

Spironolactone alleviates myocardial fibrosis via inhibition of Ets-1 in mice with experimental autoimmune myocarditis

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Abstract. Spironolactone improves cardiac structure, function and prognosis in patients with heart failure and delays the progression of cardiac fibrosis. However, the exact underlying mechanism of this process remains to be elucidated. The present study therefore aimed to explore the protective effect and underlying mechanism of the aldosterone receptor antagonist, spironolactone, on myocardial fibrosis in mice with experimental autoimmune myocarditis (EAM). The EAM model was induced in BALB/c mice via immunization with murine cardiac α -myosin heavy chain sequence polypeptides. The cardiac function of the mice was assessed using echocardiography and the levels of inflammatory cytokines were quantified using ELISA. E26 transformation-specific sequence-1 (Ets-1) expression was knocked down using lentivirus-mediated small interference RNA. Total collagen deposition was assessed using Masson's trichrome and Ets-1, TGF- β 1, Smad2/3, collagen I and III protein expression levels were detected using immunohistochemistry and western blotting. MMP-2 and MMP-9 mRNA expression levels and activity was determined using reverse transcription-quantitative PCR and gelatin zymography, respectively. The results of the present study demonstrated that spironolactone significantly improved myocardium hypertrophy, diastolic cardiac function and decreased myocardial inflammation and collagen deposition induced by EAM. Spironolactone treatment significantly inhibited Ets-1 and smad2/3 phosphorylation. In addition, inhibition of Ets-1 reduced the expression and activity of MMP-2 and MMP-9 and decreased cardiac fibrosis in EAM mice. The results indicated that the improvement of myocardial fibrosis by spironolactone may

be associated with the TGF- β 1/Smad-2/3/Ets-1 signaling pathway in EAM mice.

Introduction

Myocarditis is an inflammatory disorder that is associated with an increased risk of developing dilated cardiomyopathy (DCM) (1,2). Collagen deposition, ventricular dilation and heart failure are indicating characteristics of myocarditis progression to DCM. Experimental autoimmune myocarditis (EAM) animal models are used for the investigation of the pathophysiologic mechanism behind the transition of myocarditis to DCM. Inflammatory cytokines, including IL-1 β , IL-6 and TGF- β serve a key role in myocardial collagen remodeling (3). A previous study reported the potential role of inflammatory cytokines in the transition of myocarditis to DCM via the regulation of MMP expression (4). In addition, evidence suggests that the aldosterone receptor causes cardiac oxidative stress, inflammation and fibrosis (5). Spironolactone, a nonselective aldosterone receptor inhibitor, has been reported to relieve the process of cardiac fibrosis and remodeling following cardiac injury in numerous experimental and clinical studies (6-8). However, the mechanism of spironolactone in myocarditis remains to be elucidated.

E26 transformation-specific (Ets) transcription factor family members share a highly conserved DNA-binding domain and are involved in cell differentiation, proliferation, metastasis, apoptosis and tissue remodeling (9). Ets sequence-1 (Ets-1), has been demonstrated to enhance fibrotic processes in the heart and in other organs. In addition, Ets-1 also regulates TGF- β -induced tissue fibrosis and participates in the tissue fibrosis process by regulating the expression of genes encoding enzymes involved in matrix degradation (10). Ets-1 activation regulates TNF- α -induced MMP-9 (11). However, the effect of spironolactone on Ets-1 in cardiac fibrosis has remains to be elucidated.

The aim of the present study was to investigate the underlying mechanisms of how spironolactone protects against post-myocarditis remodeling. It was hypothesized that spironolactone could improve myocardial fibrosis via the inhibition of Ets-1 via TGF- β signaling pathways in EAM mice. Furthermore, another aim was to identify

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a potential novel therapeutic approach for patients with myocarditis/DCM.

Materials and methods

Animals. A total of 50 female BALB/c mice (age, 6-8 weeks; weight, 18-20 g) were purchased from the Experimental Animal Center of Shandong University (Jinan, China). All experimental procedures were performed in accordance with animal protocols approved by The Second Hospital of Shandong University Animal Care Committee (approval no. KYLL-2020-(KJ) A-0134). All mice were housed in a pathogen-free animal facility, which was maintained at 22-24°C at 50-60% humidity with 12:12 h light-dark cycle. The mice had easy access to food and water before the experiments. For cardiac diameter and cardiac function assessment, mice were anesthetized with 3% isoflurane, which was subsequently maintained at 1.3%. At the end of study, mice were anesthetized by intraperitoneal injection of pentobarbital sodium (100 mg/kg) and euthanized via exsanguination.

Induction of EAM and treatment. For EAM model construction, murine cardiac α -myosin heavy chain [α 614-629 (Ac-SLKLMTLFTSYASAD-OH); GL Biochem (Shanghai), Ltd.] was dissolved in PBS (1 mg/ml) and emulsified 1:1 with complete Freund's adjuvant (CFA; MilliporeSigma). Each mouse was subcutaneously injected with 200 μ l of emulsion (containing 200 μ g of murine cardiac α -myosin) by inguinal injection or subaxillary injection on day 0 and 7 to induce EAM (12). The control group mice were treated with CFA mixed with PBS following the same procedure as for the EAM group mice. To knockdown Ets-1 expression, a small interfering (si)RNA against mouse Ets-1 was transfected into mouse hearts and a scramble siRNA was employed as a control. The target sequence for Ets-1 was 5'-GCUACCUUCAGU GGUUUCATT-3' and scramble siRNA sequence was 5'-UUC UCCGAACGUGUCACGUTT-3' (Shanghai GenePharma Co., Ltd.). Mice were randomly divided into five groups: i) Control group (n=10); ii) non-treated EAM group (n=10); iii) spironolactone-treated EAM group (n=10); iv) saline-treated EAM group (n=10); and v) siRNA-Ets-1-treated EAM group (n=10). Spironolactone treatment therapy started on day 7 following the emulsion injection. The myocardial injection method was used to transfect cells based on previous study (13). A total of 1×10^7 UT/30 μ l lentivector with siRNA-Ets-1 was injected in multiple sites in the left ventricle of the EAM group mice at day 7. Mice were fed orally via gastric gavage for 4 weeks from day 7 to day 36 after immunization. The calculated daily dosage of 50 mg/kg/day spironolactone per mouse was based on a previous study by Wehr *et al* (14). Assessment and analysis of the establishment of EAM in the mouse model were performed on day 36. No mice succumbed throughout the duration of the present study.

Biochemical quantification. Serum was collected for ELISA. Blood samples were collected via eyeball removal and were stored at 4°C overnight to let the serum separate from the blood cells. Aldosterone was quantified using a Aldosterone ELISA kit (cat. no. ZC-38593; Shanghai Zhuo Cai Technology Co., Ltd.) Serum levels of IL-6 and TNF- α were also quantified

using IL-6 and TNF- α ELISA kit (cat. no. ZC-37988 and ZC-39024, respectively; Shanghai Zhuo Cai Technology Co., Ltd.), according to the manufacturer's protocol.

Echocardiography. Echocardiography was performed on the mice as previously described (15,16). Wall thickness and left ventricular (LV) dimensions, including LV internal dimensions (LVIDs) in diastole, LVID in systole, LV posterior wall (LVPW) of diastole, LVPW of systole and LV posterior diameter in systole, were obtained from the short-axis view. LV ejection fraction (LVEF) was assessed according to the American Society of Echocardiography Guidelines (17). Pulsed-wave Doppler echocardiography was used to measure early (E) and late (A) blood flow velocities via the mitral valve and the E/A ratio was determined.

Histology and immunohistochemistry. Heart tissues were fixed using 10% formalin at room temperature for 72 h, dehydrated with an ethanol gradient, embedded in paraffin, sectioned (thickness, 5 μ m) and stained with hematoxylin and eosin (H&E). The main steps of H&E staining were the following: Staining with hematoxylin for 10 min at room temperature, washing in tap water, staining with eosin for 3 min at room temperature, washing in distilled water and ethanol (90%), dehydration in ethanol (95%), ethanol (100%). Masson trichome staining was used to stain collagen fibers dark green, which were quantified to assess fibrosis. Masson staining takes place at room temperature and the main steps are the following: Staining with hematoxylin for 5 min, washing with tap water, staining with fuchsin for 5 min, rinsing in distilled water, incubating in phosphotungstic-phosphomolybdic acid for 5 min, dyeing aniline blue for 5 min. Paraffin sections underwent immunohistochemistry using a microwave-based antigen retrieval method. Sections were incubated with primary antibodies for rabbit polyclonal collagen I (1:100; cat. no. AF7001; Wuhan Servicebio Technology Co., Ltd.), collagen III (1:500; cat. no. GB111629; Wuhan Servicebio Technology Co., Ltd.), TGF- β 1 (1:100 cat. no. AF1027; Wuhan Servicebio Technology Co., Ltd.) and phosphorylated (p)-Ets-1 (1:200; cat. no. CBP1153; Assay Biotechnology Co., Inc.) overnight at 4°C. Following the primary antibody incubation, samples were incubated with a HRP-conjugated secondary goat anti-rabbit antibody (cat. no. PV9001; Zhongshan Bio-Tech Co., Ltd.) for 30 min at 37°C. Signals were amplified using an ABC kit (Vector Laboratories, Inc.). Sections were imaged using a confocal FV 1000 SPD laser scanning microscope (Olympus Corporation). Immunohistochemical staining was analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc.).

Western blotting. Total protein was extracted from mice myocardium using RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology). Subsequently, total protein was separated using SDS-PAGE on a 10% gel. Separated protein was then transferred onto a PVDF membrane, which was incubated overnight at 4°C with the following primary antibodies: total Ets-1 (1:1,000; cat. no. 6258; Cell Signaling Technology, Inc.), p-Ets-1 (1:500; cat. no. CBP1153; Assay Biotechnology Co., Inc.), Smad-2/3 (1:1,000; cat. no. 8685; Cell Signaling Technology,

Inc.) and p-Smad-2/3 (1:1,000 dilution; cat. no. 8828; Cell Signaling Technology, Inc.). Following the primary incubation membranes were incubated with an HRP-conjugated secondary antibody for 1 h at room temperature (1:2,500 dilution; cat. no. ZB-2306; OriGene Technologies, Inc.). Protein expression levels were normalized to β -actin (1:1,000; cat. no. 4970; Cell Signaling Technology, Inc.) as an internal control and p-proteins to that of the total protein. Bands were semi-quantified using optical densities, which were analyzed using ImageJ software (v1.8, National Institutes of Health).

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from heart tissue using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and converted into complementary DNA using a RevertAid kit (Fermentas; Thermo Fisher Scientific, Inc.), both kits were used according to the manufacturer's protocol. Reactions involved the use of a real-time PCR thermocycler (IQ5 real-time PCR cycler; Bio-Rad Laboratories) with SsoFast EvaGreen Supermix (Bio-Rad Laboratories). The program was 30 sec at 95°C, then 40 cycles of 96°C for 5 sec and 56°C for 10 sec. mRNA expression levels were normalized to the internal reference gene GAPDH. The primer sequences used for qPCR were as follows: MMP-2 forward F, 5'-ACAAGTGGTCCGCGTAAAGT-3' and reverse R, 5'-GTAAACAAGGCTTCATGGGGG-3'; MMP-9 F, 5'-GCCGACTTTTGTGGTCTTCC-3' and R, 5'-GGTACAAGTATGCCTCTGCCA-3'; and GAPDH F, 5'-AGGTCCGTG TGAACGGATTGGG-3' and R, 5'-TGTAGACCATGTAGT TGAGGTCA-3'. Relative fold change of mRNA expression levels was calculated using the $2^{-\Delta\Delta CT}$ method (18). Data are representative of three independent experiments.

Gelatin zymography. The enzymatic activity of MMP in myocardial tissues was assayed using gelatin zymography. Samples were electrophoresed via SDS-PAGE on a 10% gel containing 0.1% gelatin. After the gels were washed twice with 2.5% Triton X-100, the gels were incubated in activation buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl₂, 1 μ M ZnCl₂] at 37°C overnight. Subsequently, gels were stained with Coomassie brilliant blue R-250 solution for 3 h at room temperature. Gels were de-stained with 45% methanol and 10% acetic acid until the bands of lysis become clear. Lytic bands of gelatin digestion were represented by MMP-2 (72 kDa) and MMP-9 (92 kDa) activity.

Statistical analysis. Statistical analysis was performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA) and data are presented as the mean \pm SD. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Aldosterone serum concentration increases in EAM mice. Aldosterone synthase expression levels are high in human myocardium during acute myocarditis (5). Aldosterone serves an important role in the pathophysiology of cardiac remodeling (19). Therefore, aldosterone concentration in EAM mice myocardium was assessed using ELISA. Compared with the

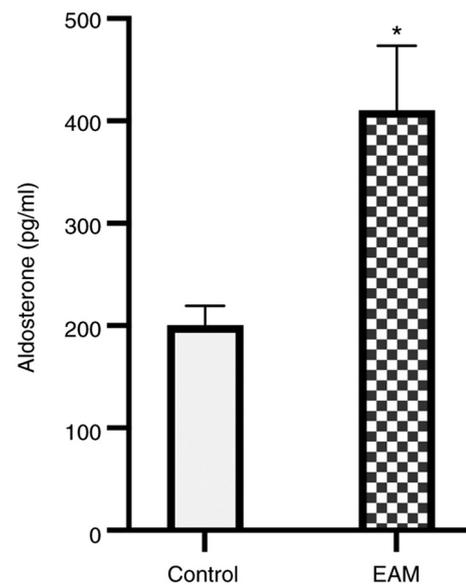


Figure 1. Plasma concentrations of aldosterone in the mice. As compared with controls, plasma aldosterone of EAM mice increased significant. * $P < 0.05$ vs. control. EAM, experimental autoimmune myocarditis. Data are representative of three independent experiments. EAM, experimental autoimmune myocarditis.

controls, aldosterone serum concentrations were significantly increased in EAM mice on day 36 ($P < 0.05$; Fig. 1).

Spironolactone decreases inflammatory cytokine levels in EAM myocardial tissue. Previous results have demonstrated that inflammatory cytokines serve an important role in the progression of post-myocarditis cardiac remodeling (20). On day 36, H&E staining demonstrated that inflammatory cell infiltration was present in the EAM group compared with the control groups. However, spironolactone treatment significantly reduced cell infiltration in the EAM group (Fig. 2A). To evaluate the levels of inflammatory cytokines in the EAM myocardium, TNF- α and IL-6 serum concentrations were quantified using ELISA. Compared with the control groups, the serum concentrations of TNF- α and IL-6 significantly increased in the EAM group ($P < 0.05$) but significantly decreased in the EAM model with spironolactone treatment ($P < 0.05$; Fig. 2B and C).

Spironolactone ameliorates myocarditis-induced myocardium hypertrophy and diastolic dysfunction. Echocardiography was used to assess cardiac function on day 36 of the experiment. It was determined that the LVEF, E/A ratio, LV chamber size and wall thickness did not differ between animals in each group. At day 36, compared with the control groups, a significantly lower E/A ratio was observed in EAM mice ($P < 0.05$), which was accompanied by a significant increase in the LVPW ($P < 0.05$). Spironolactone treatment significantly ameliorated the reduced E/A ratio and LVPW in EAM mice ($P < 0.05$). However, there was no statistically significant difference in LVEF or LVID between the four groups investigated ($P > 0.05$). These results indicated that EAM mice may exhibit LV hypertrophy and diastolic dysfunction at day 36, but there was no evidence of LV dilatation and systolic dysfunction (Table I).

Table I. Echocardiographic parameters.

	Control	EAM	Spirolactone	Saline
LVPWd (mm)	0.48±0.07	0.71±0.11 ^a	0.57±0.09 ^b	0.73±0.10 ^a
LVPW _s (mm)	0.83±0.17	1.31±0.29 ^a	1.02±0.18 ^b	1.26±0.22 ^a
LVIDd (mm)	4.51±0.24	4.32±0.36	4.59±0.35	4.40±0.28
LVIDs (mm)	3.70±0.38	3.55±0.31	3.74±0.24	3.54±0.25
LVEF (%)	64.65±11.25	68.37±9.81	71.50±13.18	69.38±11.65
E/A	1.40±0.18	0.69±0.25 ^a	1.10±0.36 ^b	0.79±0.27 ^a

EAM, experimental autoimmune myocarditis; LVEF, left ventricular ejection fraction; E/A, the ratio of early to late mitral inflow velocity; LVIDd, left ventricular internal dimensions in diastole; LVIDs, left ventricular internal dimensions in systole; LVPWd, left ventricular posterior wall of diastole; LVPW_s, left ventricular posterior wall of systole; LVPWs, LV posterior diameter in systole. ^aP<0.05 vs. control groups; ^bP<0.05 vs. EAM groups.

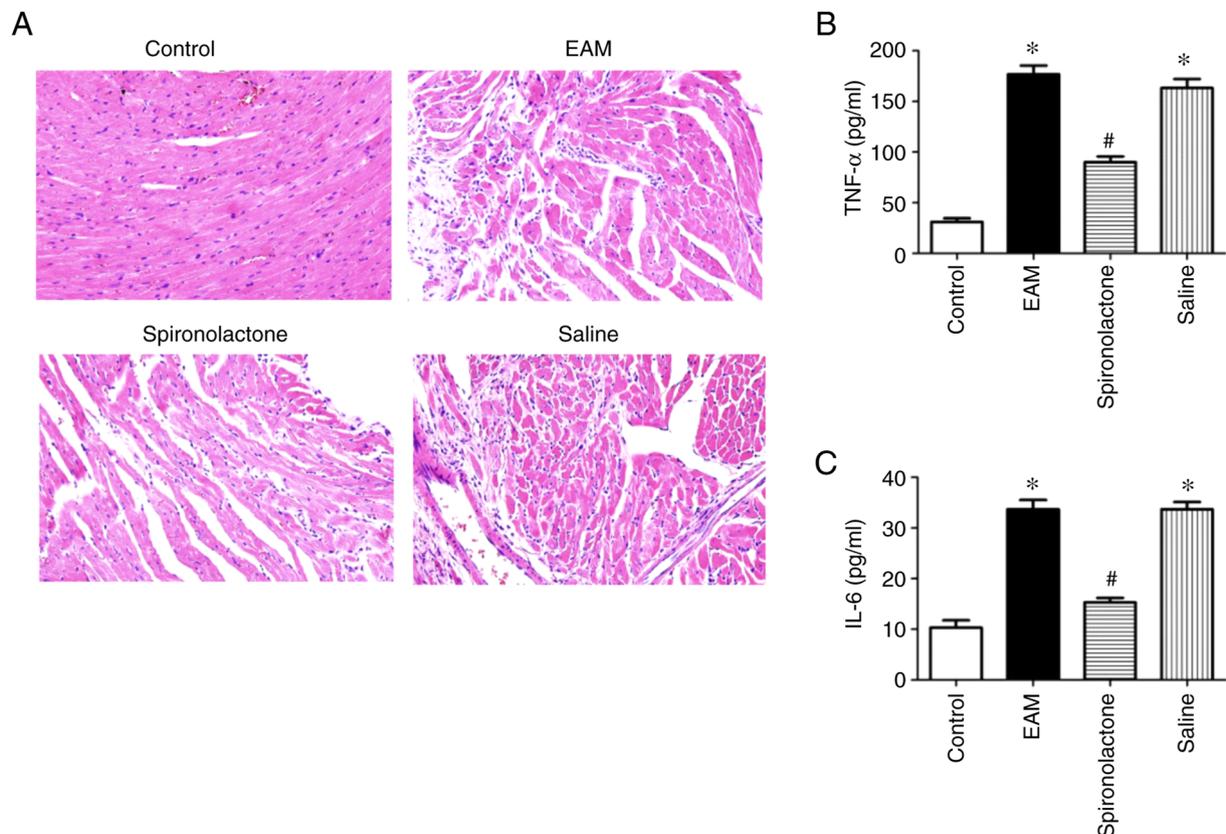


Figure 2. Spirolactone treatment reduces inflammation in mouse myocardia. (A) Representative hematoxylin and eosin staining of left ventricular (magnification, x400). The infiltration of inflammatory cells was reduced significantly by spironolactone treatment. Serum concentrations of (B) TNF- α and (C) IL-6 levels measured using ELISA. ^{*}P<0.05 vs. control and [#]P<0.05 vs. EAM. Data are representative of three independent experiments. EAM, experimental autoimmune myocarditis.

Ets-1 activation is downregulated in spironolactone-treated EAM mice. Subsequently, whether Ets-1 participated in EAM-induced cardiac fibrosis was investigated. Ets-1 protein expression levels were determined using immunohistochemistry (Fig. 3A) and western blotting (Fig. 3B). The results demonstrated that total Ets-1 and p-Ets-1 protein expression levels were significantly increased (P<0.05; Fig. 3C and D) in EAM mice myocardium. Subsequently, the effect of spironolactone on myocarditis-induced Ets-1 activation was investigated. Compared with the myocarditis mice groups,

Ets-1 protein expression levels and phosphorylation were significantly reduced by spironolactone treatment (P<0.05).

Spirolactone inhibits TGF- β 1/Smad-2/3 signaling pathway activation in EAM mice. TGF- β 1 performs a crucial role in cardiac fibrosis, whereby Ets-1 participates in matrix remodeling in response to TGF- β 1 (21). In the present study, the effect of spironolactone on the TGF- β 1/Smad-2/3 signaling pathway was explored. The protein expression level of TGF- β 1 in the myocardium was determined by immunohistochemistry. The

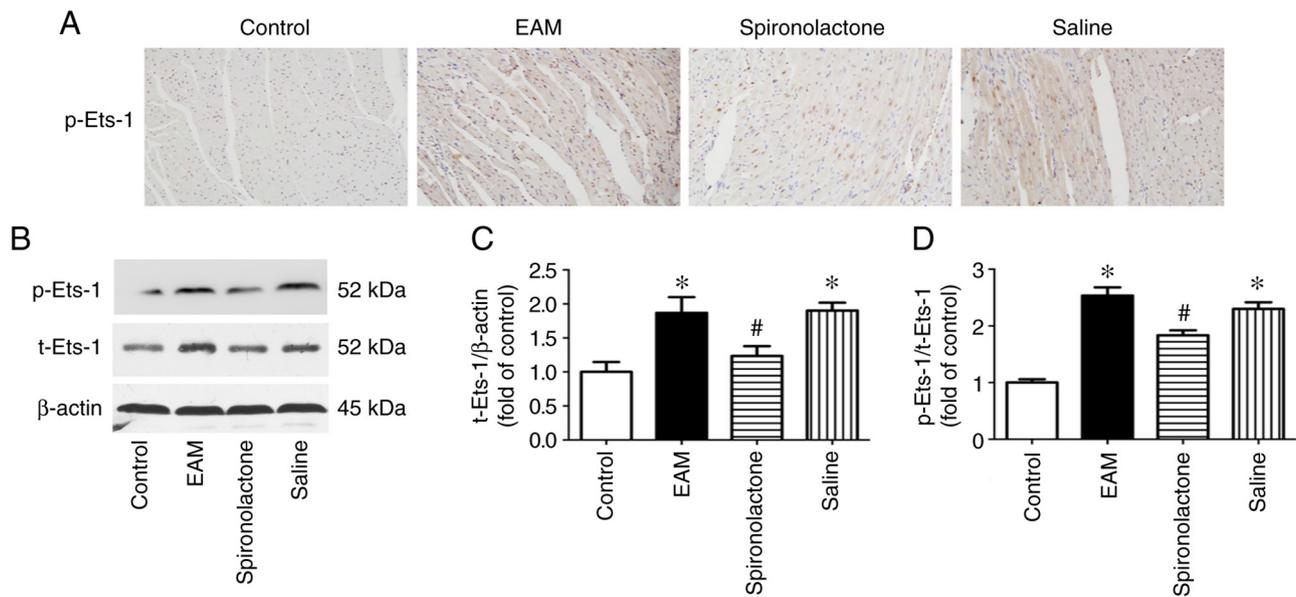


Figure 3. Spironolactone downregulated the expression of p-Ets-1 in EAM mice. (A) Immunohistochemical analysis of phosphor-Ets-1 protein expression in mice myocardium (magnification, x400). (B) Western blot analysis of protein level of (C) p-Ets-1 and (D) t-Ets-1 protein levels in myocardial tissues. * $P < 0.05$ vs. control and # $P < 0.05$ vs. EAM. Data are representative of three independent experiments. p-, phosphorylated; Ets-1, E26 transformation-specific sequence-1; t-, total; EAM, experimental autoimmune myocarditis.

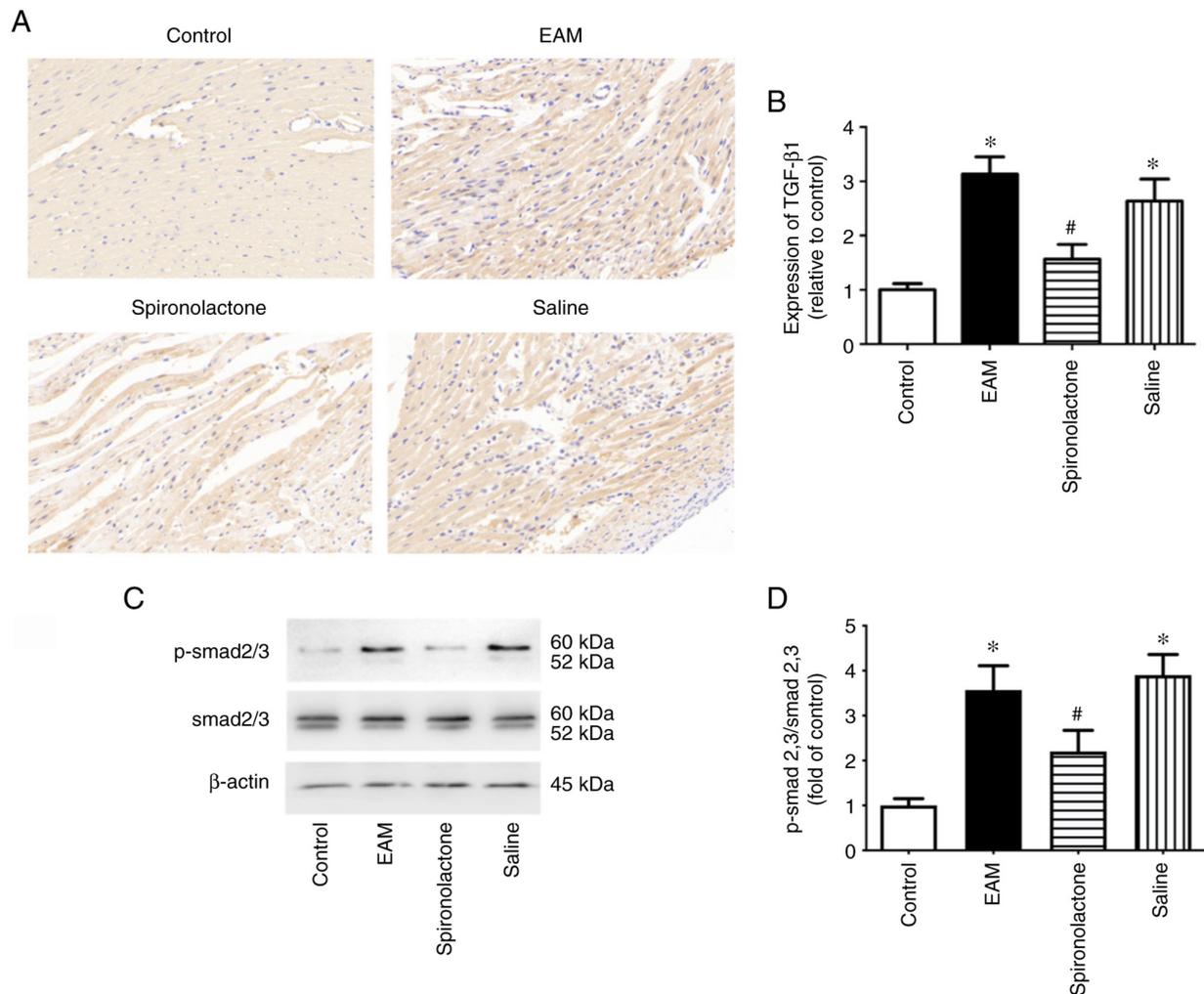


Figure 4. Effect of spironolactone on TGF- β 1 and Smad-2/3 in EAM myocardium. (A) Immunostaining of TGF- β 1 (magnification, x400). (B) Semi-quantification analysis of TGF- β 1 staining. (C and D) Western blot analysis of p-Smad2/3 and Smad2/3 expression. * $P < 0.05$ vs. control and # $P < 0.05$ vs. EAM. Data are representative of three independent experiments. EAM, experimental autoimmune myocarditis.

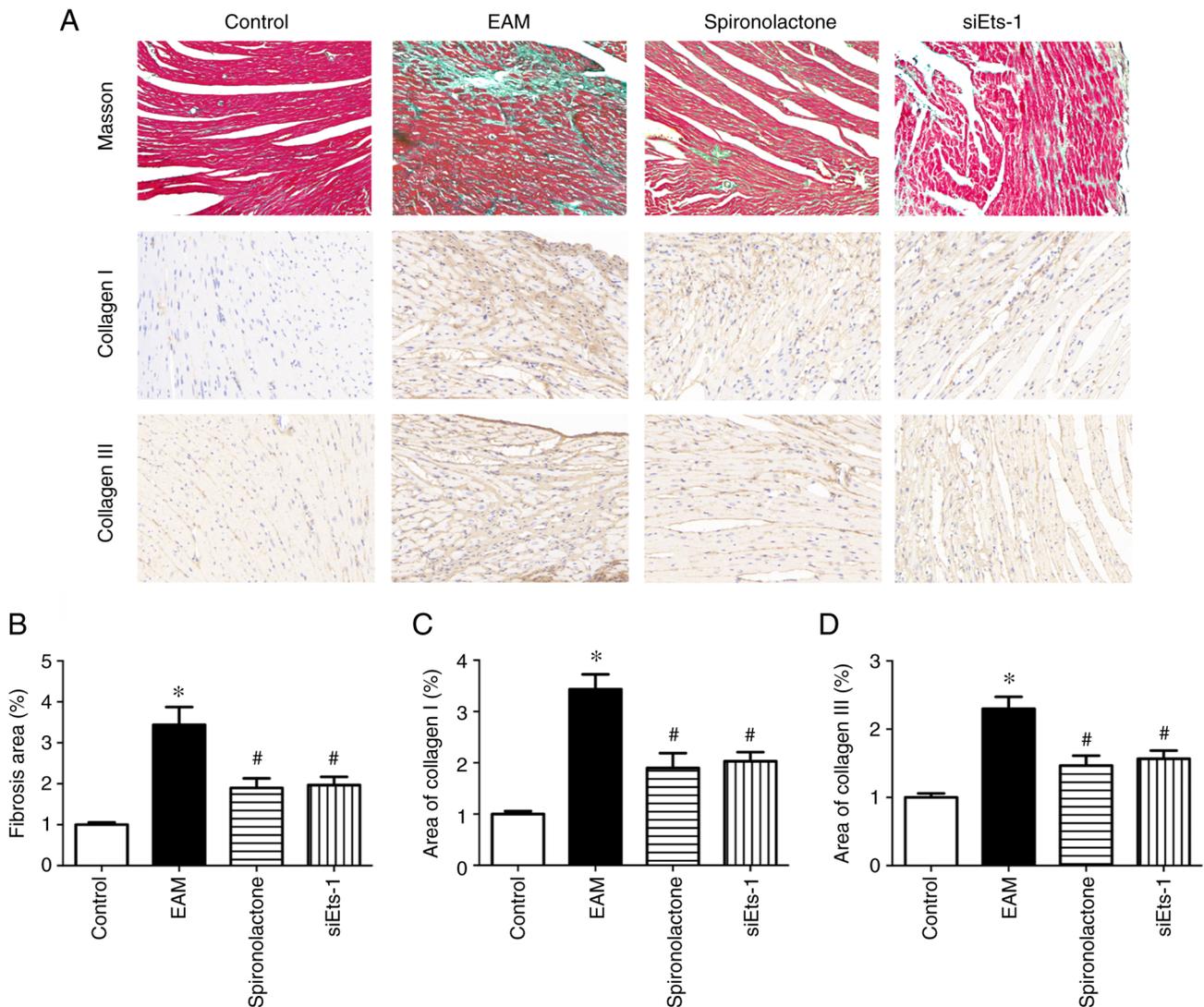


Figure 5. Inhibition of Ets-1 attenuate myocarditis-induced cardiac fibrosis. (A) Representative Masson' trichrome staining (first row; magnification, x400), immunostaining of collagen I (second row; magnification, x400) and collagen III (third row; magnification, x400). (B) Quantitative analysis of myocardial fibrosis. (C) Semi-quantification analysis of collagen I staining. (D) Semi-quantification analysis of collagen III staining. * $P < 0.05$ vs. control and # $P < 0.05$ vs. EAM. The Data were determined by randomly selecting five fields of view. Ets-1, E26 transformation-specific sequence-1; si-small interfering.

results demonstrated that TGF- β 1 protein expression levels were significantly increased in EAM mice compared with the control groups ($P < 0.05$). However, these increased protein expression levels were significantly reduced with spironolactone treatment ($P < 0.05$; Fig. 4A and B). Smad proteins are the downstream signaling molecules of the TGF family and are also involved in myocardial remodeling (22). The effect of spironolactone on p-Smad-2/3 protein expression levels in EAM mice was assessed. Western blotting demonstrated that spironolactone significantly inhibited the myocarditis-induced increase of p-Smad-2/3 in myocardial tissue ($P < 0.05$; Fig. 4C and D). These results indicated that spironolactone may inhibit Ets-1 activation via the TGF- β 1/Smad-2/3 signaling pathway in EAM mice.

Ets-1 knockdown attenuates myocardial fibrosis in EAM mice. Aldosterone antagonists are established therapeutics for patients with heart failure (23). The Masson's trichrome staining method was used to assess the degree of myocardial fibrosis. The results

demonstrated that in the EAM group fibrosis was significantly increased ($P < 0.05$; Fig. 5A and B). As reported in previous studies, the present study demonstrated that spironolactone significantly inhibited myocardium fibrosis in EAM mice ($P < 0.05$). Furthermore, in EAM mice, significantly increased protein expression levels of collagens I and III were observed in the myocardium ($P < 0.05$; Fig. 5C and D). Spironolactone treatment significantly decreased the protein expression levels of collagen I and III in the EAM group ($P < 0.05$). Ets factors are important mediators of ECM remodeling (24). To further examine the effects of Ets-1 on cardiac fibrosis induced by myocarditis, Ets-1 expression was silenced using siRNA. Compared with the control, Ets-1 protein expression levels were significantly reduced by siRNA transfection ($P < 0.05$; Fig. S1). Immunohistochemistry analysis demonstrated that the quantities of collagen I and collagen III significantly decreased in the siEts-1 group compared with EAM group ($P < 0.05$, Fig. 5). These results indicated that spironolactone may limit myocarditis-induced fibrosis that is mediated by the inhibition of Ets-1.

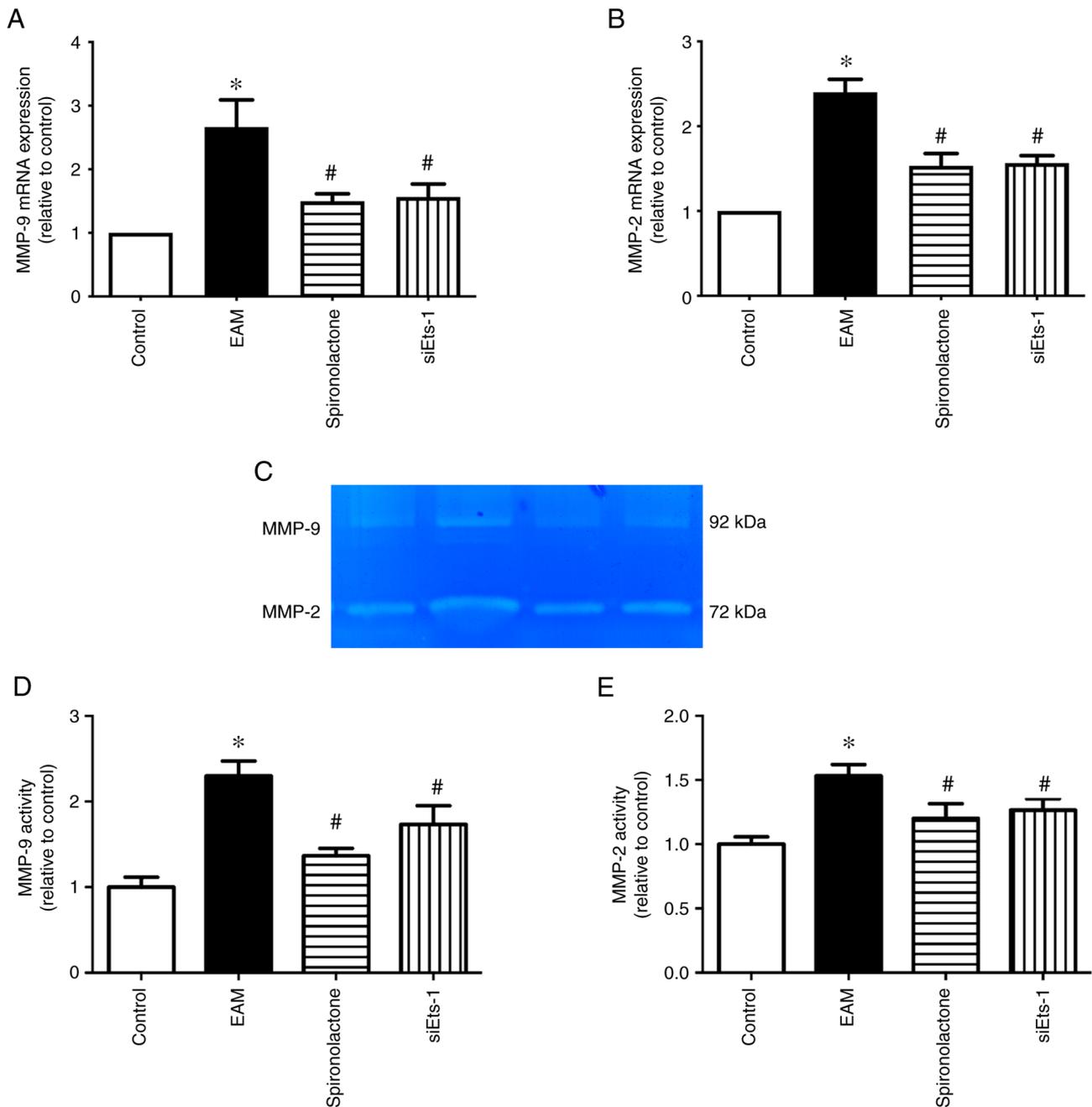


Figure 6. Effect of Ets-1 on expression and activity of MMP-2 and MMP-9 in myocarditis mice. (A and B) mRNA expression of MMP-2 and MMP-9 was measured by reverse transcription-quantitative PCR. (C) Gelatin zymography analysis of activity of (D) MMP-9 and (E) MMP-2. Data are representative of three independent experiments. * $P < 0.05$ vs. control and # $P < 0.05$ vs. EAM. Ets-1, E26 transformation-specific sequence-1.

Ets-1 inhibition decreases MMP-2 and MMP-9 expression and activity levels in EAM mice. MMPs may also serve a central role in ECM remodeling (25). RT-qPCR and gelatin zymography demonstrated that the mRNA expression levels and the activity of MMP-2 and MMP-9 increased significantly in the EAM group compared with the controls, whereas spironolactone treatment significantly attenuated this effect ($P < 0.05$; Fig. 6). Furthermore, the role of Ets-1 in MMP mRNA expression and activity in EAM mice was explored. The results demonstrated that compared with EAM mice, the increased mRNA expression levels and activity of MMP-2 and MMP-9 were significantly downregulated by Ets-1-siRNA ($P < 0.05$). These results therefore indicated that spironolactone

may inhibit the expression and activation of MMP-2 and MMP-9 via Ets-1 activation suppression.

Discussion

Myocarditis is a suspected common precursor of DCM (26). Previous studies demonstrate that increased aldosterone causes oxidative stress, inflammation and fibrosis (27). Spironolactone is a widely used antagonist of aldosterone that is used to treat chronic heart failure. Numerous clinical studies have demonstrated that the administration of an aldosterone antagonist improves LV remodeling in patients with heart failure (28,29). However, the underlying mechanism

of the protective effects of spironolactone on myocarditis remains to be fully elucidated. In the present study, the results demonstrated that aldosterone serum concentrations significantly increased in EAM mice. Spironolactone was demonstrated to significantly reduce inflammation and fibrosis and significantly improved LV diastolic functions in EAM mice. Furthermore, the results indicated that the protective effect of spironolactone via downregulated Ets-1 expression levels may be via the TGF- β 1/Smad-2/3 signaling pathway in EAM mice.

Inflammation is a key pathophysiologic factor in the EAM and is closely associated with myocardial fibrosis (1,30). Following coxsackievirus B3 infection, inflammatory cytokines, such as TNF- α , IL-1 β , IL-4 and TGF- β 1 are increased in the myocardium (31). Inflammatory cytokines may stimulate the expression of profibrotic factors like TGF- β and plasminogen activator inhibitor-1. Biomarkers of inflammation are associated with a risk of developing DCM. Li *et al* (32) report that TNF- α overexpression in the myocardium causes myocardial remodeling and LV dysfunction associated with increased MMP expression. *In vitro*, IL-1 β and IL-6 promote the remodeling of interstitial collagen by increasing total MMP activity in cardiac fibroblasts (33). These aforementioned studies indicate that inflammatory induction of IL-1 β , IL-6 and TNF- α may contribute to myocardial collagen remodeling mediated via the MMP/tissue inhibitors of metalloproteinases system. Consistent with these findings, the results of the present study demonstrated a significant increase in the TNF- α and IL-6 concentrations in EAM mice. However, spironolactone significantly reduced these levels.

Aldosterone is a multifunctional molecule that serves a significant role in heart failure. Spironolactone slows the progression of heart failure by decreasing the serum markers of fibrosis or type I and/or III collagen metabolism, which reverses changes to cardiovascular structure and function in patients at high risk of developing heart failure (34). Spironolactone prevents perivascular/interstitial fibrosis in experimental models of hypertension (35). Furthermore, spironolactone significantly reduces cardiac fibrosis and inflammation in streptozotocin-induced diabetic rats (36). In addition, the aldosterone antagonist eplerenone is an anti-inflammatory and protects viral myocarditis mice from heart remodeling (6). In the present study, the results demonstrated that EAM mice exhibited significant LV hypertrophy and diastolic dysfunction at day 36, whereas no LV dilatation or systolic dysfunction were observed. Spironolactone significantly reduced abnormal interstitial collagen accumulation and improved LV hypertrophy and diastolic dysfunction. In addition, this protective effect was attributed to the significantly decreased levels of proinflammatory cytokines such as TNF- α , IL-6 and TGF- β 1, as well as the significant inhibition of MMP-2 and MMP-9 mRNA expression and activity levels in EAM mice. Even though experimental studies and clinical trials have indicated that spironolactone slows the progression of cardiac fibrosis, the precise mechanisms have remained uncertain. To the best of the authors' knowledge, the present study was the first to demonstrate that spironolactone significantly downregulated the expression of Ets-1 in EAM mice. This result indicated that spironolactone may

improve EAM-induced cardiac dysfunction via inhibition of Ets-1 activation.

The transcription factor Ets-1 is a critical mediator of ECM remodeling. Ets-1 governs a wide spectrum of ECM-related target genes, including matrix proteins and enzymes (21). Previous studies have demonstrated that Ets-1 is a transcriptional regulator of numerous proteinases, including MMP-1, MMP-3 and urokinase-type plasminogen activator (u-PA) (21,37,38). MMPs not only modulate the degradation of matrix components but also effect collagen synthesis. Previous studies have demonstrated that increased MMP expression accompanies severe fibrosis in myocardial tissue (39). Furthermore, abnormal MMP activity results in excessive collagen deposition, which contributes to the development of myocardial fibrosis and cardiac dysfunction (40). The role of Ets-1 as a regulator of ECM in tumors and autoimmune diseases has also been well characterized (38,41), whereas the role of Ets-1 in myocardial fibrosis has rarely been reported. It has previously been reported that the inhibition of Ets-1 could diminish angiotensin II-induced cardiac fibrosis via the inhibition of the endothelial-to-mesenchymal transition (42). However, to the best of the authors' knowledge, the effect of Ets-1 on cardiac fibrosis induced by myocarditis has not previously been explored. In the present study, myocardial fibrosis and MMP-2 and MMP-9 mRNA expression levels and activity were significantly upregulated in EAM mice and these enhanced effects were significantly attenuated by the inhibition of Ets-1.

Myocardial fibrosis is a typical characteristic observed during the transition of myocarditis to DCM (43). A previous study reported the contribution of Smad-dependent signaling pathways to TGF- β -induced cardiac fibrosis (44). TGF- β 1 signaling via Smad-2/3 phosphorylation contributes to the binding of angiotensin II to the angiotensin type 1 receptor, which induces cardiac fibrosis (45). TGF- β 1 mediates MMP expression via its inhibitory element in the promoter region of the MMP genes (46). The results of the present study demonstrated that the increased TGF- β 1 and p-Smad2/3 protein expression levels were significantly inhibited by spironolactone treatment in EAM mice. Therefore, the protective effect of spironolactone may be associated with the TGF- β 1/Smad2/3 signaling pathway. A previous study also reports that Ets-1 could mediate TGF- β induced tissue fibrosis (47). Therefore, it was hypothesized that the effect of spironolactone on Ets-1 was mediated by TGF- β 1/Smad-2/3 signaling pathway in EAM mice.

In conclusion, the present study demonstrated that Ets-1 inhibition may attenuate myocarditis-induced cardiac fibrosis. The results indicated that the protective effect of spironolactone on post-myocarditis remodeling may be a result of inflammation and fibrosis suppression via the inhibition of Ets-1. Furthermore, the results indicated that Ets-1 activity induced by myocarditis may be associated with the TGF- β /Smad-2/3 signaling pathway. The present study may have helped identify a novel therapeutic approach for the treatment of myocarditis-induced DCM.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WKW designed the study, performed the experiments and wrote the manuscript. BW designed the study and analyzed the data. XHC performed the experiments and analyzed the data. YSL designed the study and wrote the manuscript. WKW and YSL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committees of the Second Hospital of Shandong University (approval no. KYLL-2020-(KJ)A-0134; Jinan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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