significantly lower compared to the AA model group (Figure 5B–5D, $p<0.05$). Furthermore, XFC exhibited the greatest inhibitive effects on the expression of p-p38, p-p65, and TLR4 in cardiac tissues from the AA model rats.

## Discussion

Cardiac I/R injury is characterized by a few myocardial episodes induced by myocardial reperfusion and coronary recanalization after myocardial ischemia [15,16]. I/R injury cause a series of complex pathological and physiological changes [17]. Therefore, we investigated the effects of Xinfeng capsule on cardiac injury and cardiomyocyte apoptosis. We also assessed the associated mechanisms underlying the protective effects of Xinfeng capsule.





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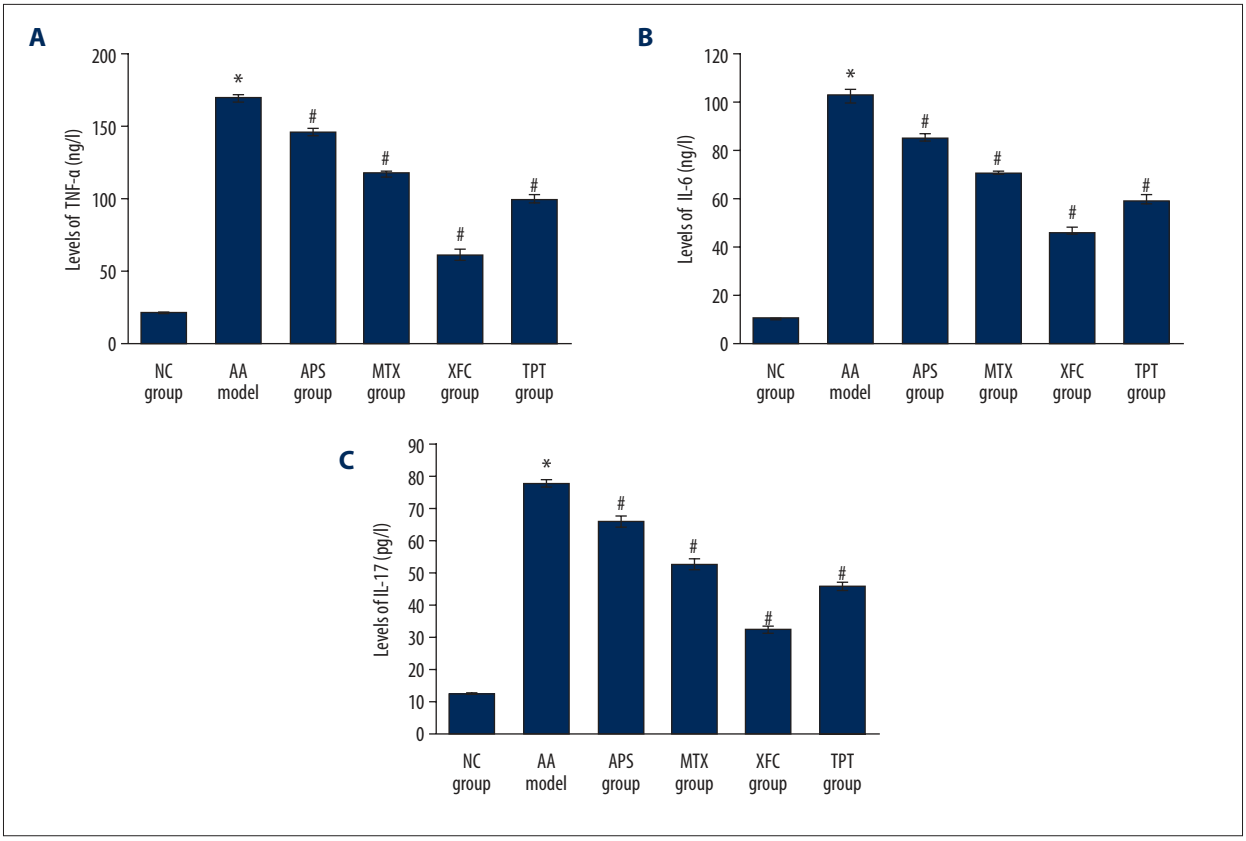


**Figure 3.** Proinflammatory cytokine levels in serum were evaluated by ELISA. (**A**) Statistical analysis of TNF-α levels. (**B**) Statistical analysis of IL-6 levels. (**C**) Statistical analysis of IL-17 levels. * $p<0.05$ *vs.* NC group, # $p<0.05$ *vs.* AA model group.

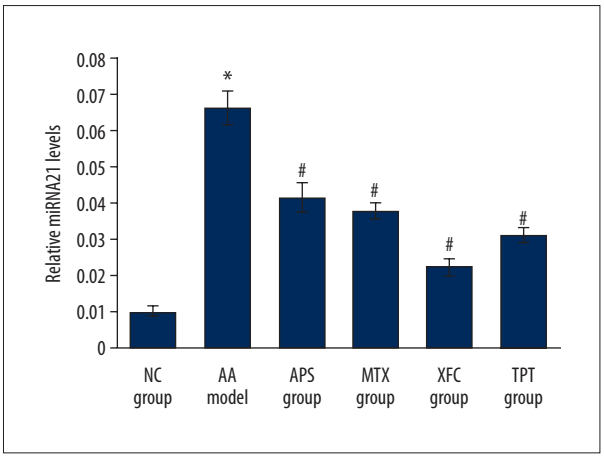


**Figure 4.** miRNA-21 expression assessed by qRT-PCR in every group. * $p<0.05$ *vs.* NC group, # $p<0.05$ *vs.* AA model group.

In response to intracellular physiological or pathological stimuli and cell injuries, the miRNAs were activated and endogenously overexpressed [18]. Previous studies [19, 20] reported that miRNA-21 expression was induced in heart tissues or myocardial cells after I/R injury, and finally caused myocardial cell injuries. Based on qRT-PCR assay findings, levels of miRNA-21 in the AA model group were significantly higher than in the NC group. Nevertheless, miRNA-21 levels in the XFC group and other drug treatment groups were significantly lower than in the AA model. These findings suggest that XFC inhibits miRNA-21 expression.

Apoptosis (programmed cell death) is widely considered to be induced by genetic factors [21], and finally causes cell death. Therefore, cell apoptosis was detected in groups by using TUNEL staining. The findings showed that late and early apoptosis in the APS, MTX, XFC, and TPT groups were significantly lower than in the AA model group. XFC treatment exhibited the best apoptosis-inhibitive effects, which suggests that XFC could be extensively applied for suppressing cardiomyocytes apoptosis in AA rat models.

TLR family molecules were previously reported to be a series of important regulators that participate in inflammation and immune processes [22]. The TLR-associated molecules activate many signaling pathways, such as the MAPKs/p38 signaling pathway and NF-κB/p65 signaling pathway. TLR molecules then modulate the transcription of inflammation-associated genes [23], such as TNF-α associated signaling pathway genes and interleukin family genes. In the present study, serum levels





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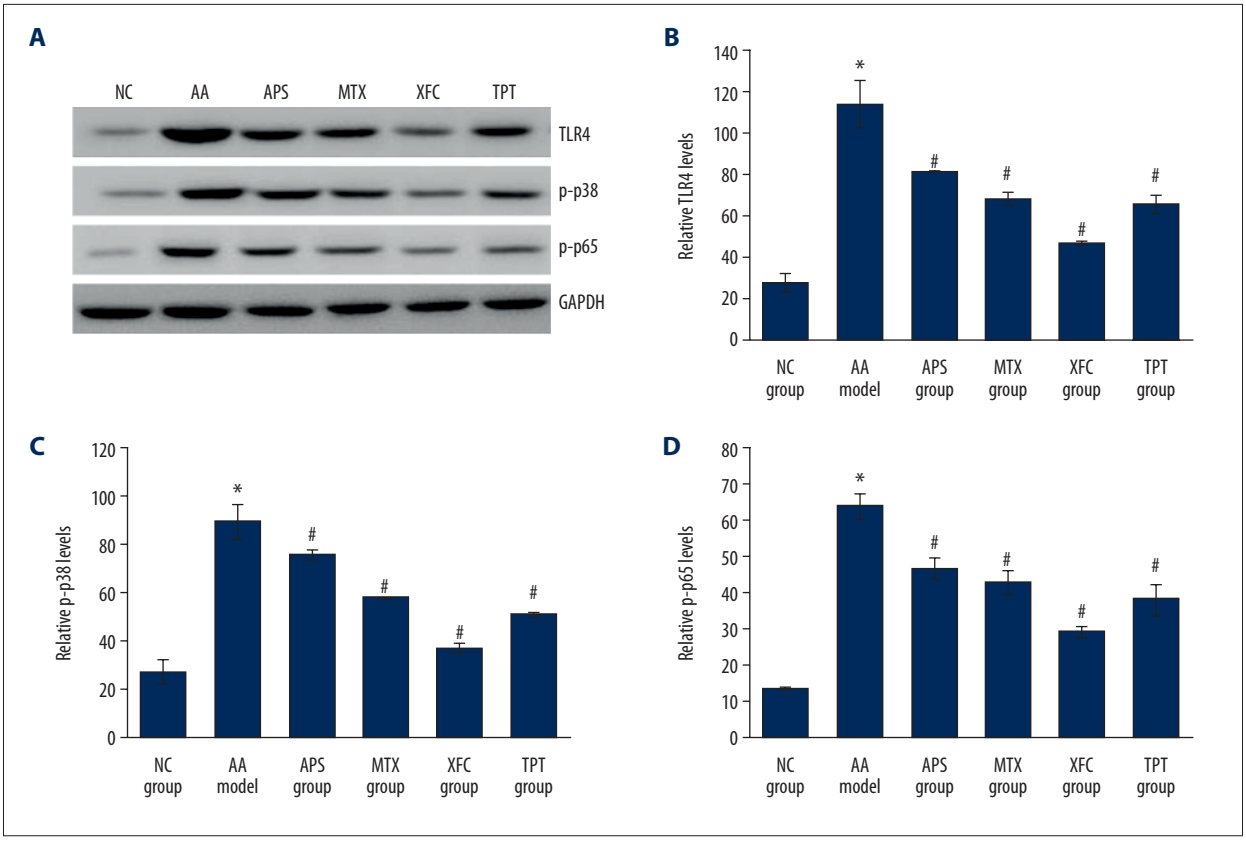


**Figure 5.** Examination of the TLR4, p-p38, and p-p65 expression by Western blot assay. (**A**) Western blot assay bands for every group. The statistical analyses were also performed for TLR4 (**B**), p-p38 (**C**), and p-p65 (**D**) in every group. * $p<0.05$ *vs.* NC group, # $p<0.05$ *vs.* AA model group.

of p-p38, p-p65, and TLR4 in groups were examined. Western blot results showed that levels of p-p38, p-p65, and TLR4 in the AA model group were significantly higher compared to the NC group. However, p-p38, p-p65, and TLR4 levels in the XFC group were significantly lower than in the AA model group, which suggests that XFC protects against cardiac injury through triggering the TLR4/p-38/p65 signal pathway.

Previously published studies [24,25] demonstrated that the cardiac damage caused by rheumatoid arthritis is related to proinflammatory cytokines, including TNF-α, IL-17, and IL-6. Thus, cytokine levels in cells treated with XFC were examined in this study. The results indicated that the levels of TNF-α, IL-17, and IL-6 were significantly lower in the XFC group than in the AA group. The above findings suggest that XFC inhibits the inflammatory response and may be effective in treating rheumatoid arthritis.

## Conclusions

XFC significantly improved proinflammatory response, decreased the number of cells staining TUNEL-positive, reduced proinflammatory cytokine levels, and decreased TLR4, MAPK/p38, and NF-κB/p65 levels in AA model rats. In summary, Xinfeng capsule, a Chinese traditional medicine, protects against cardiac injury in an AA rat model by modulating proinflammatory cytokines expression via the TLR4/MAPK/NF-κB signaling pathway.

### Conflict of interest

None.





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