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Panax notoginseng saponins ameliorates coxsackievirus B3-induced myocarditis by activating the cystathionine-γ-lyase/ hydrogen sulfide pathway

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Abstract

This study is to determine the therapeutic effects of *Panax notoginseng* saponins (PNS) on coxsackievirus B3 (CVB3)-induced myocarditis, and whether cystathionine-γ-lyase (CSE)/ hydrogen sulfide (H_2S) pathway is involved. Mouse model of myocarditis was induced by CVB3 infection and the mice were subjected to vehicle (saline) or drug treatments (sodium bisulfide (NaHS), propargylglycine (PAG) or PNS). The results showed that there were inflammatory cell infiltrations, interstitial edemas, as well as elevated inflammatory cytokines, in CVB3-induced myocarditis. PAG administration increased, whereas NaHS treatment decreased the severity of the myocarditis. PNS treatment dramatically alleviated these myocardial injuries and decreased the viral mRNA expression by the enhanced expression of CSE/H_2S pathway. Moreover, the therapeutic effects of PNS on myocarditis were stronger than NaHS. Finally, the effect of PNS on CSE/H_2S pathway and cardiac cell protection were verified in cultured cardiac cells. PNS may be a promising medication for viral myocarditis therapy.

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Disclosures

None

Human subjects/informed consent statement

No human studies were carried out by the authors for this article

Animal Studies

The work was conducted with the approval and in accordance with the guidelines of the Wenzhou Medical University. All institutional and national guidelines for the care and use of laboratory animals were followed and approved by the university institutional committees.

Keywords

Myocarditis; *Panax notoginseng* saponins; hydrogen sulfide; cystathionine-γ-lyase

Introduction

Viral myocarditis is a main cause of cardiomyopathy, which may lead to heart failure, arrhythmia, and even sudden death[1]. Many supportive therapeutic strategies such as anti-virus, immune suppression, and anti-oxidation therapies, have been tried to reverse the underlying active myocardial inflammation; however, they are not efficient enough to improve the patient survival in clinical applications [1–3]. Viral myocarditis remains a challenging disease, and effective therapies are still needed in practice. Therefore, elucidation of the fundamental mechanisms responsible for the development of myocarditis is very important [4–6].

Recently, hydrogen sulfide (H_2S) has been shown to play key roles in physiological and/or pathological processes including inflammation, oxidative stress, apoptosis, and vaso-relaxation [7]. Moreover, high levels of H_2S, as well as the activation of cystathionine-γ-lyase (CSE)/H_2S pathway, have also been found in myocardial tissues, making this pathway a potential target in the treatment of inflammatory heart diseases [8,9]. Indeed, activation of CSE/H_2S pathway could effectively protect hearts against ischemic injuries [8–11]. In addition, CSE/H_2S pathway is also involved in viral myocarditis [12].

Panax notoginseng saponins (PNS) is the major active ingredient in the traditional Chinese herb *Panax notoginseng*, which has been used to treat cardiovascular diseases for more than 1000 years. PNS has been found to have extensive effects on the cardiovascular system by regulating multiple signaling pathways [13]. For example, PNS could attenuate atherosclerosis via reciprocal regulation of lipid metabolism and inflammation [14], and inhibit ischemia-induced apoptosis in cardio-myocytes by the activation of the phosphoinositide Kinase-3 (PI3K)/Akt pathway [15]. In vivo, injection of PNS is able to reduce myocardial injuries induced by ischemia [16].

Although PNS has a strong protective effect on myocardial injures, the therapeutic effects of PNS on viral myocarditis have not been reported. In addition, the effect of PNS on CSE/H_2S pathway in hearts with viral myocarditis is still unclear. In the present study, we try to investigate the effects of PNS on myocarditis, and to determine whether the CSE/H_2S pathway is involved in PNS-mediated therapeutic effect on myocarditis in CVB3-infected mice.

Methods

Reagents and kits

CVB3 Nancy strains and Hela cells were purchased from ATCC (Rockville, MD, USA). PNS was purchased from Guangxi Wuzhou Pharmaceutical (Group) Co., Ltd. (Wuzhou, Guangxi, China). DL-propargylglycine (PAG) and sodium bisulfides (NaHS) were purchased from Sigma (St. Louis, MO, USA). DMEM, FBS, and Penicillin/Streptomycin

were purchased from Gibco (Grand Island, NY, USA). BCA kit was from Thermo Scientific (Rockford, IL, USA). IL-6 and TNF-α ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). H_2S ELISA kit was purchased from Shanghai Biological Technology Co., Ltd. (Shanghai, China). The cDNA synthesis kit and SYBR green were purchased from Boston Biomedica Inc. (West Bridgewater, Mass., USA). GAPDH and CSE antibodies were purchased from GeneTex (San Antonio, TX, USA). ECL kit was purchased from Advanstar (Cleveland, Ohio, USA). Anti-rabbit IgG was purchased from Millipore (Bedford, MA, USA). The primers for CVB3 (F-5′-GTATGCTGCGACTAGACGTTGT-3′, R-5′-TTCTCTTCTCTGCGTTTCCTGT-3′), CSE (F-5′-CTGATACGACTTTCTGTGGGC-3′, R-5′-AGTTCTGCGTATGCTCCGTAA-3′), and GAPDH (F-5′-GGTTGTCTCCTGCGACTTCA-3′, R-5′-TGGTCCAGGGTTTCTTACTCC-3′) were synthesized by Sangon Biotech (Shanghai, China). Lipofectamine™ 2000 transfection reagents were obtained from Invitrogen (Carlsbad, CA).

Cell culture and detection of apoptosis

H9c2 cells (ATCC CRL1446, a cardiac cell line) were grown at a density of about 105 cells/cm2 and cultured as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, glutamine (2 mM), nonessential amino acids (1%), penicillin (100 IU), and streptomycin (100 j,g/ml) under an atmosphere of 5% CO2 in air saturated with water vapor at 37°C. The medium was replaced by fresh medium every 2 days. Cell apoptosis was induced by H_2O_2 (200 μM) for 6 h and was measured by terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) staining as described in our previous study [17]. Briefly, cardiac myocytes cultured on coverslips in 24-well plates were fixed in 4% paraformaldehyde. The TUNEL staining was done using the *in situ* cell death detection kit (Roche) according to the manufacturer's protocol. The numbers of TUNEL-positive cells and the total cells were counted under a fluorescence microscope.

RNA Interference

siRNA for CSE (siRNA-CSE) and control siRNA (siRNA-control) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamin 2000 and siRNAs (50 nM) were mixed and kept still for 20 min at room temperature, and then the mixtures were added to cardio-myocytes cultured in 6-well plates for transfection. Knockdown of CSE was confirmed by using Western blotting.

Virus propagation

Hela cells were maintained in DMEM medium, supplemented with 10% FBS at 37°C in 5% CO_2. After reaching at least 80% confluence, CVB3 was added. When cytopathic affection (CPE) was evident, the virus was released from these cells by three freeze-thaw cycles. Samples were centrifuged at 3000 rpm at 4 °C for 10 min, and the supernatant was collected. Then, the virus titer was calculated by Reed-Muench formula.

Animals

All animal protocols were approved by the Animal Care and Use Committee of Wenzhou Medical University and were consistent with the Guide for the Care and Use of Laboratory Animals (updated (2011) version of the NIH guidelines). A total of 140 Balb/c male mice from two repeated experiments (five-weeks-old, weighing 20.9 g ± 2 g) obtained from the Experimental Animal Research Center (Zhejiang, China), were randomly assigned into 5 groups: control group (n=20), viral myocarditis model group (n=30), NaHS-treated group (n=30), PAG-treated group (n=30), and PNS-treated group (n=30). Mice in the control group were injected daily with phosphate-buffered saline. All the other groups were infected by injecting 0.1 ml CVB3 ($10^{-6.5}TCID_{50}$), and then the mice received intraperitoneal injections of PBS, NaHS (50 μmol/kg), PNS (100 mg/kg) [18], or PAG (40 mg/kg), for 10 consecutive days. The mice were sacrificed on day 4 and day 10 respectively after infection. Blood specimens and heart tissue were collected from these mice for following assays.

Histopathology

Histopathological analysis was performed as described [19]. In brief, hearts were isolated after perfusion to remove blood. The ratio of heart weight to tibia length (HW/TL) was calculated to assess the degree of edema. The heart tissue was then fixed in 10% paraformaldehyde, and embedded in paraffin. Then, the tissue was sectioned, stained with hematoxylin and eosin and were sealed with neutral balsam. % inflamed lesion areas of heart tissue were measured in these heart sections.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the levels of IL-6, TNF-α, H_2S in serum, cardiac homogenate, and cell culture supernatant according to the manufacturers' instructions. The concentrations were calculated with reference to the standard curves.

RNA extraction and RT-PCR

Total RNA from heart tissues and cardiac cells was extracted with Trizol reagent, according to the manufacturer's instructions. After denaturing at 95°C for 5 min, reverse transcription was performed with a total volume of 20 μL, at 70°C for 5min, 37°C for 5 min, 42°C for 60 min, and 70°C for 10 min, followed by 40 cycles of PCR reaction consisting of 95°C (10s) and 60°C (40s). The PCR reaction was directly monitored by ABI StepOne Plus Sequence Detection System (Applied Biosystems), and all the results were normalized against GAPDH.

Western blot analysis

The proteins were extracted from the cardiac tissue and cardiac cells. The concentrations were determined by the BCA protein assay kit. Then an equal amount of protein extract (50μg) was separated by 10% SDS-PAGE and stacked by 5% SDS-PAGE. They were then transferred onto polyvinylidene difluoride (PVDF) membrane. Then the blots were probed with primary antibody, followed by secondary antibody. Bands were visualized by using

enhanced-chemiluminescence (ECL) kit, and then quantified by densitometry using AlphaEaseFC software.

Statistical analysis

Data was expressed as mean ± SE. Statistical analysis was performed with SPSS 19 software (SPSS Inc., Chicago, IL, USA). Three or more groups were compared by one-way ANOVA and post-hoc analysis with the least significant difference, Student-Newman-Keuls test and Dunnett's test. Statistical significance was defined as $P < 0.05$.

Results

Animals

There were no deaths in control group. Eight out of 30 mice were died in viral myocarditis model group (Mortality rate: 26.67%); Six mice out of 30 mice were died in NaHS-treated group (Mortality rate: 20.00%); Ten mice out of 30 mice were died in PAG-treated group (Mortality rate: 33.33%); Five mice out of 30 mice were died in PNS-treated group (Mortality rate: 16.67%). The sacrificed animals of each group at two time points are: day 4: control group (n=10), viral myocarditis model group (n=10), NaHS-treated group (n=10), PAG-treated group (n=10), and PNS-treated group (n=10); day 10: control group (n=10), viral myocarditis model group (n=12), NaHS-treated group (n=14), PAG-treated group (n=10), and PNS-treated group (n=15).

PNS alleviates myocardial injuries in CVB3-induced myocarditis

Histopathological analysis demonstrated that, compared with the control group, there were obvious myocardial injuries in the CVB3-induced model group on day 4 after infection, which became more severe on day 10, in terms of the inflamed area in heart tissue sections, and interstitial edema (HW/TL ratios) (Fig. 1). PAG treatment aggravated these injuries in myocarditis models. In contrast, the treatment with NaHS or PNS significantly alleviated these myocardial injuries, with a greater extent in the PNS-treated group (Fig. 1).

The concentrations of serum IL-6 and TNF-a were utilized as inflammatory markers for the severity of myocarditis. As shown in Fig. 2, compared with basal levels, IL-6 and TNF-a increased two-fold in the CVB3 group on day 4 and day 10 following infection. Administration of PAG increased the serum levels of IL-6 and TNF-α, while PNS and NaHS reduced these inflammatory cytokines in serum (Fig. 2).

These results suggest that there were obvious inflammatory cell infiltration, interstitial edema, as well as elevated inflammatory cytokines in CVB3-induced myocarditis. PAG administration increased, while NaHS treatment decreased the severity of the myocarditis and its blood inflammatory biomarkers. Interestingly, treatment with PNS could alleviate these myocardial injuries, even to a greater extent than the NaHS treatment.

PNS activates the CSE/H_2S pathway in CVB3-induced myocarditis

To find out whether the CSE/H2S pathway was involved in the therapeutic effects of PNS on CVB3-induced myocarditis, the H2S levels in serum and heart tissue were detected by

ELISA, and the mRNA and protein expression levels of CSE in heart tissues were assessed with qRT-PCR and Western blot, respectively. Our results indicated that CVB3-infection reduced the levels of H_2S in both serum and heart tissue (Fig. 3). The administration of PAG, which inhibited CSE activity, resulted in even lower levels of H_2S both in serum and heart tissue. The treatment of NaHS, the H_2S donor, increased H_2S levels in myocarditis models, which was further increased by PNS treatment (Fig. 3).

The detection of CSE expression indicated that CVB3-infection decreased the expressions of CSE at both mRNA and protein levels, compared to the control group on both the time points, which were further declined by the PAG treatment (Fig. 4). However, both CSE mRNA level and CSE protein level in PNS-treated group were higher than those in viral myocarditis model group. For the treatment of NaHS, no effects on CSE expression at either mRNA or protein level were observed in the myocarditis models. Moreover, for either H_2S or CSE expression, there were no significant differences between data on day 4 and day 10 after infection (Fig. 3 and 4). These results suggested that PNS treatment could enhance the expression of H_2S and CSE in CVB3-induced myocarditis, which might contribute to its therapeutic effects in these models.

PNS activates the CSE/H_2S pathway in the cultured cardiac cell line

To provide direct evidence that PNS could enhance the CSE/H_2S pathway in cardiac cells, the cardiac cell line H9c2 cells were applied. The cells were treated with vehicle (saline) or PNS (10 μg/ml). At 4h after treatments, cell culture supernatants were collected to determine the levels of H2S by ELISA. Then, the RNAs of H9c2 cells were isolated and CSE mRNA was measured by qRT-PCR. As shown in Fig. 5, PNS treatment significantly increased the levels of H2S and CSE in cultured H9c2 cells.

PNS mediated cardiac cell protection is related to CSE

To test whether PNS-mediated protective on cardiac cells was related to CSE, the expression of CSE was knocked down as shown in Fig. 5C and 5D. H_2O_2-induced cardiac cell apoptosis was significantly inhibited by PNS (Fig. 5E). Interestingly, PNS-mediated protective effect on cardiac apoptosis was blocked in CSE-knock-downed cells (Fig. 5E).

PNS decreases the viral mRNA expressions in CVB3-induced myocarditis

To determine whether PNS has effects on virus propagation in CVB3-induced myocarditis, the mRNA levels of CVB3 were determined by qRT-PCR. Our data showed that, compared with the control group, the expression of CVB3 mRNA was increased by the treatment of PAG on both day 4 and day 10 after infections (Fig. 6). When treated with H_2S, the virus replication was significantly inhibited, as indicated by the obviously decreased CVB3 mRNA level. Furthermore, PNS treatment resulted in an even more dramatic reduction of the CVB3 mRNA expression in the myocarditis model, at the two time points (Fig. 6). These results suggested that PNS could significantly decrease the viral mRNA expressions in CVB3-induced myocarditis.

Discussion

Myocarditis is defined as inflammation of the myocardium, always followed by cardiomyocyte necrosis and/or degeneration [20, 21]. Viral myocarditis is a common cause of acute heart failure, and CVB3 is the most common virus in the disease etiology [22]. H_2S, the third novel gasotransmitter in addition to NO and CO, has been considered as a biologically relevant signaling molecule with potentials in the development of various diseases [23–28]. Previous studies have shown that H_2S plays key roles in some biological processes, including inflammation, oxidative stress, apoptosis, and vaso-relaxation [7, 29]. Recently, several researchers have found that CSE, cystathionine-β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST) are the key enzymes to produce H_2S, using L-cysteine as substrate [30, 31]. Moreover, CSE is the major H_2S-forming enzyme in myocardial tissues [8]. The predominant role of CSE/H_2S pathway has also been demonstrated by the high levels of CSE mRNA expression and H_2S concentration in rat myocardium [9]. Our study provides several lines of evidence indicating the CSE/H_2S pathway is involved in the pathogenesis of viral myocarditis.

H_2S has been thought of as toxic gas with a foul odor for a long time; however its role in viral myocarditis is still unclear. H_2S has been demonstrated to exert significant cardio-protective effects through its anti-apoptotic, anti-inflammatory, and antioxidant effects [32]. Fox *et al.* found that H_2S might represent a novel endogenous mechanism of cytoprotection in inflamed joints, suggesting a potential anti-inflammation therapy for viral myocarditis [33].

CSE is the major H_2S-forming enzyme in myocardial tissues [8, 9, 34, 35]. Several researchers found that the increased CSE expressions could reduce inflammation and/or oxidative stress in the myocardium through hydrogen sulfide generation [36, 37]. In our study, the mice received intraperitoneal injections of a high dosage of CVB3 (0.1 ml/ $TCID_{50}10^{-6.5}$) would induce a large area of inflammatory cell infiltration, myocardial necrosis, and interstitial edema. In addition, recent studies have shown that intermittent hypoxia would induce cellular damages, decrease endothelial CSE expression and reduce endogenous H_2S production [38]. Accordingly, we hypothesized that lower expression of CSE in infected myocardium might be due to the protein degradation and cell damage. In line with our results, Hua *et al.* demonstrated that the exogenous administration of H_2S was protective to the infected myocardium, while the inhibition of endogenous H_2S exerted harmful effects (39).

Our data also showed that the administration of NaHS alleviated myocardial injuries, inflammatory cell infiltration, as well as interstitial edema, and inhibited virus replication, while PAG treatment aggravated myocardial injuries and enhanced the virus replication as shown by the viral mRNA expression. Virus titers should be detected to further verify the discovery in future studies. NaHS is a H_2S donor, which would exert a protective effect on myocardial injuries through increasing H_2S levels. Conversely, PAG is an irreversible CSE inhibitor, which could aggravate myocardial injuries through inhibiting the expressions of CSE, as well as H_2S. Our findings give strong evidence indicating that the CSE/H_2S

pathway plays a significant role in viral myocarditis and up-regulating H_2S production has a protective effect at the early stage of the disease.

PNS have been demonstrated to have extensive effects on the cardiovascular system [19]. Our results showed that PNS activated CSE expression at both mRNA and protein levels, along with the increased H_2S levels in our animal model in vivo. To provide direct evidence that PNS could enhance the CSE/H_2S pathway in cardiac cells, the cardiac cell line H9c2 cells were applied. We found that PNS treatment could indeed increase the levels of H2S and CSE in cultured H9c2 cells. In addition, PNS could protect the cardiac cells from H_2O_2-mediated apoptosis. It should be noted the concentration of H_2O_2 (200 μM) we used is only for the apoptosis cell model. It may not be related to the real the concentration of H_2O_2 (200 μM) in hearts with virus myocarditis. The protective effect of PNS is related to CSE as the effect was inhibited in CSE-knock-downed cells. Moreover, PNS effectively ameliorated myocardial injuries, inhibited inflammatory cell infiltration and reduced interstitial edema. These observations indicated that PNS could ameliorate viral myocarditis by activating CSE/H_2S pathway. Compared with the H_2S donor, NaSH, PNS was more effective to activate the restrained expression of CSE and H_2S in these models. Furthermore, our data showed that both PNS and NaHS could reduce CVB3 mRNA levels, while PAG resulted in enhanced viral levels. Therefore, we conclude that PNS may influence virus propagation in the early stage. Luo and Esfandiarei found that CVB3 replication could be reduced by inhibition of the PI3K/Akt and ERK1/2 pathways [40, 41]. Moreover, these pathways have been found to be related to the effects of PNS [15, 42–44]. In addition, H_2S could also influence these pathways in biological processes [45–48]. But whether or not H_2S and/or these pathways participate in the viral proliferation-inhibiting effects of PNS still merits in-depth studies.

In conclusion, our results show that PNS could ameliorate myocardial injuries, inflammatory cell infiltration, and interstitial edema by activating the CSE/H2S pathway, and could inhibit CVB3 replication during the early stage of CVB3-induced myocarditis. Based on these results, PNS would be a potential medication for viral myocarditis treatment.

There are some limitations in this study. First, in this study, the treatment of infected mice began the day of virus infection. Although it was good for a pre-clinical study to test the therapeutic effect of PNS and the potential mechanisms involved, clinically, the treatments for myocarditis are regularly performed after the viral myocarditis has become established. Thus, additional set of studies should be performed to determine the therapeutic effect of PNS on myocarditis after virus infection. Second, the dose-response and the side effects should be determined before it could be used in clinical. Third, the myocarditis was induced in mice that were 5 weeks old. The mouse immune system may not be mature until 7–8 weeks of age. Thus, the study and the discoveries should be repeated using adult mice. In addition, although we found that CSE and H_2S may be involved in PNS-mediated cardiac protection, other mechanisms should also be identified in future studies.

Clinical relevance of the study

Viral myocarditis remains a challenging disease, and novel effective therapies based on fundamental mechanisms are needed in clinical practice. In this study, we have identified that CSE/H2S pathway may be a critical molecular mechanism in the development of viral myocarditis. PNS could ameliorate myocardial injuries, inflammatory cell infiltration, and interstitial edema and inhibit CVB3 replication by activating of the CSE/H2S pathway in CVB3-induced myocarditis. Thus, PNS would be a new therapeutic medication for viral myocarditis treatment.

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Abbreviations

PNS	*Panax notoginseng* saponins
CVB3	coxsackievirus B3
CSE	cystathionine-γ-lyase
H_2S	hydrogen sulfide
NaHS	sodium bisulfide
PAG	propargylglycine
PI3K	Phosphoinositide Kinase-3
qRT-PCR	Quantitative real-time reverse transcription polymerase chain reaction

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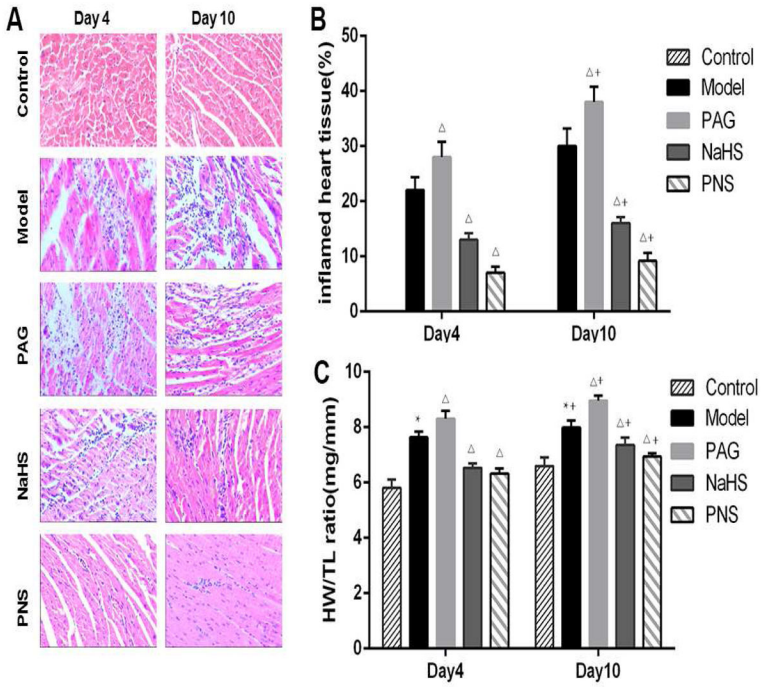

Fig. 1. Histological analysis of the infected hearts

(A) Representative H&E staining images (×400) of heart sections from normal control mice (control, n=10 on day 4 and 10 on day10), mice with viral myocarditis (model, n=10 on day 4 and 12 on day10), mice with myocarditis and PAG treatment (PAG, n=10 on day 4 and 10 on day10), mice with myocarditis and NaHS treatment (NaHS, n=10 on day 4 and 14 on day10), and mice with myocarditis and PNS treatment (PNS, n=10 on day 4 and 15 on day10; (B) % inflamed heart tissue in heart sections from different groups; (C) The ratio of heart weight to tibia length (HW/TL) from different groups. * P<0.05 compared with the control group; $^{\Delta}P < 0.05$ compared with the model group; $+P < 0.05$ within the same group compared with the time point day 4.

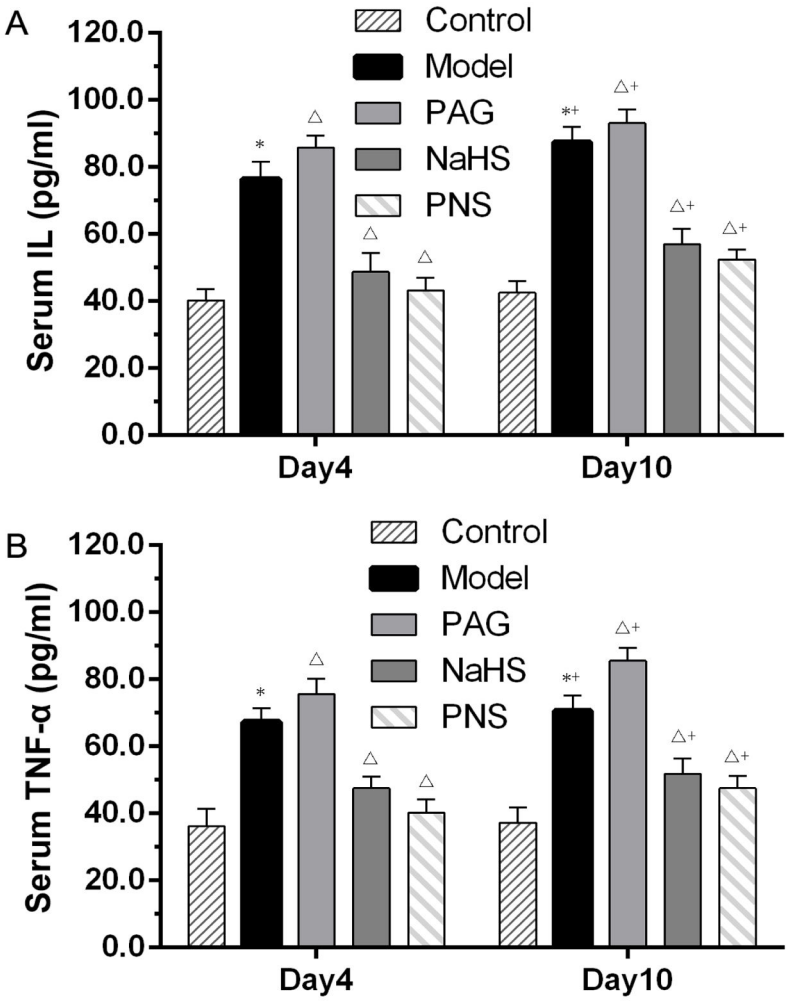

Fig. 2. Levels of proinflammatory cytokines in serum in CVB3-induced myocarditis

ELISA analysis was used to detect the IL-6 (A) and TNF-α (B) in serum in each group. **P* < 0.05 compared with the control group. The animal numbers of each group are described in Fig. 1. $^{\Delta}P < 0.05$ compared with the model group; +*P* < 0.05 within the same group compared with the time point day 4. Note: control, n=10 on day 4 and 10 on day10; mice with viral myocarditis (model), n=10 on day 4 and 12 on day10; mice with myocarditis and PAG treatment (PAG), n=10 on day 4 and 10 on day10; mice with myocarditis and NaHS treatment (NaHS), n=10 on day 4 and 14 on day10; and mice with myocarditis and PNS treatment (PNS), n=10 on day 4 and 15 on day10.

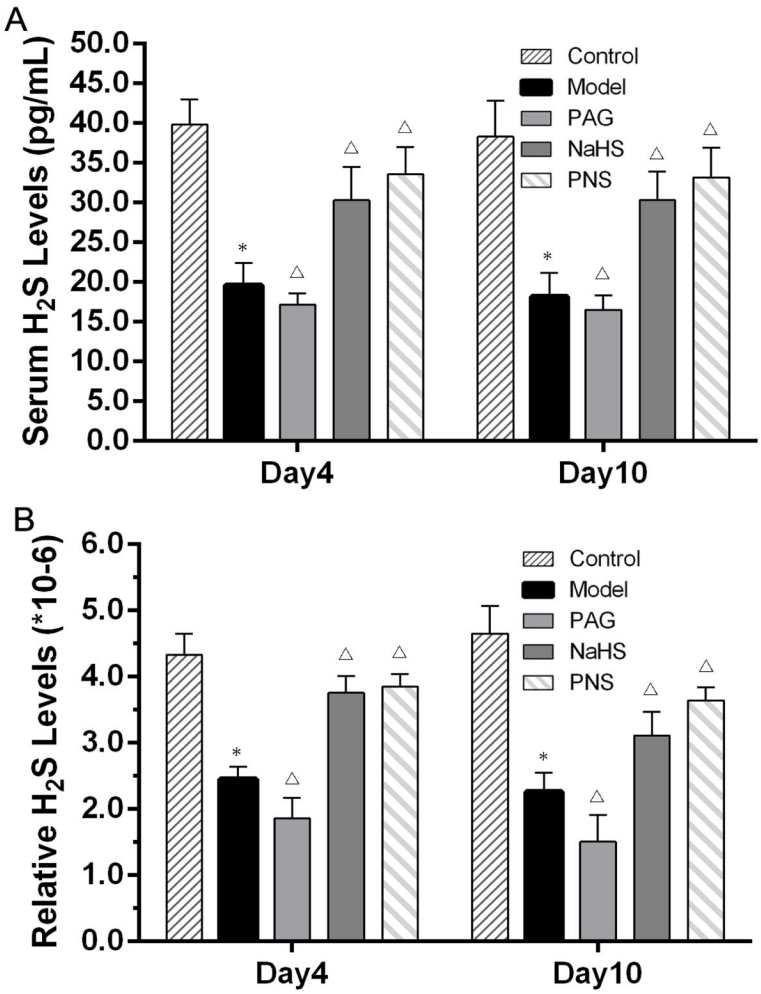

Fig.3. Serum H_2S levels in CVB3-induced myocarditis

(A) ELISA was performed to detect the H_2S levels in serum; (B) Serum H_2S levels expressed relative to total protein contents. **P*<0.05 compared with the control group. $^{\Delta}$*P*<0.05 compared with the model group; +*P*< 0.05 within the same group compared with the time point day 4. Note: control, n=10 on day 4 and 10 on day10; mice with viral myocarditis (model), n=10 on day 4 and 12 on day10; mice with myocarditis and PAG treatment (PAG), n=10 on day 4 and 10 on day10; mice with myocarditis and NaHS treatment (NaHS), n=10 on day 4 and 14 on day10; and mice with myocarditis and PNS treatment (PNS), n=10 on day 4 and 15 on day10.

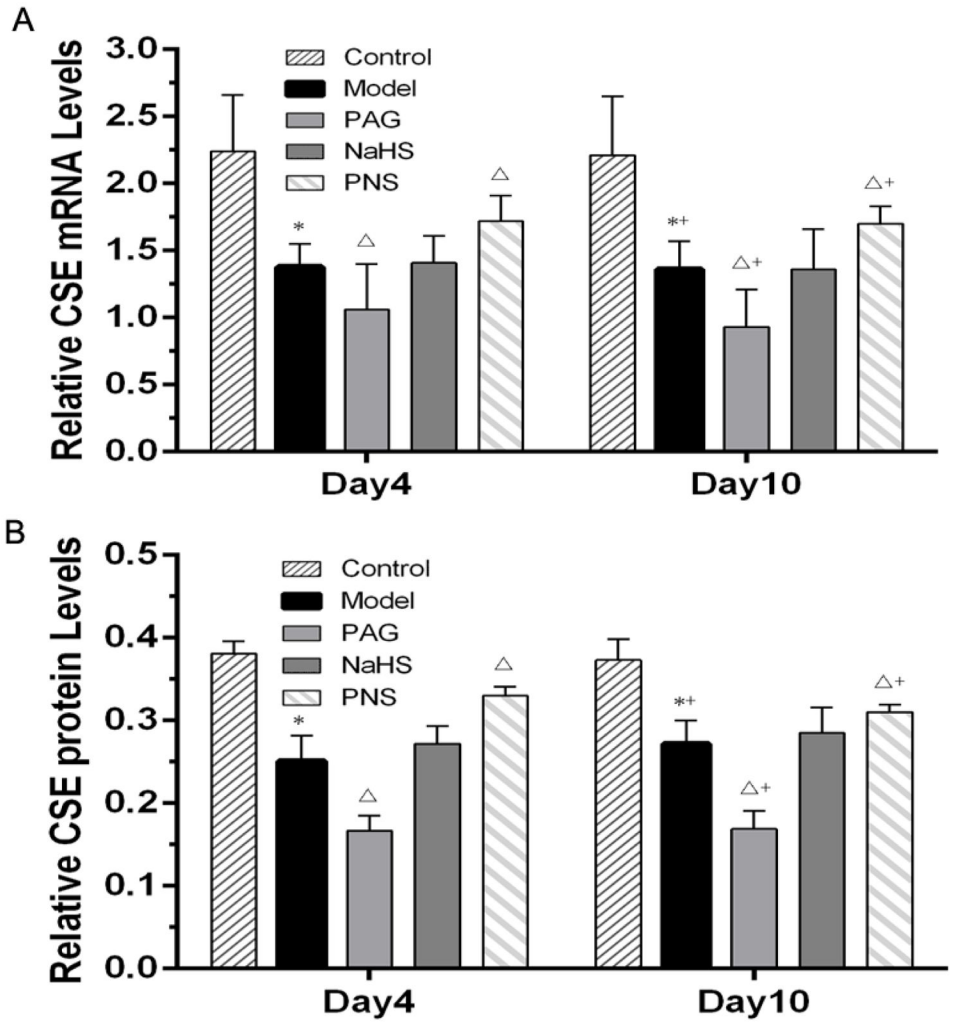

Fig. 4. CSE mRNA and protein levels in CVB3-induced myocarditis

(A) CSE mRNA level was measured by qRT-PCR; (B) Western blot analysis of CSE; **P*<0.05 compared with the control group; $^{\Delta}P$<0.05 compared with the model group; +*P* < 0.05 within the same group compared with the time point day 4. Note: control, n=10 on day 4 and 10 on day10; mice with viral myocarditis (model), n=10 on day 4 and 12 on day10; mice with myocarditis and PAG treatment (PAG), n=10 on day 4 and 10 on day10; mice with myocarditis and NaHS treatment (NaHS), n=10 on day 4 and 14 on day10; and mice with myocarditis and PNS treatment (PNS), n=10 on day 4 and 15 on day10.

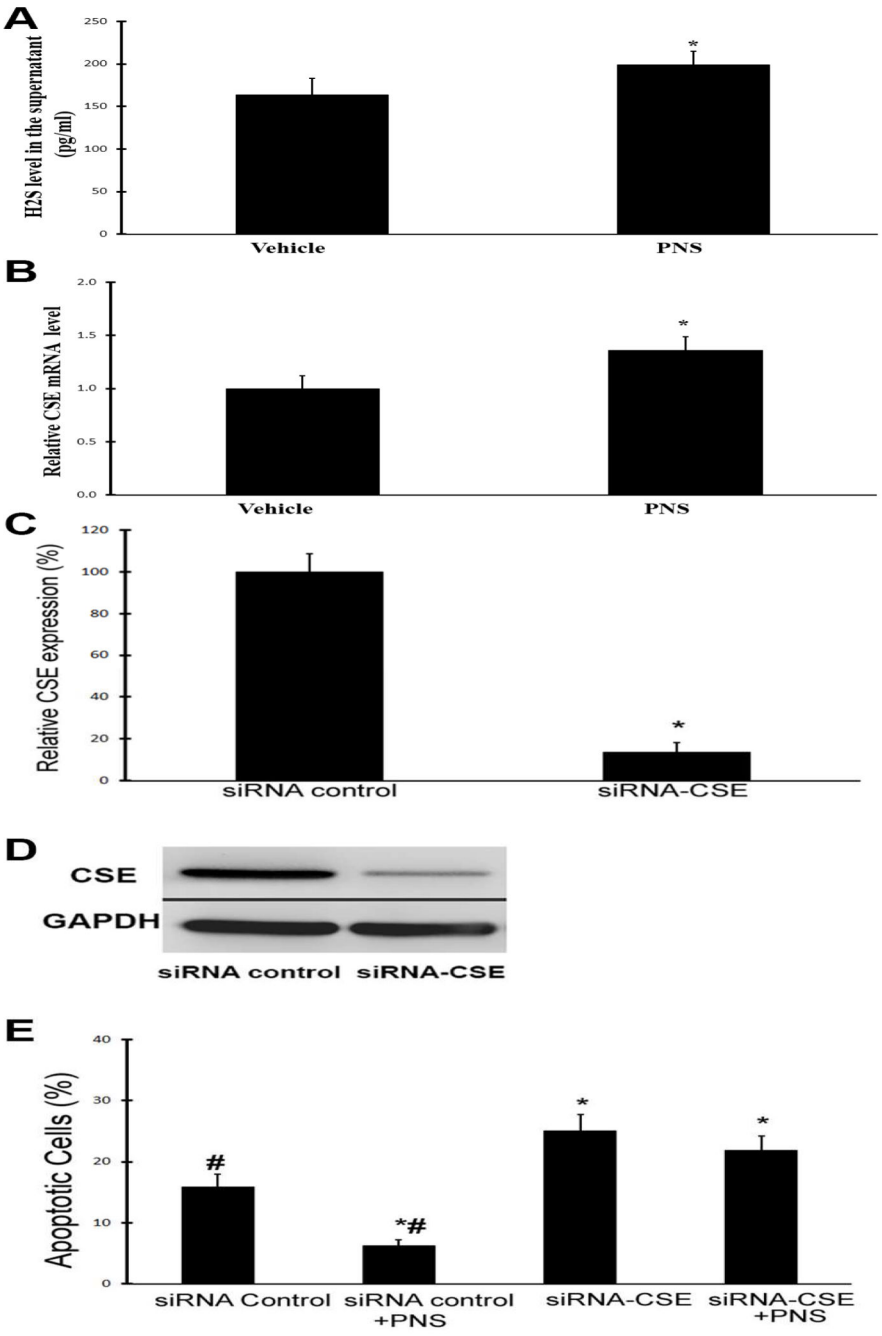

Fig. 5. PNS activates the CSE/H_2S pathway in cultured cardiac cell line and protect the cardiac cells from apoptosis via CSE

A &B: Cultured cardiac cell line H9c2 cells were treated with vehicle (saline) or PNS (10 μg/ml). At 4h after treatments, cell culture supernatants were collected to determine the levels of H2S by ELISA. Then, the RNAs of H9c2 cells were isolated and CSE mRNA was measured by qRT-PCR. A. PNS increases the level of H2S in the supernatant of cultured H9c2 cells. B. PNS increases the level of CSE mRNA in cultured H9c2 cells. n=6; **P*<0.05 compared with vehicle group. C. CSE is knocked down by its siRNA (siRNA-CSE, 50 nM) in cardiac cell. n=6; **P*<0.05 compared with siRNA control group (siRNA control). D.

Representative Western blots of CSE. E. H9c2 cells were treated with siRNA control (50 nM), siRNA control plus PNS (10 μg/ml) (siRNA control+PNS), siRNA-CSE (50 nM), and siRNA-CSE plus PNS (10 μg/ml). Cell apoptosis was induced by H_2O_2 (100 μM) for 6 h and was measured by TUNEL. n=6, *$p<0.05$ compared with siRNA control; # $p<0.05$ compared with siRNA-CSE group.

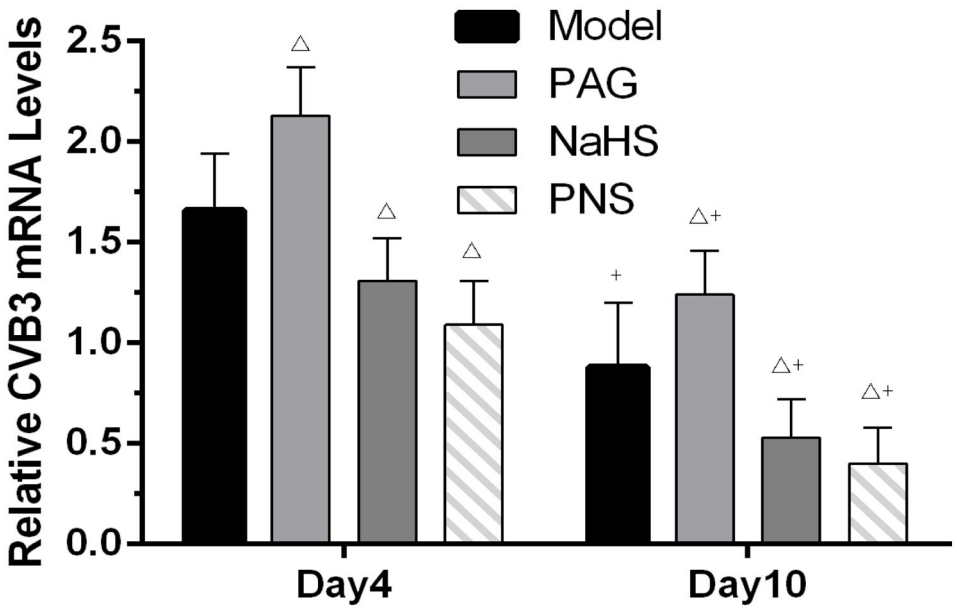

Fig. 6. CVB3 mRNA levels in CVB3-induced myocarditis measured by qRT-PCR

$^{\Delta}P< 0.05$ compared with the model group; $+P<0.05$ within the same group compared with the time point day 4. Note: mice with viral myocarditis (model), n=10 on day 4 and 12 on day10; mice with myocarditis and PAG treatment (PAG), n=10 on day 4 and 10 on day10; mice with myocarditis and NaHS treatment (NaHS), n=10 on day 4 and 14 on day10; and mice with myocarditis and PNS treatment (PNS), n=10 on day 4 and 15 on day10.