Vagus nerve stimulation attenuates septic shock-induced cardiac injury in rats

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Running title: Vagus nerve stimulation attenuates septic shock

Abstract

Objectives: This research aimed to evaluate whether vagus nerve stimulation (VNS) could effectively prevent septic shock-induced cardiac injury in rats and investigate the potential mechanisms.

Methods: Female Sprague-Dawley rats were divided into the Sham group (sham cecal ligation and puncture [CLP] plus vagal nerve trunk separation), the Vehicle group (CLP plus vagal nerve trunk separation), and the VNS groups (CLP plus vagal nerve trunk separation plus VNS). The left ventricular function was analyzed by echocardiography. Histologic examinations of the cardiac tissues were performed through hematoxylin and eosin staining and TUNEL staining.

Results: The Vehicle group had worse cardiac function, higher levels of cardiac injury markers, and enhanced myocardial apoptosis than the Sham group. The rats in the VNS groups had enhanced cardiac function, lower levels of cardiac injury markers, and inhibited myocardial apoptosis than those in the Vehicle group. Elevated interleukin-1β and tumor necrosis factor-αlevels and activated nuclear factor kappa B (NF-κB) signal in septic shock rats were inhibited by the performance of VNS.

Conclusions: This study suggests that VNS contributes to the reduction of myocardial apoptosis and improvement of left ventricular function to attenuate septic shock-induced cardiac injury in rats. The performance of VNS inhibits the inflammatory responses in heart tissues via the regulation of NF-κB signal.

Key words: Vagus nerve stimulation; Septic shock; Cardiac injury; Inflammatory response

Introduction

Shock is a syndrome characterized by tissue hypoperfusion due to various causes [1]. Stimulated by pathological factors such as trauma, blood loss, and infection, severe tissue hypoperfusion leads to tissue ischemia and hypoxia, causing extensive activation of the inflammatory system, releasing a series of pro-inflammatory cytokines, triggering systemic inflammatory responses, leading to intricate pathophysiological process [2]. Septic shock and hemorrhagic shock are two shock states caused by different etiologies and share a common pathophysiological process [3].

After pathogenic microorganisms such as bacteria invade the body, they trigger a systemic inflammatory response through certain specific pathways. Septic shock or sepsis is essentially an uncontrolled self-destructive systemic inflammatory process [4]. Septic shock is a severe stage of sepsis [5]. Between the immune system and the nervous system, vagus nerve and acetylcholine form the cholinergic anti-inflammatory pathway, which regulates inflammatory responses [6].

Septic shock causes cardiac injury [7]. Cardiac function has direct association with the prognosis of septic shock patients [8]. Endotoxins and inflammatory factors in the serum are the main causes of cardiac injury in the patients with septic shock.

Vagus nerve stimulation (VNS) has been reported to be cardioprotective [9]. Selective efferent VNS is effective in attenuating myocardial ischemia/reperfusion injury [10]. Although VNS has been widely reported for its application in septic shock models, there are rare reports on studying the heart as a target organ.

In this study, the septic shock rat model was replicated by cecal ligation and puncture (CLP), and the myocardial protective effect of VNS on rats with septic shock was investigated.

Methods

Animals

All experiments were approved by the ethics committee of Cangzhou Central Hospital. The animal experimentation was in accordance with the Guide for the Care and Use of Laboratory Animals (1985). Animals were divided into three groups as follows: the Sham group (sham CLP + vagal nerve trunk separation); the Vehicle group (CLP + vagal nerve trunk separation); and the VNS groups (CLP + vagal nerve trunk separation + VNS).

Rats were anesthetized by 40 mg/kg pentobarbital sodium through intraperitoneal injection. The common carotid artery and vagus nerve were separated. The arterial pressure was continuously monitored by connecting a pressure conduction system and a monitor. A 2-3 cm long incision was made in the middle of the anterior abdomen and the cecum was exposed. In CLP, the cecum was ligated at the root with 3.0 silk thread and punctured through a 50-mL syringe needle to produce two pairs of holes. Rats in the Sham group were treated with open and closed abdomens, without CLP. When the mean arterial pressure dropped to 2/3 of the initial blood pressure, the modeling was considered successful.

Continuous stimulation (5 V, 2 Hz, 1 millisecond pulse width) was delivered by a stimulator through a pair of Teflon-coated silver hooks. The stimulation was performed immediately after CLP and lasted for 20 min.

Rats were euthanized at 12 h after operation for echocardiographic evaluation and sample collection.

ELISA

Sample of venous blood weas collected in tubes containing EDTA. The serum was collected after the centrifugation at 1500 rpm for 10 min. The concentrations of creatine kinase-MB (CK-MB) and cardiac troponin I (cTnI) were assessed by commercial ELISA kits (Elabscience, China). The heart tissue was homogenized and centrifuged at 10000 g for 10 min to collect the supernatants. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) levels were assessed by commercial ELISA kits (Thermo Fisher, USA).

Immunohistochemistry

The heart tissue was fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 4 μm thick sections. Slide was stained with hematoxylin-eosin (HE) to observe muscle and connective tissues. The level of cardiac apoptosis was detected by TUNEL staining kit (Roche Applied Science, United States). The nucleus was shown with DAPI (Beijing Biodee Biotechnology, China).

qRT-PCR

Total RNA was isolated by Trizol reagent. PrimeScript™ RT Reagent Kit (TaKaRa, Japan) was used for the first strand cDNA synthesis. qPCR was carried out using Ssofast Evergreen Supermix (BioRad, Canada). The primer sequences (5' to 3') used in this study were:

Bax F: CCAAGAAGCTGAGCGAGTGTCTC

Bax R: AGTTGCCATCAGCAAACATGTCA

Bcl-2 F: GGAGCGTCAACAGGGAGATG

Bcl-2 R: GATGCCGGTTCAGGTACTCAG

Actin F: CAGGGTGTGATGGTGGGTATGG

Actin R: AGTTGGTGACAATGCCGTGTTC

Western blot

Western blot was performed with standard protocol. Heart tissues were homogenized for protein sample collection. Protein was separated by SDS–PAGE and transferred to the PVDF membranes. The membranes were blocked and then incubated overnight at 4°C with primary antibodies. The primary antibodies included: anti-β-actin (Santa Cruz biotechnology, USA), anti-TNF-α (Abcam, USA), anti-Bcl-2 (Cell Signaling Technology, USA), anti-p65 (Cell Signaling Technology, USA), anti-Bax (Cell Signaling Technology, USA), anti-IL-1β (Abcam, USA), anti-cleaved caspase-3 (Cell Signaling Technology, USA), and anti-p-NF-κB p65 (Cell Signaling Technology, USA). The membranes were incubated with secondary antibodies and the signals were visualized by the chemiluminescence kit.

Statistical analysis

Data were analyzed by the SPSS Version 16.0 and presented as mean ± SD. Brown-Forsythe ANOVA followed Dunn's multiple comparisons test was used to analyze the data. Values were significantly different when $p < 0.05$.

Results

The influence of VNS on the cardiac function in septic shock rat model

Cardiac function parameters were measured at 12 hours post-CLP. In the Vehicle group, the rats had significantly lower left ventricular fractional shortening (LVFS) and left ventricular ejection fraction (LVEF) than those in the Sham group (Fig. 1A and B). In the VNS group, the rats had higher LVEF and LVFS than in the Vehicle group (Fig.

1A and 1B). Meanwhile, left ventricular end systolic diameter (LVESD) and left ventricular end diastolic dimension (LVEDD) in the Vehicle group were significantly higher than in the other groups (Fig. 1C and 1D).

The influence of VNS on the markers of cardiac injury in septic shock rat model

CK-MB and cTnI are serum markers of cardiac injury. The Vehicle group had remarkably higher serum cTnI and CK-MB levels than the Sham group (Fig. 2A and 2B). The VNS group had remarkably lower levels than the Vehicle group (Fig. 2A and 2B).

The influence of VNS on the pathological changes in the myocardial tissue of septic shock rat model

HE staining results were shown in Fig. 3A. In normal rats, the results of myocardial tissue HE staining showed normal myocardial cell morphology, neatly arranged myocardial fibers, and clear cell boundaries. After the induction of septic shock, the HE staining showed disordered myocardial fibers, nuclear lysis or loss, and unclear cell boundaries. However, these pathological changes in septic shock rat model were significantly improved by the treatment of VNS. The results of TUNEL staining were shown in Fig. 3B. In the Vehicle group, the apoptotic ratio was significantly higher than in the other groups (Fig. 3C).

The influence of VNS on the cardiac apoptosis in septic shock rat model

The Bax mRNA level in the Vehicle group was significantly higher than in the other groups, while the Bcl2 mRNA level was significantly lower (Fig. 4A and 4B). In the Vehicle group, the rats had significantly higher cleaved caspase-3 and Bax levels than

those in the Sham group (Fig. 4C-E). In the VNS group, the rats had significantly lower protein levels of cleaved caspase-3 and Bax than the rats in the Vehicle group (Fig. 4C-E). The Bcl2 protein level in the Vehicle group was significantly lower than in the other two groups (Fig. 4C and 4F).

The influence of VNS on the cardiac inflammatory response in septic shock rat model

The concentrations of IL-1β and TNF-α in heart tissues in the Vehicle group were significantly higher than in the other groups (Fig. 5A and 5B). The Vehicle group had significantly higher levels of IL-1β, TNF-α, and p-p65 in heart tissues than the Sham group (Fig. 5C-F). The VNS group had significantly lower levels of IL-1β, TNF-α, and p-p65 in heart tissues than the Vehicle group (Fig. 5C-F).

Discussion

In patients with septic shock, depending on the extent of the inflammatory response, the clinical presentation include the heart, lung, and liver failure, acute kidney injury, coagulopathy and death [11]. During sepsis, the cardiac dysfunction can be indicated by diastolic dysfunction or systolic heart failure [12]. Various molecular mechanism participate in the sepsis-induced myocardial injury, including pro-inflammation cytokines, nitric oxide (NO), and prostanoids [7].

It has been proved that the autonomic nerve system is a major regulator of immune function [13]. The pro-inflammatory activity is regulated through the sympathetic branch and the inflammatory response is attenuated by the parasympathetic part. The electrical stimulation of the vagus nerve has been proved to attenuate the inflammatory responsiveness [14]. Vagus activation promotes the release of acetylcholine. The

binding of acetylcholine to α7 nicotinic acetylcholine receptors on inflammatory cells strongly inhibits the release of cytokine and attenuates inflammatory responses [15].

VNS is an effective and safe therapeutic strategy for neurological disease [16]. VNS is being explored as treatment for a variety of autoimmune and chronic inflammatory disorders, due to its demonstrated anti-inflammatory properties [17]. VNS may attenuate the inflammatory response through activation of the cholinergic anti-inflammatory pathway (CAP) [18]. VNS helps to arrest the progression to sepsis by attenuating the inflammation through the restore of balance between parasympathetic and sympathetic tone [19]. VNS decreases the mortality in LPS-induced endotoxemia model by attenuating vagally induced release of acetylcholine [20]. In respiratory regions of the developing rat brainstem, VPS can inhibit the upregulation of IL-6 and TNFα caused by LPS [21]. In an oxazolone -induced colitis model, VNS reduces colonic inflammation and improves survival in this sepsis model [22]. Since it has indeed become an important target of therapeutic research strategies for inflammatory diseases and sepsis, VNS is investigated as an adjunct therapy in COVID-19 patients in clinical trials [23]. The descending cardiac branch of the vagus is key for normal cardiac function [17]. There was an inverse relationship between inflammatory markers and vagus nerve activity, measured by heart rate variability [24]. VNS treatment shows cardiac remodeling protection effect before or during ischemia in animal model [25, 26]. The other research has proved that VNS is a promising treatment in heart failure [27, 28]. In a canine high-rate ventricular pacing model, chronic VNS helps to regulate heart rate and improves heart function [29]. In a rat ischemia/reperfusion model, VNS improved cardiac function and reduced infarct size [30].

As a complication of septic shock, cardiac injury is characterized by left ventricular dilatation and decreased ejection fraction [31]. The left ventricular hemodynamic

parameters were measured to assess the cardiac function. The LVEF and the LVFS were significantly decreased by the induction of septic shock, while the LVEDD and LVESD values were significantly increased, which show obvious characteristics of cardiac insufficiency. The VNS treatment significantly increased the LVEF and the LVFS, and decreased the LVEDD and LVESD values.

In normal rats, the results of myocardial tissue HE staining showed normal myocardial cell morphology, neatly arranged myocardial fibers, and clear cell boundaries. After the induction of septic shock, the HE staining showed disordered myocardial fibers, nuclear lysis or loss, and unclear cell boundaries. However, these pathological changes in septic shock rat model were significantly improved by the treatment of VNS.

CK-MB and cTnI are markers that can reflect the degree of myocardial injury [32]. Significantly elevated serum cTnI and CK-MB levels in septic shock rat model also indicated significant myocardial damage. The treatment of VNS in septic shock rat model markedly reduced the serum levels of cTnI and CK-MB. These findings suggested that VNS could ameliorate septic shock-induced cardiac dysfunction and myocardial damage.

Cardiac injury leads to the activation of program cell deaths [33]. In myocardial apoptosis, the main molecular markers are anti-apoptosis Bcl-2 and increased proapoptosis Bax [34]. Caspase 3 is a crucial molecule in apoptosis execution. The overexpression of caspase 3 causes decreased cardiac function [35]. It has been proved that the downregulation of Bcl-2 expression is prevented by the application of VNS in rat model [34].

HE staining results showed that enhanced pathological changes in the Vehicle group were alleviated in the VNS group. TUNEL positive cells in myocardial tissue were significantly increased after the induction of septic shock. The apoptosis in heart tissue

were significantly decreased in VNS-treated septic shock rat model. The expression of Bax and the cleaved caspase-3 was significantly decreased by the treatment of VNS. However, the levels of Bcl-2 were significantly increased by the treatment of VNS. Thus, our results suggested that VNS reduced myocardial injury through the anti-apoptotic effect.

Increased TNF-α levels in serum and heart tissue indicate that TNF-α is an endogenous mediator in LPS-induced shock [36]. TNF-α and IL-1β act synergistically to cause sepsis-associated myocardial depression in human [37]. In lipopolysaccharides (LPS)-induced septic rats, both the inflammatory response markers IL-1β and TNF-α have increased levels in serum and cardiac tissues [7]. In this research, IL-1β and TNF-α levels in the heart tissues of septic shock rat model were significantly elevated. After the treatment of VNS, IL-1β and TNF-α levels in the heart tissues of septic shock rat model were significantly decreased.

During the cardiac injury, the release of damage-associated molecules induce inflammatory responses by signaling through the NF-κB [38]. NF-κB signaling contributes to the sepsis-induced cardiac dysfunction [39]. NF-κB signal activity in the heart tissues of septic shock rat model was significantly elevated. After the treatment of VNS, NF-κB signal activity in the heart tissues of septic shock rat model were significantly decreased. Thus, our results indicated that VNS reduced cardiac inflammatory response through the regulation of NF-κB signal.

The ex vivo analysis of cardiac excitation-contraction coupling was not provided in this study. Although VNS has been widely reported for its application in septic shock models, there have been rare reports on studying the heart as a target organ. Therefore, we have not found a suitable in vitro model for septic shock-induced myocardial injury. Perhaps stimulating myocardial cells with LPS can partially simulate this process, but

it is even more challenging to establish a model where the vagus nerve and myocardial cells interact, and to influence myocardial cells through VNS. Clearly, there are currently no relevant techniques available. Unfortunately, we have not found a suitable approach to conduct related experiments.

Conclusions

This study suggests that VNS contributes to the reduction of myocardial apoptosis and improvement of left ventricular function to attenuate septic shock-induced cardiac injury in rats. The performance of VNS inhibits the inflammatory responses in heart tissues via the regulation of NF-κB signal.

List of Abbreviations

VNS	vagus nerve stimulation
CLP	cecal ligation and puncture
CK-MB	creatine kinase-MB
cTnI	cardiac troponin I

Ethics approval and consent to participate

All experiments were approved by the ethics committee of Cangzhou Central Hospital. This study was performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85-23 Rev. 1985).

Consent for publication

Not applicable.

Availability of data and material

Data could be obtained upon request to the corresponding author.

Competing interests

The authors declare that they have no conflict of interest.

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None.

Authors' contributions

Data curation: Y.C.S, M.X.C and Y.C; Project administration: Y.C; Supervision: Y.C; Writing - original draft: Y.C.S, M.X.C and Y.C; Writing - review and editing: Y.C.S, M.X.C and Y.C.

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None.

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Figure legends

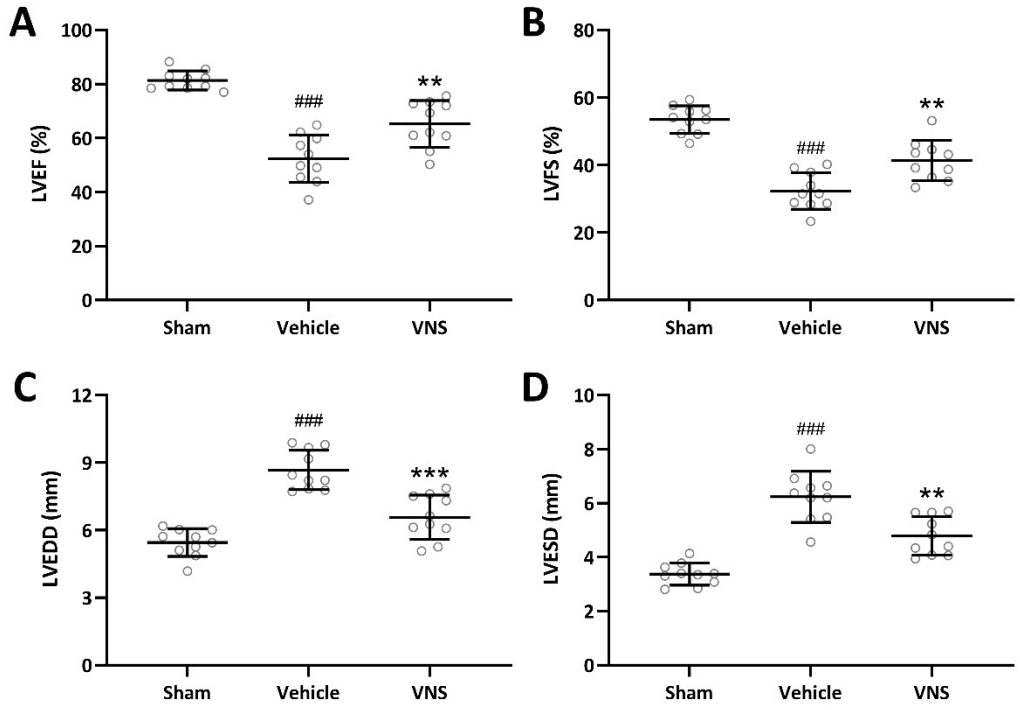

Figure 1. Protective effects of vagus nerve stimulation on left ventricular ejection fraction (LVEF) (A), left ventricular fractional shortening (LVFS) (B), left ventricular end diastolic dimension (LVEDD) (C) and left ventricular end systolic diameter (LVESD) (D) of rats from each group at 12 hours post-CLP. n = 10 for each group. ###p < 0.001 compared to Sham. **p < 0.01, ***p < 0.001 compared to vehicle. Brown-Forsythe ANOVA followed Dunn's multiple comparisons test.

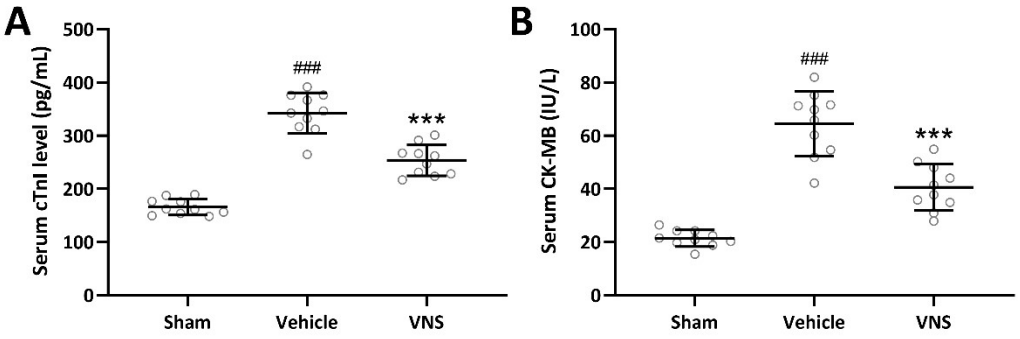

Figure 2. Protective effects of vagus nerve stimulation on serum cTnI (A) and CK-MB

(B) of rats from each group at 12 hours post-CLP. n = 10 for each group. ###p < 0.001 compared to Sham. ***p < 0.001 compared to vehicle. Brown-Forsythe ANOVA followed Dunn's multiple comparisons test.

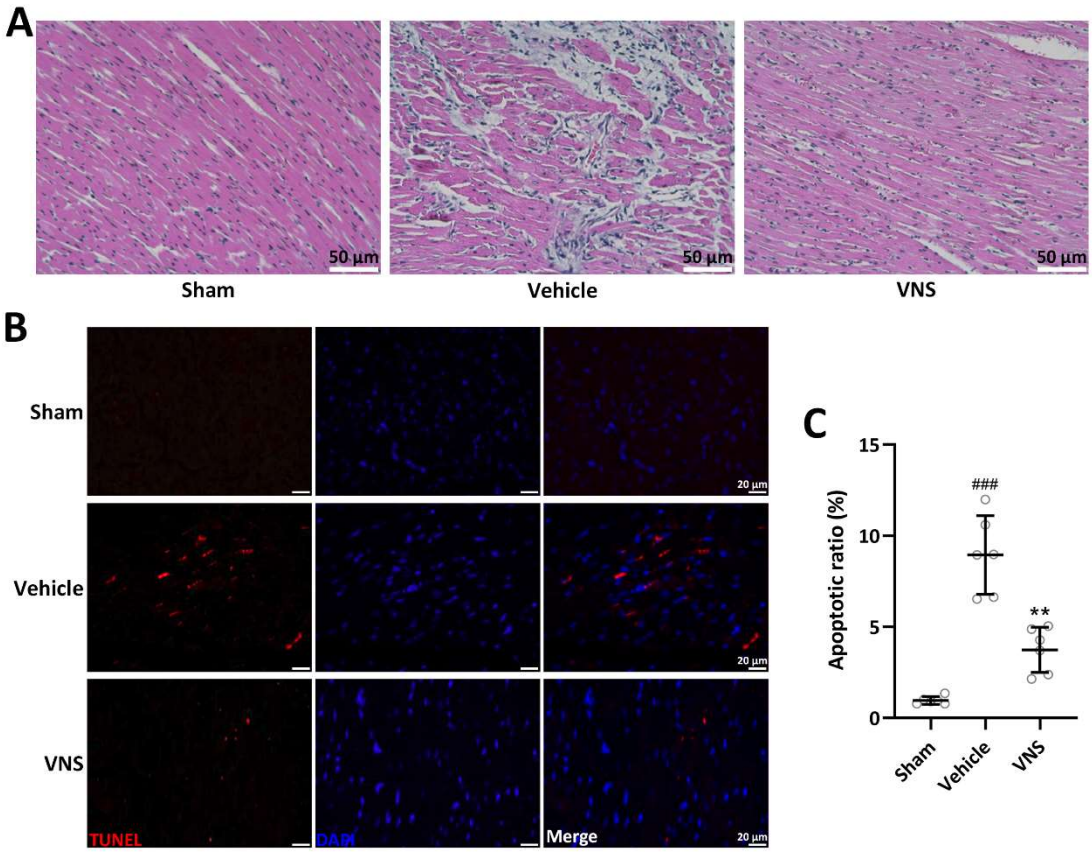

Figure 3. Protective effects of vagus nerve stimulation on cardiac structures and cardiac apoptosis in septic shock rats. (A) Representative HE staining to show the pathological changes in myocardial tissue of rats from each group at 12 hours post-CLP. (B) Representative TUNEL staining of myocardial tissue of rats from each group at 12 hours post-CLP and the quantification of apoptotic ratios (C). 8 slices were used for the quantification of the single rat and 6 rats were used for each group. ###p < 0.001 compared to Sham. **p < 0.01 compared to vehicle. Brown-Forsythe ANOVA followed Dunn's multiple comparisons test.

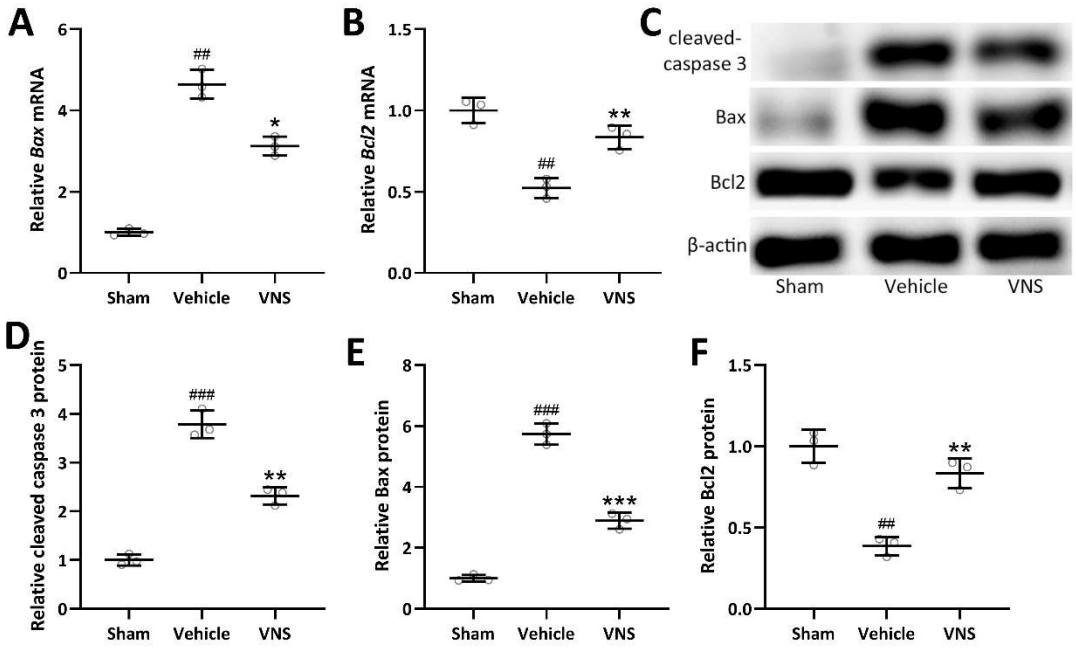

Figure 4. Protective effects of vagus nerve stimulation on cardiac apoptosis in septic shock rats. The mRNA expressions of Bax and Bcl 2 in heart tissues were detected by qRT-PCR (A, B). © Western blotting was used to measure the protein expressions of cleaved caspase-3, Bax and Bcl 2 in heart tissues and the relative expressions were normalized to sham (D-F). Tissues from 10 rats in each group were mixed and the experiments were repeated for 3 times. ##$p < 0.01$, ###$p < 0.001$ compared to Sham. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to vehicle. Brown-Forsythe ANOVA followed Dunn's multiple comparisons test.

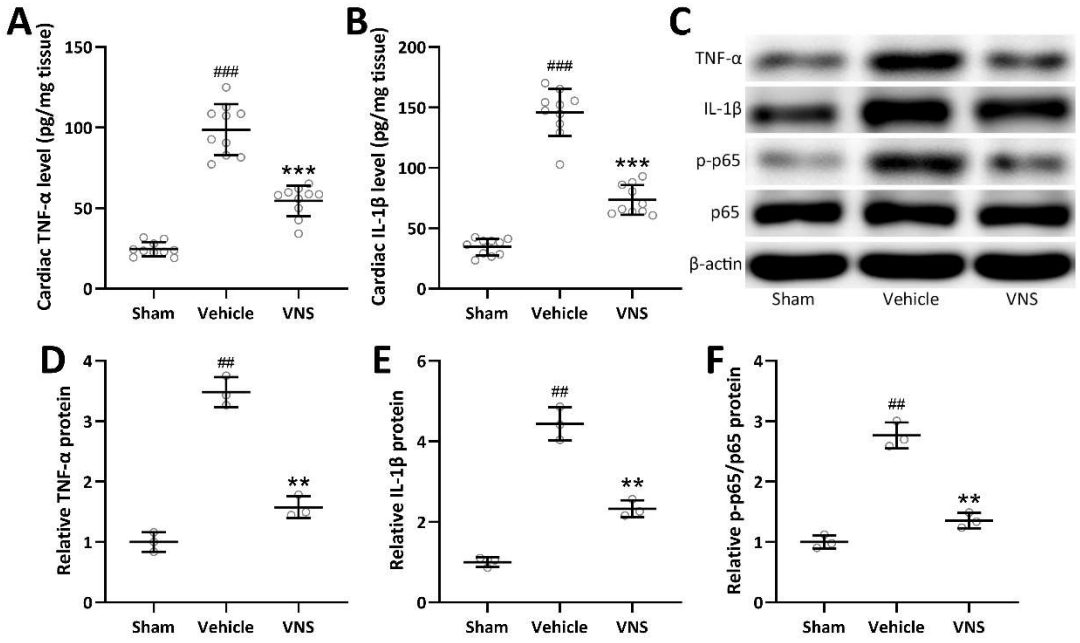

Figure 5. Protective effects of vagus nerve stimulation on cardiac inflammatory response in septic shock rats. Concentrations of TNF-α (A), IL-1β (B) in heart tissues were measured by ELISA. n = 10 for each group. (C) Western blotting was used to measure the protein expressions of TNF-α, IL-1β, p-p65 and p65 in heart tissues and the relative expressions were normalized to sham (D-F). Tissues from 10 rats in each group were mixed and the experiments were repeated for 3 times. ##$p < 0.01$, ###$p < 0.001$ compared to Sham. **$p < 0.01$, ***$p < 0.001$ compared to vehicle. Brown-Forsythe ANOVA followed Dunn's multiple comparisons test.