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SHENQI FUZHENG INJECTION IMPROVES CVB3-INDUCED MYOCARDITIS VIA INHIBITING TRAF6 EXPRESSION

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Abstract

Viral myocarditis is a main cause of heart failure in young adults, which characterized by cardiac inflammation and caused by Coxsackievirus B3 (CVB3) infection. However, efficient therapies targeting inflammation and inflammatory response pathway are still elusive. Shenqi Fuzheng injection (SQFZI) is extensively applied in the cardiovascular diseases. But whether SQFZI may affect cardiac inflammation during acute viral myocarditis remains to be elucidated. The purpose of the present study was to investigate the potential protective effect of SQFZI on CVB3-induced myocarditis. Total of 120 mice were intraperitoneally inoculated with CVB3 to establish acute viral myocarditis model. For the CVB3-infected mice model, the body weight, mortality was observed. RT-PCR, western blot and immunohistochemistry methods were selected to detect the TRAF6 expression in myocardial tissues. We found that the expression of TRAF6 mRNA and protein were markedly and persistently increased during the progression of CVB3-induced myocarditis. The serum enzymes activity, including CK, CK-MB, LDH, AST, were also enhanced in CVB3-induced myocardial tissues. Notably, injection with SQFZI remarkably reduced CVB3-induced TRAF6 production and alleviated the severity of myocarditis. This study demonstrates the protective role of SQFZI against CVB3-induced myocarditis, which may explore a new therapeutic strategy for the treatment of viral myocarditis.

Key words: Shenqi fuzheng injection, CVB3, myocarditis, TRAF6.

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INTRODUCTION

Viral myocarditis is a main cause of heart failure in young adults and often progresses to chronic myocarditis, congestive heart failure, and dilated cardiomyopathy. Coxsackievirus B3 is known to be the most common causative agent in human myocarditis (8,12). In spite of the extensive exploration was performed in the past decades, the pathogenesis of viral myocarditis is also elusive, and there is no effective therapy strategy for this disease so far. Experimental studies have indicated that although CVB3 can directly destroy myocardium (11), the overwhelming inflammatory response is primarily responsible for myocyte damage (3). What's important is that the inflammatory response is closely associated with the signal-transduction activation, especially for the toll-like receptor 4/nuclear factor kappaB signal pathway (TLR4-NF-kB) (9,26). Among the signal-transduction molecules involve in the TLR4-NF-kB pathway, the TRAF6 is the most important molecule (10,17). Clinical studies have also found increased levels of circulating tumor necrosis factor-α (TNF-α), IL-6 and other pro-inflammatory cytokines in patients with myocarditis (16), but the molecules in the TLR4-NF-kB pathway were seldom detected in the clinical. In clinical, many immunosuppressive drugs are used to control inflammation (22), but no drugs targeting to the TLR4-NF-kB pathway for the myocarditis patients. So blocking the inflammatory response signal-transduction pathway considers as a potential therapeutic strategy for viral myocarditis.

Actually, a few approaches also have been reported to regulate the signal-transduction pathway for treating viral myocarditis in mice. Ding et al. (6) reported that cinnamaldehyde can directly reduce the inflammation in VMC by inhibiting the TLR4-NF-kB signal-transduction pathway. Besides, it has been found that the extracellular heat shock cognate protein 70 plays a critical role in regulating the myocarditis innate immune response and cardiac function after ischemia-reperfusion through a TLR4-dependent pathway (33). Some clinical researches also indicated that cardiomyocytes express Toll-like receptors, especially for the TLR2, TLR4 and TLR5 signal via NF-kB, which results in the decreased contractility and a concerted inflammatory response (2). Our previous study (28) also showed that inhibition of the TLR4-NF-kB pathway could alleviate myocardial inflammation by modulating local cytokine profile. However, the key factor induced the blocking of the TLR4-NF-kB pathway in the viral myocarditis, as well as the efficient therapy targeting inflammation are still needed further development. So exploiting the new therapeutic strategies is much necessary.

Shenqi Fuzheng injection (SQFZI), being composed of traditional Chinese medicine extraction of Codonopsis pilosula and Astragalus mongholicus, which is extensively applied in the cardiovascular diseases (18,20,25). Experimental studies have indicated its critical role for improving and regulating the immune function, and preventing inflammation in vivo (29). The protective role of SQFZI has also been reported in several other diseases, including immuno-enhancement disease (27), lung cancer (7) and breast cancer (5). However, whether the SQFZI could regulate the TLR4-NF-kB signal-transduction pathway and affect cardiac inflammation during viral myocarditis are still unclear. So the present study was performed to examine the potential protective effect of SQFZI on CVB3-induced myocarditis.

In this study, the SQFZI was injected to the CVB3-induced myocarditis mice. Mice were intravenously injected with SQFZI 0.5 hours after CVB3 inoculation. TRAF6, Serum myocardial enzymes and myofibrils in cardiac tis-

sues of CVB3 infected mice with SQFZI were analyzed. The therapeutic effect of SQFZI on viral myocarditis was assessed carefully and its possible mechanism involved was explored.

MATERIALS AND METHODS

Mice and Virus

Specific pathogen free (SPF) male Balb/C mice, 6 weeks and 20 gram, were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. CVB3 (Nancy strain) was maintained by passage through HeLa cells, and obtained from Wuhan Institute of Virology, Chinese Academy of Science. Viral was routinely determined prior to infection by a 50% tissue culture infectious dose (TCID50, 10^{-7}) assay of HeLa cell monolayer. All experiments performed in this study were strictly accord to a manner to minimize suffering of laboratory mice. All animal procedures were performed according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, P.R. China, 1998).

Trail grouping

The total 120 mice were divided into 5 groups randomly: no-CVB3 infection group (CON, 20 mice, 0.1 ml Eagle's without CVB3) and 4 groups of CVB3 infected mice (0.1 ml Eagle's with 1×10^2 TCID50), including model group (CVB3, 28 mice), ribavirin group (RIB, 24 mice), low-dose SQFZI group (LSQFZI, 24 mice), high-dose SQFZI group (HSQFZI, 24 mice). 5 mice were sacrificed in the 3rd and 10th day, all of the survival mice were sacrificed in the 30th day for every group.

CVB3 infection and SQFZI injection in mice

Mice in CVB3, RIB, LSQFZI, HSQFZI group were infected by intraperitoneal injection with 1×102 TCID50 CVB3 at day 0. To examine the therapeutic effects of SQFZI, the mice received intravenous injection of SQFZI after CVB3 infection. We performed the dose-dependent (from 0.1ml to 2.0ml) experiments, and the results showed that when the amount increased to 0.4ml, the SQFZI began to protect the model mice (more than a half mice were protected), and 0.9 ml achieved the peak protective function. We also detected the total saponin of astragaloside and codonopsis pilosula by HPLC-ELSD method, and the concentration are 0.082mg/ml and 0.048mg/ml, respectively. So we the mice received intravenous injection of low-dose (0.4 ml), high-dose (0.9 ml) SQFZI 0.5 hours after CVB3 infection. Infected mice receiving Eagle's without CVB3 (saline, 0.4 ml) and RIB (containing 2.7 mg in 0.9 ml saline) were used as control.

Tissue histopathology and myocarditis grading

Three, ten and thirty days following CVB3-infection, the heart tissues were collected, sectioned and stained with hematoxylin and eosin (HE). Sections were examined by two independent investigators in a blinded manner, and the severity of myocarditis was assessed using the same procedures as previously described (13), 0-4 scale, in which 0= no inflammation; 1=less than 25% inflammation; 2=25%-50% inflammation; 3=51%-75% inflammation; 4=more than 75% inflammation.

Cardiac enzymes assays

Levels of aspartate transaminase (AST), lactate dehydrogenase (LDH), creatine kinase (CK), Creatine kinase isoenzyme (CK-MB) of cell culture supernatants and heart homogenates were detected by fully automatic biochemical analyzer following the manufacturer's instructions (Ebioscience, USA).

RNA extraction and hemi-quantitative RT-PCR

In order to evaluate the transcriptional status of TRAF6 and cardiac enzymes in the myocardic tissue, the hemi-quantitative RT-PCR assay was performed. The forward primer is 5'- GAATCACTTGGCACGACACTT-3', reverse primer is 5'- GCTCGCCCACGTACA TACTCT-3'. In parallel, we selected the individual GADPH as the internal control, with the forward primer: 5'-AAGCCCATCACCATCTTCCAG-3', and reverse primer: 5'-TGAGCCCTTCCACAATGCC-3'. The primer sequences of the cardiac anzymes were listed in the Table 1. With an RNAsimple Total RNA Kit (TIANGEN, China), total tissue RNA was prepared. Reverse transcription was performed using SuperScript™ III First-Strand Synthesis System (Invitrogen, USA) as the manufacturer's protocol. After electrophoresed on 1.5% agarose gel, the gel images

Table 1. The sequences of the primers used in the semi-quantitative RT-PCR analysis for mRNA levels of the cardiac enzyme genes.

Gene		Sequences
β-actin	Forward	GGACTTCGAGCAGGAGATGG
	Reverse	GCACCGTGTTGGCGTAGAGG
AST	Forward	CCAGGGAGCTCGGATCGT
	Reverse	GCCATTGTCTTCACGTTTCCTT
LDH	Forward	GGCCTGTGCCATCAGTATCT
	Reverse	GCCGTGATAATGACCAGCTT
CK	Forward	GCTCTCTGTGGAAGCTCTCAACA
	Reverse	GATGAGCTGCTGCTGCTCCT
CK-MB	Forward	GCAGCTCATCGACGACCACTTC
	Reverse	GGGCACTGCAGGCAATAAGTTA

of each PCR product were digitally captured with a CCD camera and analyzed with the NIH Imager beta version 2. Relative transcriptional values of each factor in hemi-quantitative RT-PCR are presented as a ratio of the signal value of the specific PCR product and that of the individual GADPH.

Western blot

The Myocardic tissue lysates were separated by 15% SDS-PAGE and electro-transferred onto nitrocellulose membranes. After blocking with 5% deffated milk in PBST (phosphate buffered saline, pH 7.6, containing 0.05% Tween-20) overnight at 4 °C, the membranes were incubated with 1:300 TRAF6 specific polyclonal antibody (pAb) (Cambridge, UK), 1:1000 CK specific monoclonal antibody (mAb) (Santa Cruz, USA), 1:1000 CK-MB specific mAb (Santa Cruz, USA), 1:500 specific LDH pAb (Santa Cruz, USA), 1:1000 AST specific mAb (Santa Cruz, USA), 1:1000 β-actin specific monoclonal antibody (mAb) for 2h at room temperature, and then incubated with 1:2000 horseradish peroxidase (HRP)-conjugated anti-rabit IgG (Santa Cruz, USA). The reactive signals were visualized by ECL kit (PE Applied Biosystems, USA).

Immunohistochemistry

The indirect streptavidin-biotin-peroxidase complex method was applied to 4-μ-thick cryostat serial sections. The sections were incubated with rabbit polyclonal antibody against human TRAF6 protein, which recognizes the TRAF6 protein. Antigen-antibody binding was demonstrated using horseradish peroxidase-labeled polymer conjugated to rabbit secondary antibodies by the dextranpolymer technique (Envision+kit/HRP, DAKO, Japan).

Statistic analysis

Quantitative analysis of immunoblot images was carried out using computer-assisted software Image Total Tech (Pharmacia, USA). Briefly, the image of immunoblot was scanned with Typhoon (Pharmacia, USA) and digitalized, saved as TIF format. The values of each target blot were evaluated. All data are presented as the mean ± SD. Statistical analysis was performed using the *T* test. Probabilities of less than 0.05 were considered to be statistically significant.

RESULTS

Cardiac inflammation was markedly enhanced in CVB3 infected mice

The mice which administered intraperitoneal injection with 1×10^2 TCID50 CVB3 at day 0 were selected to be the acute viral myocarditis model. The changes of the body weight were monitored daily until 30 days past-infection. The histological analysis of heart tissues was performed at day 0, 3, 10, 30 days, respectively. As shown in Figure 1A, the body weight of CVB3 infected mice was significantly and continuously decreased since the third day compared with the CON group, but the change was significant from the tenth day ($P<0.01$). There were no dead mice in the CON group in every day till the 30th days. The mortality of the mice in the CVB3 group were significantly increased, including 1 in third day, 7 in tenth day, 2 in thirtieth day, and total 35.71% (10/28) mortality (Figure 1B).

Histopathology of cardiac tissues showed that the myo-

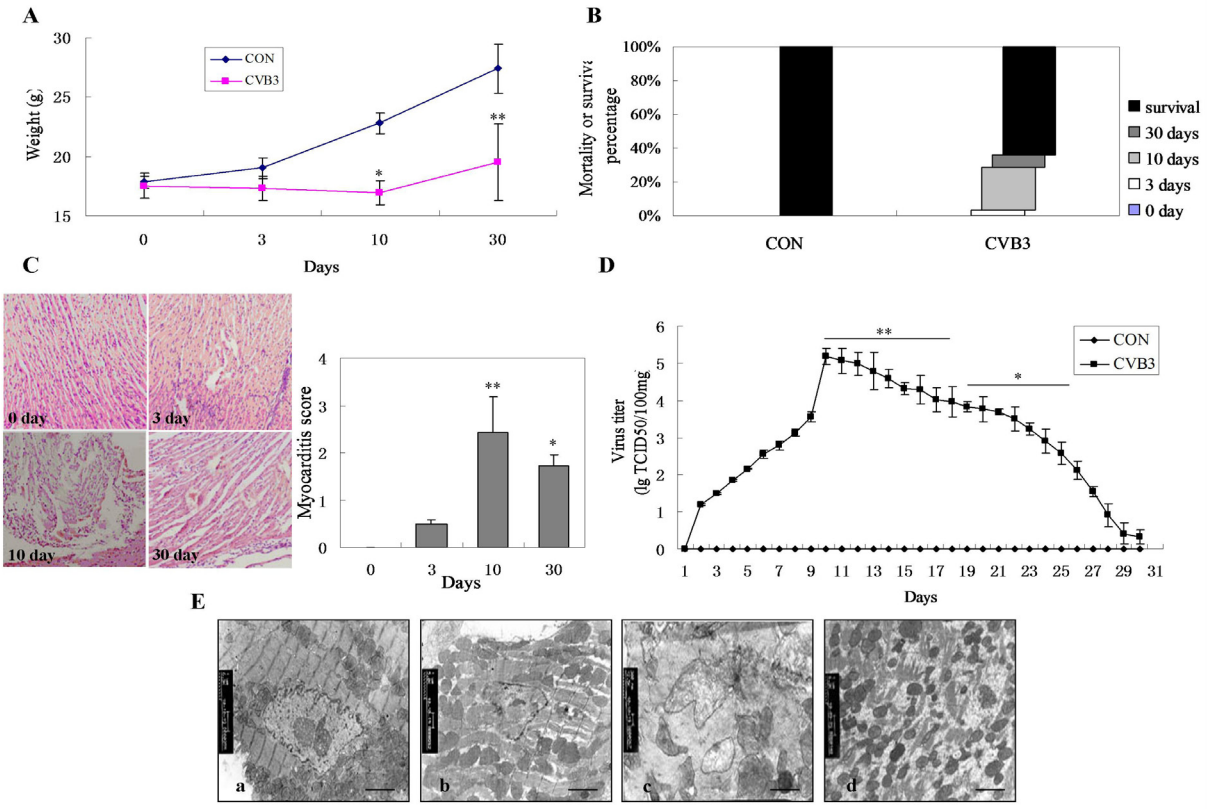

Figure 1. Expression of inflammatory response cytokines in acute myocarditis. The body weight changes (A) and mortality (B) were monitored daily until day 30 post-infection. C. Paraffin sections of heart tissues were prepared on day 0, 3, 10, 30, respectively. The cardiac inflammation was revealed by HE staining (magnification: 200×) (left). The severity of myocarditis was scored by a standard 0-4 scale according to the foci of mononuclear infiltration and myocardial necrosis (right). D. Hearts were removed aseptically, weighed, and homogenized daily post-infection for TCID50 assay. E. Electron microscope analysis for the cardiac tissues from viral myocarditis. The scale bar represents ×6300 in the EM image.

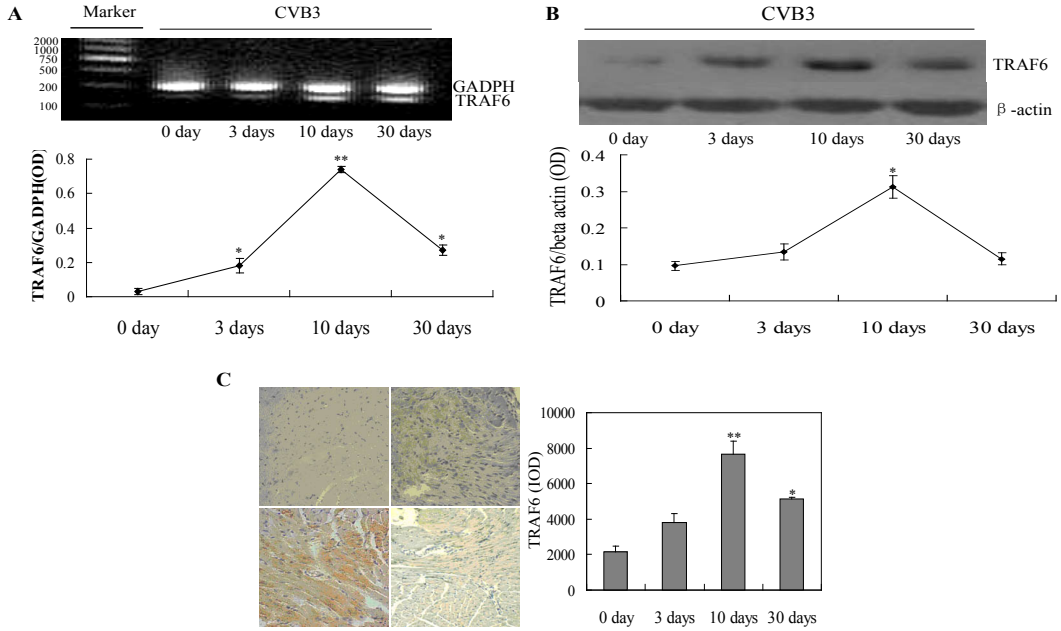

Figure 2. Activation of TRAF6 production in CVB3 infected mice. A. Heart tissue homogenates prepared at indicated time points were subjected to RT-PCR analysis with specific premier. GADPH was used as loading control. Similar results were obtained in three independent experiments. B. Western blot analysis for the TRAF6 production in heart tissue homogenates. C. immunohistochemistry analysis for the TRAF6 expression in heart tissue homogenates.

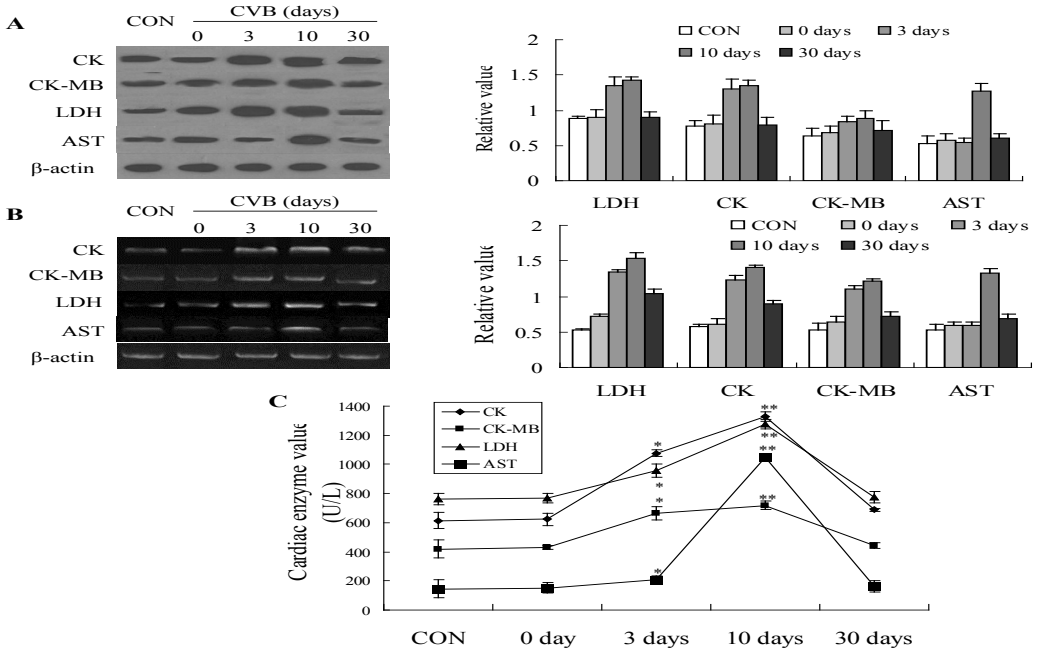

Figure 3. Enhanced level of cardiac enzyme in CVB3 infected mice. A. Western blot analysis for the CK, CK-MB, LDH and AST expression; B. RT-PCR assay for the CK, CK-MB, LDH and AST mRNA transcription; C. The activity of CK, CK-MB, LDH and AST in mouse serum on day 0, 3, 10, 30 post-infection.

cardial injury was apparently observed at the third day and increasingly severe in the following days ($P<0.05$) (Figure 1C). The virus titer detection results showed that the virus titer was peaked at tenth day, and then reduced gradually in the following days till 30 days (Figure 1D).

When examined with Electron microscope (EM), cardiac myocytes showed clumped chromatin material in the nucleus, mitochondrial cristae were disrupted, and electron dense deposits were observed within the mitochondria in CVB3 group, compared with the CON group (Figure 1E).

TRAF6 levels up-regulated and cardiac enzymes level increased in the CVB3 group

To further investigate the mechanism of the induction of pathological process of CVB3 induced viral myocarditis, the TLR4-NF-kB pathway factor TRAF6 was detected by RT-PCR, western blot and immunohistochemistry in cardiac tissues. The results showed that the TRAF6 mRNA, TRAF6 protein and TRAF6 positive staining tissues were significantly up-regulated, compared with the CON group or no CVB3 infected mice (0 day) (Figure 2A,B,C, all $P<0.01$).

Furthermore, cardiac enzymes, including AST, LDH, CK and CK-MB, were also detected by western blotting assay, RT-PCR and ELISA analysis. Both of the western blotting (Figure 3A) and RT-PCR (Figure 3B) results showed that all of the four enzymes were significantly up-regulated expression and increased the transcription, compared with the CON group ($P<0.05$). The ELISA assay results also indicated that all of the above four cardiac enzymes acti-

vity were significantly increased compared with the CON group, and the peak value appeared in the 10 days after infection (Figure 3C).

Treatment with SQFZI alleviated the CVB3-induced myocarditis

To exclude the probability that the alleviation of the myocarditis caused by the inhibition of CVB3 infection, the virus titers were detected in every group and different time point. The results showed that no significant differences were discovered among the CVB3, LSQFZI and HSQFZI group, but the virus titer of RIB decreased significantly ($P<0.05$) (Figure 4A).

In order to investigated the therapeutic effect of SQFZI on CVB3-induced acute myocarditis, mice were intravenously injected with ribavirin or 0.4 and 0.9 ml SQFZI 0.5 hours after CVB3 inoculation. Parameters of the severity of myocarditis, including body loss, survival rate, serum CK, CK-MB, LDH and AST activity. As shown in Figure 4B, mice with LSQFZI and HSQFZI treatment significant increased the body weight, and even equal to the weight of CON group, compared with CVB3 group ($P<0.01$) within 30 days post-infection. On the contrary, mice receiving SQFZI had a significantly decreased mortality (20.8 in LSQFZI group, 8.3% in HSQFZI group), compared with 35.7 in CVB3 group ($P<0.01$, Figure 4C). Consistently, serological indices of CK, CK-MB, LDH and AST activities were significant decreased in mice inoculated with SQFZI, compared with CVB or RIB treated mice (Figure 4D), indicating a significantly reduced myocardial injury.

Histological analysis of heart tissues revealed that CVB3 infected mice treated with ribavirin or CON group developed severe myocarditis on 10, 30 days after infection, with diffuse inflammation, whereas SQFZI treatment led to a significant remission of myocarditis showing few restricted mononuclear inflammation foci and tiny necrosis ($P<0.05$) (Figure 5A). EM analysis of heart tissues indicated that myocardial fibres broke was repaired, sheets re-appeared, Z line distinct, myofilament and mitochondria status improved, mitochondrial ridge re-arrangement in SQFZI treated mice group, compared with the CVB3 group (Figure 5B). All of the above results showed that SQFZI treatment could effectively protect mice from lethal myocarditis caused by CVB3 infection.

Injection of SQFZI into mice decreased CVB3-induced TRAF6 mRNA and TRAF6 protein production

To determine whether SQFZI could inhibit TLR4-NF-kB pathway factor, TRAF6, production in CVB3-infected mice, traditional Chinese medicine extraction SQFZI was employed. Groups of Balb/C mice were infected with 1×10^2 TCID50 dose of CVB3 at day 0. The SQFUZI and ribavirin were injected of LSQFUZI/HSQFUI and RIB group, respectively. Heart tissues were collected at 0, 3, 10 and 30 day, and TRAF6 expression in heart homogenates was analyzed by RT-PCR, western blot and immunohistochemistry. The results showed that SQFUI administration resulted in low and deceased TRAF6 mRNA and protein. Compared with CVB3 mice treated with CVB3 group, significant decreased of TRAF6 mRNA were observed on both the tenth and thirtieth day in the cardiac tissues of SQFUI injected mice (Figure 6A). The western blot and immunohistochemistry results also indicated that the treatment of SQFZI could significantly decreased CVB3-

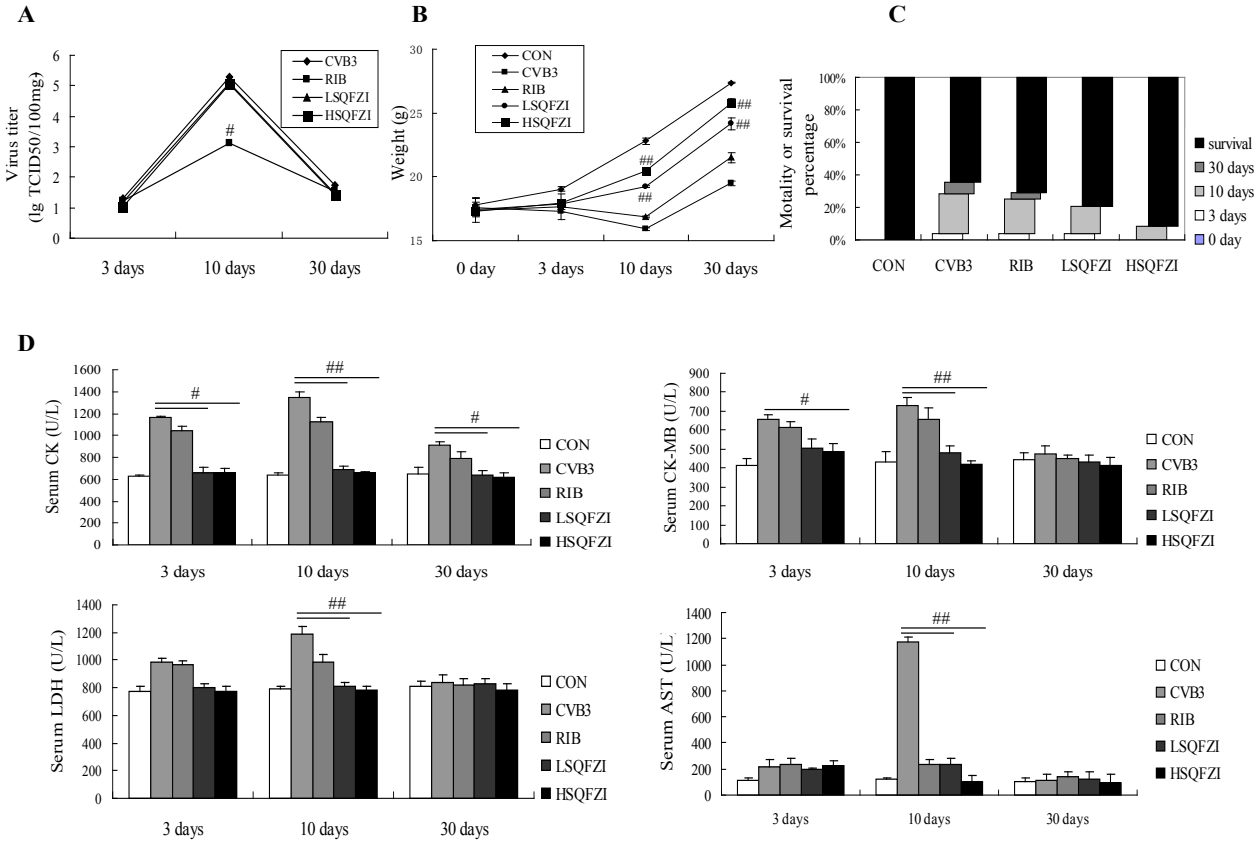

Figure 4. SQFZI administration mediated protection against CVB3-induced myocarditis. A. Hearts were removed aseptically, weighed, and homogenized daily post-infection for TCID50 assay. B and C. The body weight change (B) and majority (C) were respectively monitored daily until day 3, 10, 30 post-infection. D. Serological indices of myocarditis, the activity of CK, CK-MB, LDH and AST were respectively monitored daily until day 30 post-infection.

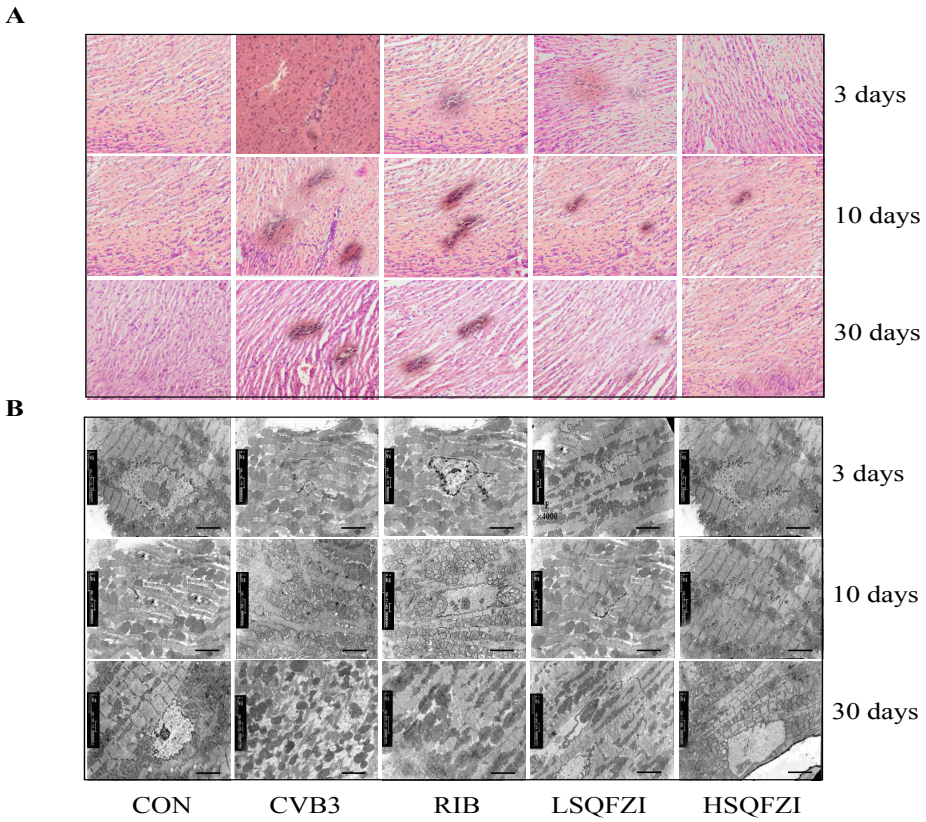

Figure 5. Administration of SQFZI improves viral myocarditis cardiac inflammation and Electron microscope structure. A. HE staining for the myocardial tissues. B. Electron microscope analysis for the myocardial tissues' micro-structure. The scale bar represents×6300 in the EM image.

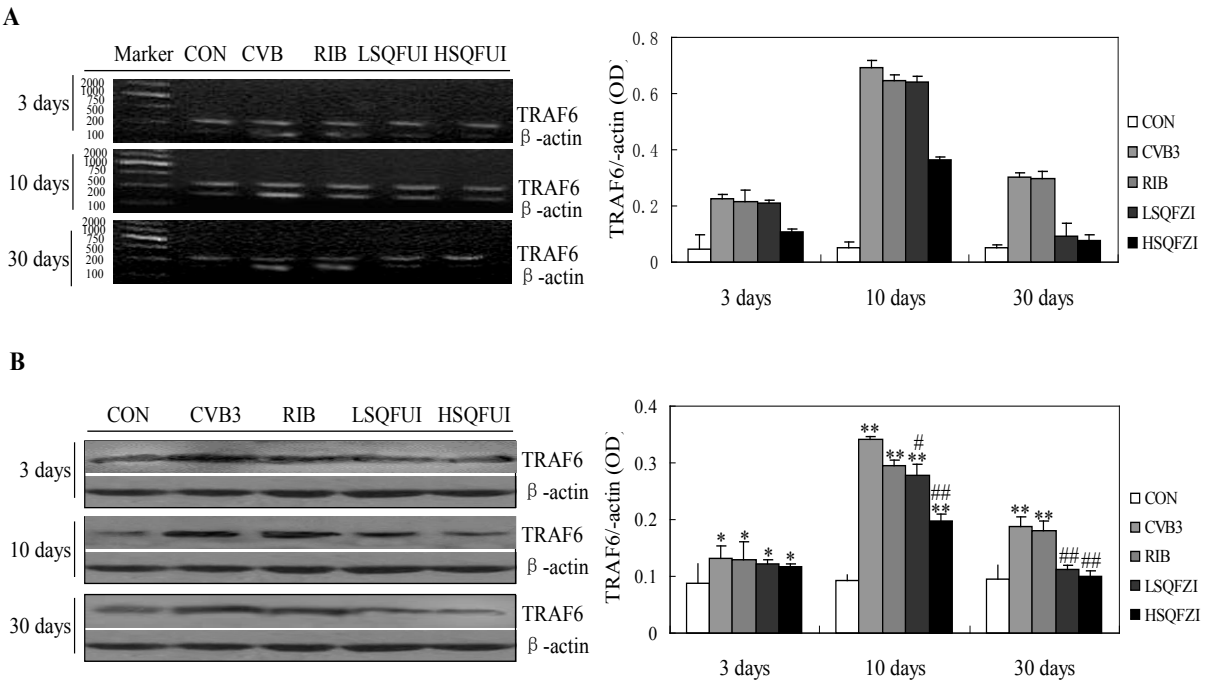

Figure 6. SQFZI decreased CVB3-induced TRAF6 mRNA and TRAF6 protein production. A. RT-PCR for the TRAF6 mRNA analysis. B. Western blot analysis for the TRAF6 protein detection.

induced TRAF6 protein production (Figure 6B, Figure 7).

DISCUSSION

In clinical, viral myocarditis is characterized by excessive inflammation of myocardium leading to heart injury following enterovirus infectious. The past studies indicated that local secretion of cytokines and chemokines by cardiomyocytes and infiltrated inflammatory cells over the course of virus infection is important in determining the pathogenesis of viral myocarditis. Gui et al. (12) proved that virus infection contributed to the increased expression of pro-inflammatory cytokines including TNF-α, IL-6, IL-1-β and chemokines. Many clinical therapeutic strategies have been targeted the expression of cytokines for the viral myocarditis patients. In the present study, the inflammatory response signal-transduction pathway, TLR4-NF-kB signal-transduction pathway was investigated. The TLR4-NF-kB pathway could regulate the cytokines expression In our study, histopathology of cardiac tissues revealed by

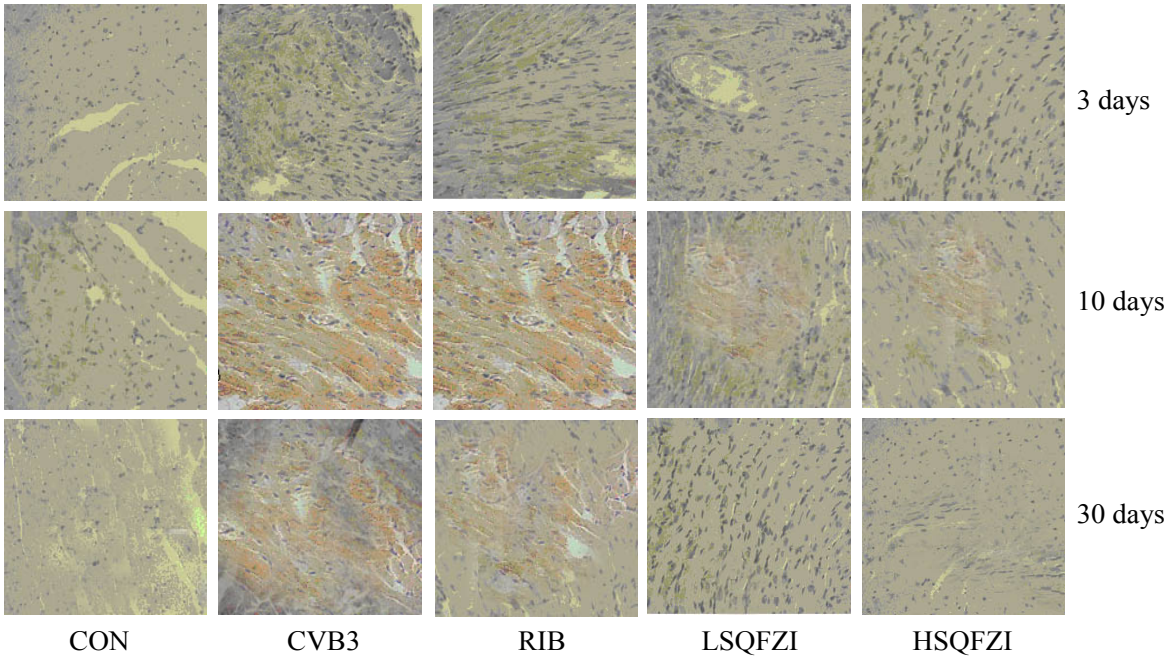

Figure 7. Immunohistochemistry analysis for the TRAF6 expression after SQFZI injection.

HE staining showed that the myocardial inflammation was increasingly severe since day 3 in CVB3-infected mice, accompanying by no weight increasing. However, the virus titer in the cardiac tissues was peaked at day 10 and then gradually reduced in the following days till day 30. Consistently, the most serious myocardial inflammation and injury, and mainly mortality were also appeared in at day 10.

In our study, the results indicated that intravenous injection with SQFZI 0.5 hours after CVB3 inoculation could significantly decreased the expression of TLR4-NF-kB signal-transduction factor in cardiac tissues on day 3, 10, 30. The SQFZI could also protect mice against viral myocarditis, as demonstrated by invariant body weight, improved survival rate (decreased mortality), contained the normal serological CK, CK-MB, LDH, AST activity and less myocardical inflammation. According to the results, we speculate that the application of SQFZI may provide a new therapeutic strategy for the treatment of viral myocarditis.

SQFZI is the compound extraction of Chinese medicine, and mainly applied in the cardiovascular diseases (25), but also applied in the some inflammatory response. Zhuang et al. (32) found that the Chinese medicinal herb complex (including Codonopsis pilosula and Astragalus mongholicus) could improve the patients' immune function, and up-regulated the counts of CD4 lymphocytes and natural killer (NK) cells. The extracts of Chinese medicinal herbs, Codonopsis pilosula, have stimulatory effect on IL-1 (23) and IL-2 (31) production by monocytes. Shao et al. (24) found that the Astragalus mongholicus polysaccharides treated DC secreted a higher level of IL-12 than untreated DC. Yuan et al's (30) study indicated that Astragalus mongholicus polysaccharides could inhibit LPS-induced production of TNF-alpha and IL-8 mRNAs, possible by suppressing the p38 signaling pathway. Furthermore, Liu et al (19) showed that Astragalus mongholicus polysaccharides might suppress $CD4^+CD25^+$ Treg activity via binding TLR4 on Tregs and trigger a shift of Th2 to Th1 with activation of $CD4^+$T cell-mediated immunity in burned mice combined with *P. aeruginosa infection.*

Inflammatory response pathway could be activated by the infection of virus. TLR4-NF-kB signal-transduction pathway has been considered as a prototypical pro-inflammatory signaling pathway, based on the activation of large pro-inflammatory genes including cytokines, chemokines, and adhesion molecules and toll-like receptor 4 molecules (15). All of the above molecules contribute to the pathogenesis of viral myocarditis. TRAF6 is a member of the TRAF family proteins that mainly function in IL-1R/TLR-mediated signaling pathways (4,14). TRAF6 could activate the TAK1, which plays a critical role in pro-inflammatory cytokine (e.g., interleukin-1-beta, tumor necrosis factor alpha) and toll-like receptor (TLR)-mediated signaling pathways (1). In addition to a well-established role of TRAF6 in IL1R/TLR-mediated signaling pathways during inflammation, a recent report has also implicated TRAF6 in muscle induced by denervation or cachexia (21). However, not much is know about its role in viral myocarditis. Our results showed that TRAF6 mediated TLR4-NF-kB pathway was significantly inhibited in CVB3-infected mice received SQFZI treatment. Our previous results (28) also indicated that SQFZI could resulted in lower expression levels of pro-inflammatory cytokines, which is consistent with the present results. Our study indicated that TLR4-NF-kB signal-transduction pathway could be novel targets for the treatment of viral myocarditis.

Interestingly, we observed that myocardial virus titer was reduced in all the groups mice on day 10, but had no significant difference among the groups (Figure 4A). So we can conclude that the SQFZI may affect the TRAF6 expression (SQFZI may block the TRAF6 expression directly or indirectly), but not by the virus inhibition pathway. Further investigations are needed to clarify the molecular mechanism utilized by SQFZI to induce the TRAF6 expression, and interfere with the TLR4-NF-kB pathway, which may enable us to exploit TRAF6 as a new weapon against viral myocarditis.

The molecular mechanism responsible for the TLR4-NF-kB inhibitory function of SQFZI has never been clarified. In the present report, SQFZI was firstly employed to therapy the CVB3-induced viral myocarditis to clarify the inhibitory function of SQFZI. Gui et al (12) found that endogenous TRAF6 was ubiquitylated in CVB3-infected cardiac myocytes. In our study, the levels of TRAF6 were

reduced in SQFZI injected cardiac myocytes, but elevated in CVB3-infected mice cardiac myocytes, suggesting that SQFZI may deubiquitalate TRAF6 to inhibit CVB3 activated TLR4-NF-kB signaling pathway. To our knowledge, this is the first study to demonstrate that SQFZI is required to inhibit CVB3 activated TLR4-NF-kB signaling by restricting endogenous TRAF6 ubiquitylation. But we believe that there are also some other targeting molecules for SQFZI involving in its inhibitory effect on CVB3 activated TLR4-NF-kB signaling, and further investigations will be discussed.

Our results showed that injection with SQFZI into mice 0.5 hours after CVB3 inoculation effectively alleviated the severity of viral myocarditis. SQFZI performed its function through down regulating the CVB3 induced TLR4-NF-kB signaling, and consequent reduction of TRAF6 production. Our results suggested that SQFZI could interfere innate signaling pathway in CVB3 infected cardiomyocytes since the early stage and attenuate subsequent excessive TRAF6 expression in the heart. But we could not exclude the possible effect of SQFZI on the biological behavior of cardiomyocytes and the immune cells, which may also account for the protective effect of SQFZI on viral myocarditis. Our data suggested that SQFZI had anti-inflammatory effect via inhibiting TLR4-NF-kB signaling on cardiomyocytes in CVB3 infected mice. Certainly, the precise molecular mechanisms by which SQFZI prevent mice from CVB3-induced myocarditis deserve further and successive studies.

In conclusion, we study for the first time that injection of SQFZI into mice could abrogate CVB3-induced cardiac inflammation and alleviate the severity of myocarditis. The suppression of CVB3-induced viral cardiac inflammation by SQFZI was caused by the inhibiting of TRAF6 in the TLR4-NF-kB signaling pathway. So our findings may provide an insight into better understanding of the underlying immune-pathological mechanism in CVB3-induced myocarditis. Our results also constitute the first pre-clinical data indicating that SQFZI can control CVB3-indeced viral myocarditis. This strategy may be applied as a feasible therapeutic strategy for the CVB3-induced myocarditis in clinical.

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REFERENCES

1. Adhikari, A., Xu, M. and Chen, Z.J., Ubiquitin-mediated activation of TAK1 and IKK. *Oncogene* 2007, **26**: 3214-3226.

2. Boyd, J.H., Mathur, S., Wang, Y., Bateman, R.M. and Walley, K.R., Toll-like receptor stimulation in cardiomyoctes decreases contractility and initiates an NF-kappaB dependent inflammatory response. *Cardiovasc. Res.* 2006, **72**: 384-393.

3. Calabrese, F. and Thiene, G., Myocarditis and inflammatory cardiomyopathy: microbiological and molecular biological aspects. *Cardiovasc. Res.* 2003, **60**: 11-25.

4. Chung, J.Y., Park, Y.C., Ye, H. and Wu, H., All TRAFs are not created equal: common and distinct moleculat mechanism of TRAF-mediated signal transduction. *J. Cell. Sci.* 2002, **115**: 679-688.

5. Dai, Z., Wan, X., Kang, H., Ji, Z., Liu, L., Liu, X., Song, L., Min, W. and Ma, X., Clinical effects of shenqi fuzheng injection in the neoadjuvant chemotherapy for local advanced breast cancer and the effects on T-lymphocyte subsets. *J. Tradit. Chin. Med.* 2008, **28**: 34-38.

6. Ding, Y., Qiu, L., Zhao, G., Xu, J. and Wang, S., Influence of cinnamaldehyde on viral myocarditis in mice. *Am. J. Med. Sci.* 2010, **340**: 114-120.

7. Dong, J., Su, S.Y., Wang, M.Y. and Zhan, Z., Shenqi fuzheng, an injection concocted from Chinese medicinal herbs, combined with platinum-based chemotherapy for advanced non-small cell lung cancer: a systematic review. *J. Exp. Clin. Cancer Res*. 2010, **29**: 137.

8. Esfandiarei, M. and McManus, B.M., Molecular biology and pathogenesis of viral myocarditis. *Ann. Rev. Pathol.* 2008, **3:** 127-155.

9. Fiorotto, R., Scirpo, R., Trauner, M., Fabris, L., Hoque, R., Spirli, C. and Strazzabosco, M., Loss of CFTR affects biliary epithelium innate immuntity and causes TLR4-NF-kB-mediated inflammatory response in mice. *Gastroenterology* 2011, **141**: 1498-1508.

10. Frantz, S., Ertl, G. and Bauersachs, J., Mechanisms of disease: Toll-like receptors in cardiovascular disease. *Nat. Clin. Pract. Cardiovasc. Med*. 2007, **4:** 444-454.

11. Fuse, K., Chan, G., Liu, Y., Gudgeon, P., Husain, M., Chen, M.Y., Yeh, W.C., Akira, S.Z. and Liu, P.P., Myeloid differentiation factor-88 plays a crucial role in the pathogenesis of Coxsackievirus B3-induced myocarditis and influences type I interferon production. *Circulation* 2005, **112**: 2276-2285.

12. Gu, J., Yue, Y., Chen, R.Z., Xu, W. and Xiong, S.D., A20 (TNFAIP3) alleviates CVB3-induced myocarditis via inhibiting NF-kB signaling. *PLoS One* 2012, **7**: e46515.

13. Kishimoto, C., Kawamata, H., Sakai, S., Shinohara, H. and Ochiai, H., Enhanced production of macrophage inflammatory protein 2 (MIP-2) by in vitro and in vivo infections with encephalomyocarditis virus and modulation of myocarditis with an antibody against MIP-2. *J. Virol.* 2001,**75**:1294-1300.

14. Landstrom, M., The TAK1-TRAF6 signaling pathway. *Int. J. Biochem. Cell. Biol.* 2010, **42**: 585-589.

`5. Lawrence, T., The nuclear factor NF-kB pathway in inflammation. *Cold Spring Harb Perspect Biol.* 2009, **1**: 1-13.

`6. Levine, B., Kalman, J., Mayer, L., Fillit, H.M. and Packer, A.M., Elevated circulating levels of tumor necrosis factor in sever chronic heart failure. *N. Engl. J. Med.* 1990, **323:** 236-241.

17. Linde, A., Mosier, D., Blecha, F. and Melgarejo, T., Innate immunity and inflammation-New frontiers in comparative of cardiovascular pathology. *Cardiovasc. Res.* 2007, **73**: 26-36.

18. Liu, H., Wu, X., Zhang, Y. and Lian, W., Effect of Shenqi Fuzheng injection (SFI) on immune function in patients with congestive heart failure. *Zhong Yao Cai* 2005, **28:** 854-856.

19. Liu, Q.Y., Yao, Y.M., Yu, Y., Dong, N. and Sheng, Z.Y., Astragalus mongholicus polysaccharides attenuate postburn sepsis via inhibiting negative immunoregulation of CD4+CD25+ T cells. *PLoS One* 2011, **6**: e19811.

20. Liu, X.P., Hao, X. and Xu, G.X., Clinical observation on treatment of heart failure with mixed diastolic and systolic dysfunction by shenqi fuzheng injection. *Zhongguo Zhong Xi Yi Ji He Za Zhi* 2004, **24**: 739-740.

21. Paul, P.K., Gupta, S.K., Bhatnagar, S., Panguluri, S.K. and Darnay, B.G., Targeted ablation of TRAF6 inhibits skeletal muscle wasting in mice. *J. Cell Biol.* 2010, **191**: 1395-1411.

22. Schultz, J.C., Hiliard, A.A., Cooper, L.T. and Rihal, C.S., Diagnosis and treatment of viral myocarditis. *Mayo Clin. Proc.* 2009, **84**: 1001-1009.

23. Shan, B.E., Yoshida, Y., Sugiura, T. and Yamashita, U., Stimulating activity of Chinese medicinal herbs on human lymphocytes in vitro. *Int. J. Immunopharmacol.* 1999, **21**: 149-159.

24. Shao, P., Zhao, L.H., Zhi, C. and Pen, J.P., Regulation on maturation and function of dendritic cells by Astragalus mongholicus polysaccharides. *Int. Immunopharmacol.* 2006, **6**: 1161-1166.

25. Song, Y., Li, R. and Gong, X.J., Clinical study on effect of Shenqi

Fuzheng injection on erythrocyte immunity in patients after cardiopulmonary bypass. *Zhongguo Zhong Xi Yi Ji He Za Zhi* 2007, **27**: 545-547.
26. Suganami,T., Tanimoto-Koyama, K., Nishida, J., Itoh, M., Yuan, X., Mizuzrai, S., Kotani, H., Yamaoka, S., Miyake, K., Aoe, S., Kamei, Y. and Ogawa, Y., Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. *Arterioscler Thromb. Vasc. Biol.* 2007, **27**: 84-91.
27. Wang, J., Tong, X., Li, P., Cao, H. and Su, W., Immuno-enhancement effects of Shenqi Fuzheng injection on cyclophosphamide-induced immunosuppression in Balb/C mice. *J. Ethnopharmacol*. 2012, **139**: 788-795.
28. Wang, Z. and Chen, J.S., Progress in the study of TLR-NF-kB signaling pathway associated with mycoarditis. *Med. Recapitulate* 2011, **17**: 41-43.
29. Wei, Y.F., Wang, S.Y. and Ren, L.L., Efficacy of shenqi fuzheng injection combined with chemotherapy in treatment of acute leukemia and its effect on T-lymphocyte subsets, serum IFN-gamma, IL-10 and IL-2. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 2005, **25**: 303-306.
30. Yuan, Y., Sun, M. and Li, K.S., Astragalus mongholicus polysaccharide inhibits lipopolysaccharide-induced production of TNF-alpha and interleukin-8. *World J. Gastroenterol.* 2009, **15**: 3676-3680.
31. Zeng, X.L., Li, X.A. and Zhang, B.Y., Immunological and hematopoietic effects of codonopsis pilosula on cancer patients during radiotherapy. *Zhongguo Zhong Xi Yi Jie He Za Zhi*, 1992, **12**: 607-608.
32. Zhuang, S.R., Chen, S.L., Tsai, J.H., Huang, C.C., Wu, T.C., Liu, W.S., Tseng, H.C., Lee, HS., Huang, M.C., Shane, G.T., Yang, C.H., Shen, Y.C., Yan, Y.Y. and Wang, C.K., Effect of citronellol and the Chinese medical herb complex on cellular immunity of cancer patients receiving chemotherapy/radiotherapy. *Phytother. Res.* 2009, **23**: 785-790.
33. Zou, N., Ao, L., Cleveland, J.C., Yang, X., Su, X., Cai, G.Y., Banerjee, A., Fullerton, D.A. and Meng, X., Critical role of extracellular heat shock cognate protein 70 in the myocardial inflammatory response and cardiac dysfunction after global ischemia-reperfusion. *Am. J. Physiol. Heart Circ. Physiol.* 2008, **294**: H2805-2813.