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Original Article

Effect of emodin on the expression of TLR4 and P38MAPK in mouse cardiac tissues with viral myocarditis

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Abstract: Toll-like receptor 4 (TLR4) and P38 mitogen activating protein kinase (P38MAPK) play important roles in pathogenesis of viral myocarditis (VMC). This study established a mouse VMC model, on which the dynamic expression level of TLR4 and P38MAPK was observed, along with the effect of emodin and its potential mechanism of cardiac protection. A total of 90 male BALB/c mice were randomly assigned into control, model and emodin groups (N=30 each). Coxsackie virus B3 (CVB3) was injected peritoneally to establish VMC model. 3 mg/kg/d emodin was given by gavage for 14 days. The mortality rate of mice was recorded. Viral titer was determined at day 7 by sacrificing 5 mice from each group. Fluorescent quantitative PCR was used to quantify mRNA copy number of CVB3. Eight animals were selected at day 7 and day 14 from each group to observe the cardiac morphology by H&E staining. RT-PCR was used to detect TLR4 mRNA, while P38MAPK expression was measured by Western blotting. Model group had significantly elevated expression of P38MAPK and TLR4 mRNA levels compared to control group ($P<0.05$). Emodin treatment significantly decreased mortality rate, viral titer, copy number of CVB3 mRNA, cardiac pathology score, and mRNA expression of P38MAPK and TLR4 in heart tissues ($P<0.05$). During pathogenesis of VMC, TLR4 and P38MAPK signal transduction pathways may exert important roles. Emodin may alleviate cardiac injury of CVB3-infected mice via depressing TL4 and P38MAPK expression, thus protecting cardiac tissues.

Keywords: Viral myocarditis, toll-like receptor 4, P38 mitogen activating protein kinase, coxsackie virus B3

Introduction

Viral myocarditis (VMC) is one common cardiovascular disease, and can induce heart failure or systemic shock [1, 2]. VMC is commonly caused by cardiotropic viruses such as Coxsackie virus group A, Coxsackie virus group B, polio virus, Echo virus, among which the most frequent strain is Coxsackie virus B (CVB) [3, 4]. Currently the pathogenesis of VMC is still unknown yet [5, 6]. Previous study showed its correlation with direct viral injury and immune dysfunction. After acute viral infection, cardiac muscle cells were damaged. Cytotoxic T cell-induced cytotoxicity plays an important role in VMC-related myocardial injury. Mitogen activating protein kinase (MAPK) signal pathway is activated in cardiac tissues after viral infection. P38MAPK signal pathway is one important component of MAPK family, and is related with viral infection and cardiac fibrosis [7, 8]. Toll-like receptor 4 (TLR4) is major receptor recognizing microbes in innate immunity. After viral injury, components of myocardial cells were recognized by TLR4 on membrane of myocardial membrane. The activation of TLR4 induces MAPK and nuclear factor (NF)-κB, both of which can aggravate cardiac muscle cell injury via affecting cytokine production and potentiating viral replication [9, 10]. In the pathological process of VMC, TLR4 and P38MAPK may play important roles. Emodin is one effective component of traditional Chinese Medicine rhubarb. It is one derivative of anthraquinone family. It has multiple pharmaceutical activities including immune modulation and anti-inflammation. *In vitro* study demonstrated that emodin can protect against Coxsackie virus B3 (CVB3). *In vivo* study also confirmed the role of emodin in inhibiting viral

Table 1. Primer sequence

Target gene		Sequence (5'-3')	Fragment length (bp)
TLR4	Forward	GCCGTTGGTGTATCTTTGA	
	Reverse	AGTTGCCGTTTCTTGTTC	275
β-actin	Forward	CTCTTTGATGTCACGCACGATTC	
	Reverse	GTGGGCCGCCCTAGGCACCA	398

replication and potentiating anti-oxidation [11, 12]. This study established a mouse VMC model, on which the dynamic change of expression levels of TLR4, P38MAPK were monitored, along with the effect of emodin, in order to investigate the protective function of emodin on myocardial tissues in VMC mice and possible mechanisms.

Materials and methods

Animals

90 healthy male BALB/c mice (4~6 weeks old, body weight 14~16 g) were provided by Laboratory Animal Center, Shandong University (Cert. No. SYXK-2013-0025) and were kept in an SPF-grade facility with food and water ad libitum. Animals were randomly assigned into control, model and emodin groups (N=30 each). CVB3 virus was injected intraperitoneally to prepare VMC model, while equal volume of blank medium was applied in control group.

Mice were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the Second Hospital of Shan Dong University.

Drugs and reagents

CVB3 Nancy strand strain was provided by Viral Institute, Medicine Academy of Shandong Province (viral dosage =10^7 $TCID_{50}$/0.1 ml). Emodin powder (purity >98%) was provided by the State Institute of Pharmacy and Biological Products). Injection water was provided by Kelun Pharmacology (China). Pentobarbital was provided by Shanghai Chemical Institute, Chinese Pharmacology Corp. (China). DMSO was provided by Sigma (US) and was used to dilute emodin for 3 mg/ml solutions. Rabbit anti-mouse phosphorylated P38MAPK monoclonal antibody was purchased by CST (US). Trizol kit and reverse transcription kit were purchased from Invitrogen (US). Goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) was purchased from CST (US). Primers were synthesized by Elbus (China).

Animal model

Moue VMC model was prepared by intraperitoneal injection of CVB3 as previously described [13]. In model and emodin groups, 0.1 ml of Eagle's solutions containing 100 $TCID_{50}$ CVB3 was injected intraperitoneally, while control group received equal volume of blank medium.

Drug delivery

30 min after inoculation, emodin (3 mg/kg/d) was introduced via gavage for 14 consecutive days, while control and model groups received equal volume of distilled water. Mortality rate of mice were continuously monitored during the experimental period.

Viral titer measurement

At 7 d, five mice from each group were sacrificed. Heart tissues were extracted to determine the viral titer. In brief, cardiac tissues were homogenized and centrifuged. The supernatant was saved and diluted serially (10^{-1} to 10^{-6}). 0.2 mL dilutions were then added into 96-well plate containing Hela cells (N=4 for each concentration). Infected well was identified with more than 50% of infected cells. Based on Reed-Muench method lg$TCID_{50}$ value was identified. Fluorescent quantitative PCR was employed to measure the copy number of CVB3 mRNA. Total RNA was then extracted from cardiac tissues. UV spectrometer was used to measure D_{260}/D_{280} ratio (optimal: 1.8~2.0). Amplification products were analyzed in agarose gel electrophoresis in triplicates. Relative expression level was then calculated.

HE staining

Eight mice were chosen at day 7 and day 14 from each group and were sacrificed. Heart tissues were extracted for H&E staining and observation under a light field microscope to observe the pathological implication. Myocardial pathology score was calculated according to previous documents [14]. Five high magnification fields were randomly chosen from each tissue slide to calculate the area of necrotic tissues and the ratio of inflammatory infiltration area against the whole field. The score

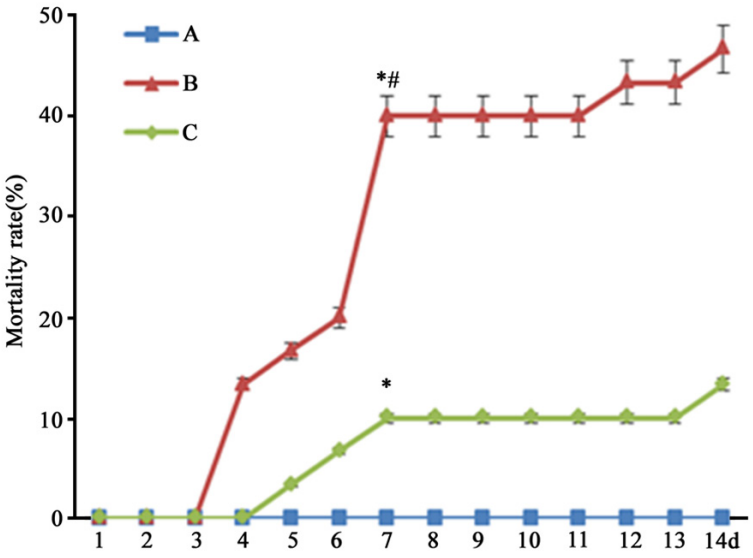

Figure 1. Mortality rate of VMC mice under emodin treatment. *P<0.05 compared to control group; #P<0.05 compared to model group.

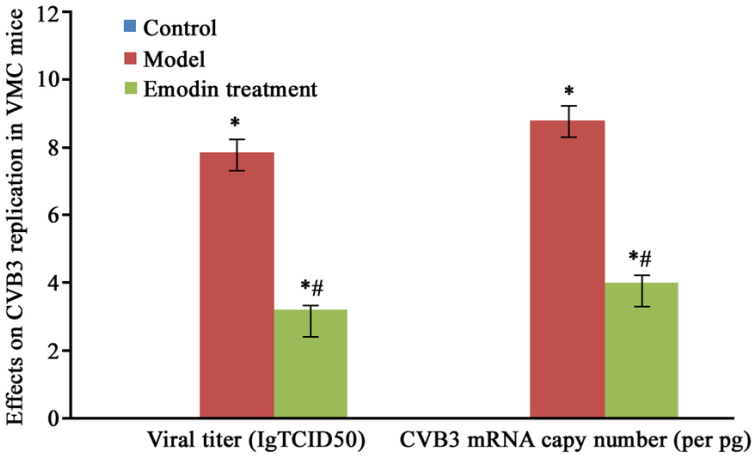

Figure 2. Effect of emodin on viral replication. *P<0.05 compared to control group; #P<0.05 compared to model group.

was determined as 0 (no necrosis), 1 (less than 25% of necrotic region), 2 (25% to 50% of total area), 3 (51% to 75% total filed area) and 4 (more than 75% necrotic regions).

RT-PCR

Total RNA was extracted from cardiac tissues for *in vitro* reverse transcription into cDNA, which was then used in RT-PCR according to the instruction of Trizol kit. Products were quantified by UV electrometer. Primer sequences were shown in **Table 1**. Amplification products were analyzed in agarose gel electrophoresis in triplicates and were expressed in relative concentration, which was the ratio of gray density value of target gene against that of β-actin.

Western blotting

Mouse cardiac tissues were lysed in lysis buffer, which was then centrifuged to collect supernatants, which were quantified by BCA kit. Protein samples were then separated in SDS-PAGE, and were transferred to PVDF membrane, which was blocked in blocking buffer for 1 h. Primary antibody (1:200 dilution) was then added for 4°C overnight incubation. After washing in PBST for three times, secondary antibody was then added for 1 h incubation, followed by TBST washing. ECL reagent was then applied for develop, followed by exposure in dark room. Quantity One software was used to analyze protein bands, which were expressed as relative expression level. OD value of phosphorylated P38MAPK protein bands was measured against that of internal reference.

Statistical analysis

SPSS19.0 software was used to analyze all data, of which those fitted normal distribution were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare means across groups. Between-group-comparison was performed in LSD test. A statistical significance was defined when P<0.05.

Results

General condition of VMC mice

Control group had normal drinking and feeding patter, with normal fur color. In model group, however, the fur began to decrease and coil

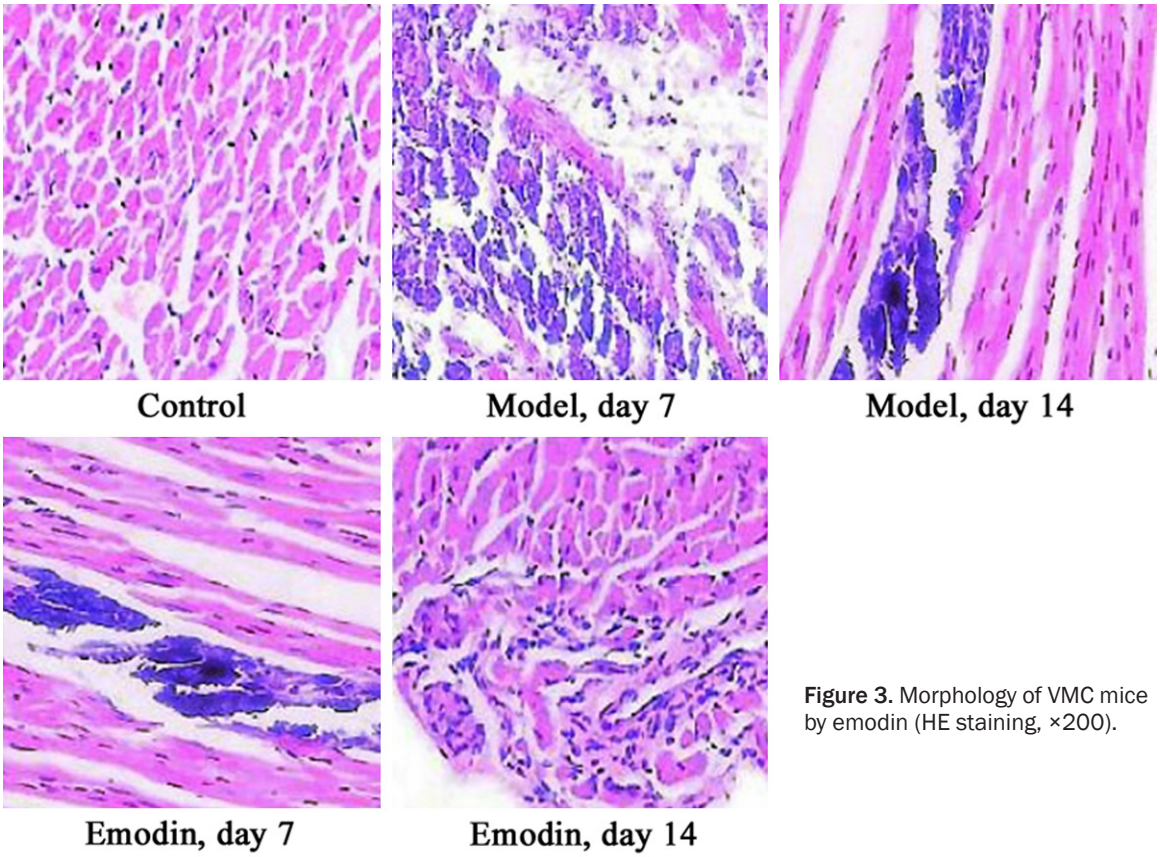

Figure 3. Morphology of VMC mice by emodin (HE staining, ×200).

after 3 days of viral inoculation. Animals then showed insensitivity to stimuli, and showed mortality after 4 days, with the peak of death around day 7. Emodin group had similar symptoms at day 4 of inoculation and reached a peak of death at day 7. Mortality rates of all groups were shown in **Figure 1**.

Effects of emodin on viral replication

No virus has been detected in cardiac muscle tissues of control group. Compared to model group, emodin treatment group had significantly depressed copy number of mRNA of CVB3 as well as viral titer ($P<0.05$, **Figure 2**).

Effects of emodin on myocardial morphology and inflammatory score

In control group, no inflammatory infiltration can be found in myocardial tissues, which had regular arrangement of cardiac muscle cells, with sharp and clear muscle fiber and evenly distributed cytoplasm. Model mice, however, had inflammatory infiltration at day 7 after viral inoculation, accompanied with cardiac fiber necrosis and elevated inflammatory score ($P<0.05$). At day 14, the condition of inflammatory infiltration was alleviated than day 7, leaving small patched of tissue necrosis and higher inflammatory score ($P<0.05$ compared to control group). Emodin treatment alleviated the pathology condition of myocardial tissues at day 7 and day 14, as shown by lower inflammatory score than model group ($P<0.05$, **Figures 3** and **4**).

TLR4 mRNA expression level in myocardial tissues in VMC mice

In control group, TLR4 mRNA had relatively lower expression. Model group had elevated TLR4 mRNA expression ($P<0.05$ compared to control group). TLR4 expression level reached a peak at day 7 and decreased at day 14. Compared to model group, emodin treatment suppressed TLR4 mRNA expression level in myocardial tissues ($P<0.05$, **Figure 5**).

P38MAPK expression level in cardiac muscle tissues

Model mice had elevated level of phosphorylated P38MAPK compared to control group,

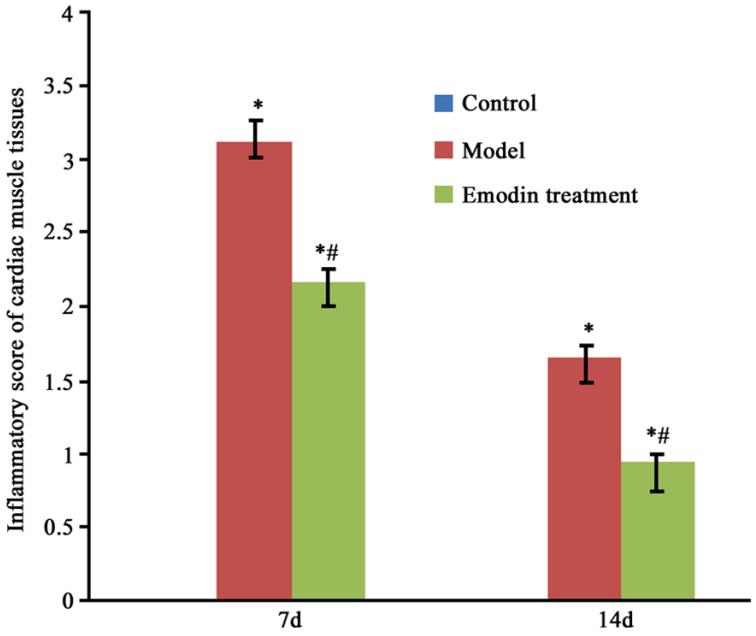

Figure 4. Effect of emodin on inflammatory score of myocardial tissues in VMC mice. *P<0.05 compared to control group; #P<0.05 compared to model group.

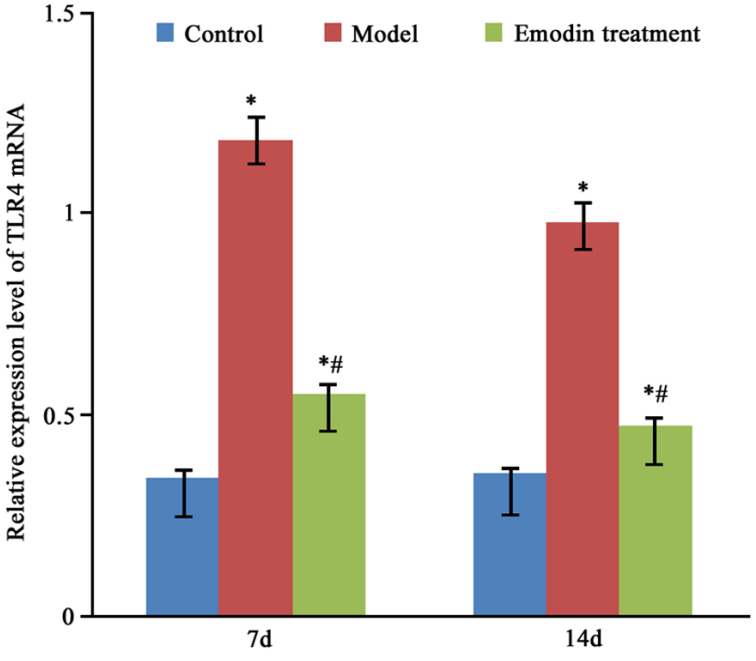

Figure 5. TLR4 mRNA expression level in cardiac muscle tissues in VMC mice. *P<0.05 compared to control group; #P<0.05 compared to model group.

and reached the peak at day 7, with lower level at day 14. Compared to model group, emodin treatment had down-regulation of P38MAPK expression level in cardiac muscle tissues (P<0.05, **Figure 6**).

Discussion

As one common cardiovascular disease in pediatrics, VMC has asymptotic onset and may develop into dilation heart disease. Currently the pathogenesis mechanism of VMC is still unknown yet, with previous knowledge showed its correlation with viral infection, progressive auto-immune heart disease and other myocardial injury [15, 16]. Both TLR and MAPK signal pathways play a role in various aspects of VMC including viral infection, stress response and inflammatory injury. Study showed higher TLR4 mRNA in endocardial membrane of VMC patients compared to healthy individuals. TLR4 mRNA expression is known to be related with viral replication, as it can recognize LPS. Sepsis may induce NF-κB signal pathway via stimulating TLR4, thus initiating inflammatory injury [17, 18]. After viral invasion, TLR4 is initiated, and activate signal cascade reaction via binding onto its ligands for recognizing pathogen-related molecular modality inside innate immunity, thus inducing cytokine production. The signal transduction of TLR4 signal can be sub-divided into myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent pathways, the former of which require the formation of MyD88-TLR signal transducing complex to activate downstream tumor necrosis factor receptor associated factor 6 (TRAF6), whose activation induces MPAK and NF-κB signal pathways. MAPK pathway involves P38, which causes inflammatory response and immune reaction, further causing myocardial injury [19, 20]. MyD88-independent pathway

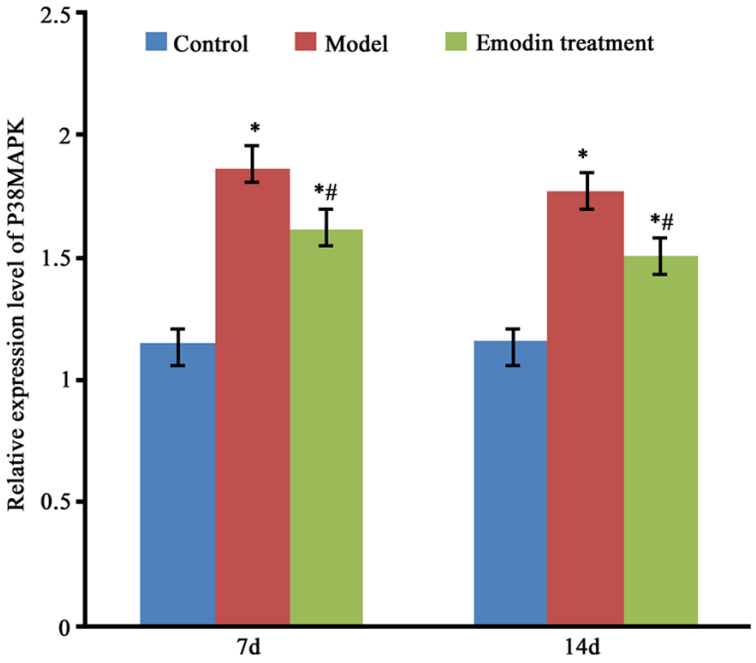

Figure 6. The expression of P38MAPK in cardiac muscle tissues in VMC mice. *P<0.05 compared to control group; #P<0.05 compared to model group.

can also activate both MAPK and NF-κB signal pathways via interferon or other modulatory factors [20, 21]. Pathogenic microbes thus can activate multiple signal pathways via different adaptor proteins for an inflammatory cascade reaction, inducing lymphocyte infiltration and early stage immune response effect, which may cause further inflammation [21]. Some studies showed that emodin could alleviate myocardial tissue inflammation via inhibiting IL-23/IL-17 inflammatory axis and decreasing CVB3 replication [12]. This study observed the dynamic change of P38MAPK and TLR4 expression in myocardial tissues in VMC mice, and the intervention effect by emodin, in an attempt to investigate possible functional mechanism of emodin on the progression of VMC. Results showed significantly decreased mortality rate, viral titer, copy number of CVB3 mRNA, and pathology score of cardiac tissues in emodin treated mice compared to model group, suggesting certain protective effect on cardiac muscles by emodin in VMC mice, possibly via inhibiting viral replication inside cells and thus suppression myocardial tissue injury.

As one of MAPK signal pathways, P38MAPK can be translocated into the nucleus after phosphorylation, and participates in inflammation and cell apoptosis. TRL4 signal pathway is correlated with the pathogenesis mechanism of dilated or viral myocarditis. In this study, both P38MAPK and TLR4 mRNA levels were significantly elevated in model group as compared to control ones. These values reached a peak at day 7 and decreased at day 14. In emodin treated animals, P38MAPK and TLR4 mRNA levels were significantly down-regulated, suggesting potentially important role of those two signal transduction pathways during VMC pathogenesis. The early activation of P38MAPK signal pathway might be related with viral replication and cardiac muscle injury induced by viral binding or inflammatory factors. Emodin might alleviate myocardial tissue injury in CVB3 infected mice via down-regulating TLR4 and P38MAPK. Previous study showed no correlation between P38MAPK phosphorylation and inflammatory score in myocardial cells [19], probably due to the endogenous production of phosphorylated P38-MAPK in myocardial cells. Such phosphorylated P38MAPK molecules participate in the regulation of cytokine-related gene transcription and expression by its nuclear translocation. TLR4 could aggravate injury of myocardial cells via enhancing viral replication for affecting the prognosis of VMC. The inhibition of TLR4 gene and blockage of related signal pathways could prevent myocardial cell necrosis and apoptosis. In summary, TLR4 and P38MAPK might play important roles during pathogenesis and progression of VMC. Emodin has certain protection on myocardial tissues and alleviates myocardial tissue injury in CVB3-infectoin mice possibly via down-regulating TLR4 and P38MAPK levels.

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Disclosure of conflict of interest

None.

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