GYY4137 ameliorates sepsis-induced cardiomyopathy via inhibiting NLRP3 inflammasome activity and reduction of oxidative stress in the myocardium

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Research Article

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Abstract

BACKGROUND: Sepsis-induced cardiomyopathy often leads to bad prognosis of patients, and even death. However, there is no effective therapeutic strategies for sepsis-caused cardiomyopathy. GYY4137 has positive therapeutic effects in many diseases. The aim of this study was to explore the mechanism underlying the sepsis-induced cardiomyopathy and investigate the protective role of the hydrogen sulfide (H$_2$S) donor GYY4137 in sepsis-induced cardiomyopathy.

METHODS: Recruitment of patients with clinical sepsis and measurement of serum H2S concentrations. GYY4137 was administrated in mouse model of sepsis-induced cardiomyopathy (SICM) was generated by LPS and cecum ligation and puncture (CLP), and then plasma levels of hydrogen sulfide and cytokines were measured, and inflammatory cell infiltration in myocardial tissue was determined by immunohistochemistry and immunofluorescence staining. RNA sequencing assay was performed to monitor the RNA expression profile. Cardiomyocytes medium was co-cultured with Macrophage, and the protective mechanism of GYY4137 was confirmed by WB, RT-PCR and immunofluorescence experiments. Establishment of SICM model using $\text{Nlrp3}^{-/-}$ mice to validate the role of GYY4137.

RESULTS: Compared to non-septic patients, septic patients had lower serum hydrogen sulfide concentrations and a negative correlation between hydrogen sulfide concentrations and NT-pro BNP. H$_2$S levels were reduced in the serum of sepsis-molded mice, GYY4137 reduced macrophage infiltration in the sepsis model. GYY4137 reduces inflammatory cell infiltration in septic myocardial tissue. GO analysis suggested that GYY4137 involved in inflammatory regulation. GYY4137 can inhibit NLRP3 inflammasome activity in macrophages and reduce the secretion of inflammatory factors, while GYY4137 can reduce the production of reactive oxygen species in cardiomyocytes, thus exerting a protective effect on the myocardium. The protective effect of GYY4137 was disappeared after $\text{Nlrp3}$ knockout.

CONCLUSION: GYY4137 can reduce myocardial injury in sepsis by inhibiting the inflammatory response and reducing the production of myocardial reactive oxygen species via NLRP3 pathway. It can provide new therapeutic ideas for clinical practice.

Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection[1]. It is one of the most common causes of death in critically ill patients worldwide, reflecting from that the sepsis mortality rate in the whole population is 25.8%, while it reaches 35.3% in hospitalized patients[2]. Sepsis causes multiple organ injury including heart, kidney and lung. The prevalence of sepsis-induced cardiomyopathy (SICM) in patients with sepsis ranges from 10–70%, indicating that heart is the most frequently attached organ [3]. Although currant morbidity as well as mortality rates have decreased, sepsis remains to be a major global health risk[4].
SICM is with the typical manifestation of systolic dysfunction, and eventually develops into circulatory disturbances. Currently, as we known, multiple issues cause the occurrence of SICM, and the major causes are subgrouped into two parts, including the classical pathogen-associated molecular patterns (PAMPs) and the damage-associated molecular patterns (DAMPs)[5]. DAMPs are consisted with stimulation by cytokines[6], imbalance of nitric oxide (NO) [7], mitochondrial dysfunction[8], dysregulation of calcium handling in cardiac myocytes[9], and damage to the glycocalyx of endothelial cells[10], one of which leads to the development of SICM. Among these, the most severe characteristic of sepsis is a typical systemic inflammatory response. Sepsis causes the impaired microorganisms and releases the proinflammatory factors, cytokines, and others, leading to the activation of immune cells and immune system, reflecting from the strong immune response [11]. Inflammatory response is a vital step in pathological condition and impaired function, such as acute lung injury and acute kidney injury. Meanwhile, numerous signaling pathways are irritated by the inflammatory response, resulting in a series of storm response among multiple organs [12]. In another word, these pathogenic factors are potential therapeutic targets. Unfortunately, there is no specific treatment for SICM, and the current treatment is still a comprehensive systemic treatment based on sepsis, with positive inotropic drugs or ventricular rate control depending on the volume response to reduce myocardial oxygen consumption, thus reducing sepsis induced myocardial damage[13].

As we known, the biological gaseous molecules including NO and H₂S are associated with cardiovascular diseases. Furthermore, both of NO and H₂S are approved to be benefit for cardiovascular diseases treatment. H₂S, a gas transmitter produced from L-cysteine catalyzed by cystathionine γ-lyase (CSE) or cystathionine β-synthase (CBS)[14], is an important signaling molecule in the cardiovascular and nervous systems. As one member of gas signaling molecules, H₂S has been demonstrated to have an essential role in a variety of states. H₂S can promote angiogenesis by inhibiting mitochondrial electron transport and oxidative phosphorylation[15]. Meanwhile H₂S also has a positive effect in anti-aging[16]. Additionally, H₂S can inhibit the inflammatory response by inhibiting the activation of Nrf2 and NF-κB, alleviating the impaired function by sepsis [17].

Considering there is no an effective therapeutic strategy available to prevent or treat sepsis-induced cardiomyopathy, we had to explore the mechanism underlying the process of SICM and identify a potential therapeutic target for treatment. Therefore, in our study, we investigated the protective role of H₂S in SICM by constructing a SICM model on NLRP3 knockout mice with GYY4137 as the H₂S donor, in order to provide new ideas for the treatment of SICM.

Materials And Methods

Human serum samples

The collection of human serum samples was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. All patients’ serum specimens were collected from the Geriatric ICU of the First Affiliated Hospital of Nanjing Medical University, and all samples were leftover specimens
from the laboratory department without additional blood collection operations on patients. Patients with combined autoimmune underlying, hematologic, and oncologic diseases were excluded.

**Animals and treatment**

All animal husbandry and experiments were approved by the Animal Welfare Ethics Committee of Nanjing Medical University (Number ID: NJMU-2012006), and efforts have been made to minimize unnecessary use and discomfort of animals.

Male 8-week-old C57BL/6 mice were purchased from the Animal Core Facility of Nanjing Medical University, and male 8 week old Nlrp3−/− mice were purchased from the Model Animal Research Center of Nanjing Medical University. All animals were housed at the Nanjing Medical University Core Facility, with temperature and humidity controlled at 25°C and 60 ± 2%, respectively, tap water and food readily available. All experiments and data analyses were performed by researchers who were unaware of treatment assignments.

**Animal models and therapy administration**

All animals were randomly assigned to each subgroup. The sepsis models were generated in two different ways: (1) Endotoxin lipopolysaccharide (LPS) model. LPS (sigma, USA) and hydrogen sulfide donor GYY4137 (sigma, USA) were dissolved in saline. LPS injected into the mice by intraperitoneal injection at a dose of 10 mg/kg. After 30 min of LPS injection, GYY4137 was injected intraperitoneally at a dose of 50 mg/Kg. The control group was injected intraperitoneally with an equal amount of saline. Eighteen hours after establishing the animal model, mice were euthanized under isoflurane anesthesia, and mouse serum and tissues were quickly collected, freshly frozen in liquid nitrogen, and stored at -80°C until analysis. (2) Cecum ligation and puncture (CLP) model. After mice were anesthetized with isoflurane, they were placed supine on the experimental table with the abdomen completely exposed, and a median incision was made along the anterior abdomen to expose the cecum. It was then ligated 1 cm from the distal end. Subsequently, a single penetrating puncture of the ligated cecum was performed with a 22-gauge needle, and then the cecum was retracted and the abdominal wall was sutured layer by layer. In the sham-operated group, the abdominal wall was incised and the cecum was exposed, but no ligation or puncture was performed.

After 30 minutes of establishing the animal model, GYY4137 was injected through the peritoneal cavity at a dose of 50 mg/Kg, and the control group was injected with an equal amount of saline. Only the WT mouse part of the experiment was used, and serum and tissue specimens were collected by isoflurane euthanasia 18 hours after surgery, and the NLRP3−/− mouse part of the experiment was added, and post-serum tissue specimens were collected after euthanasia 24 hours after surgery for subsequent analysis. Hydrogen sulfide donor GYY4137 (sigma, USA) were dissolved in saline and injected into the mice by intraperitoneal injection at a dose of 10 mg/kg.

**Cell culture and treatment**
Cardiomyocyte cells H9c2 and macrophage cells RAW264.7 were purchased from the Cell Bank of the Chinese Academy of Sciences and cultured in DMEM medium containing 10% fetal bovine serum. Cells were maintained in routine culture at 37°C with 5% CO2. Cell density reached 80% when the cells were cultured for passaging.

In culturing RAW264.7 cells, 24 hours after the administration of LPS and the intervention of GYY4137, the cell culture supernatant was collected and centrifuged at 3000 rpm for 20 minutes to remove the precipitate, and half of the supernatant was heated at 65°C for 30 minutes to inactivate the cytokines in the supernatant. After co-culture, a portion of the supernatant was separately aspirated and added to the medium in which H9c2 cells were cultured. In another experiment, in, after LPS stimulation, GYY4137-pretreated RAW264.7 were cultured with/without MCC950, an inhibitor of NLRP3. 24 hours later, the supernatant was collected and then incubated with H9c2.

**Echocardiography**

Transthoracic echocardiography was performed using a Vevo 2100 high-resolution microsound system (Fujifilm Visual Sonics Inc.). The same operator qualified in ultrasonography performed the procedure and ensured that the operator was invisible to the grouping as well as the genotype. For echocardiography, using isoflurane anesthesia, the left ventricular ejection fraction (EF), short-axis shortening (FS) were recorded by M-mode images of the left ventricle, and the parameters measured for at least three beats per mouse were measured and averaged.

**Histology**

Mouse heart tissue was fixed in paraformaldehyde and embedded in paraffin. 4 µm thick sections were sliced, and then dewaxed, rehydrated, and stained with hematoxylin eosin and immunohistochemical staining. Frozen sections of tissue were embedded using OCT and cut into 4µm thick sections for H&E staining, immunofluorescence, immunohistochemistry and TUNEL staining.

**Immunofluorescence and Immunohistochemistry**

Immunofluorescence staining of cardiac tissue was accomplished by using cardiac paraffin sections. Paraffin sections were dewaxed, rehydrated, and subjected to antigen repair in 10 mM citrate buffer (pH 6.0). After closure with 3% BSA (Sigma, USA), the sections were incubated with mouse anti-CD68 (1:100, Bio-Rad, Cat# MCA1957) in a humidified chamber at 4°C overnight, followed by incubation with Alexa Fluor® 594 secondary antibody (1:500, Invitgen) for 2 hours at room temperature, followed by DAPI (ThermoFisher) to stain the nuclei.

Take the paraffin-embedded myocardial tissues of mice, and after dewaxing, separately use CD3 (1:150, Servicebio, Cat#GB13014) labeled T cells, CD45 (1:2000, Servicebio, Cat#GB11066) labeled B cells, and Ly6G (1:500, Servicebio, Cat#GB11229) labeled neutrophils[18]. Images were acquired with a fluorescence microscope (BX53, Olympus, Japan).
Measurement of reactive oxygen species levels

We measured the levels of ROS in mouse myocardial tissues as well as cardiomyocytes using dihydroethidium (superoxide anion fluorescent probe, Cat#S0063, Beyotime, China) staining method. For heart tissue, hearts embedded with OCT were cut into 4-μm post-slice, washed three times with PBS, and incubated with 5 μM DHE at 37°C for 30 minutes. For cardiomyocytes, the medium of drug-treated cells was replaced with DHE medium containing 5 μM and incubated at 37°C for 30 min. After the DHE staining of tissues and cells was finished, the cells were washed three times again with PBS, and then the nuclei were stained with DAPI (ThermoFisher), and images were obtained with a fluorescent microscope (BX53, Olympus, Japan).

TUNEL staining

Using TUNEL staining to label apoptotic cells. Paraffin sections of tissues were dewaxed and rehydrated, apoptotic cells were labeled using POD (Roche, Cat# 11684817910), and color developed using DAB (Servicebio, Cat# G21111), sealed using hematoxylin-stained nuclei, and images were acquired using a light microscope (BX53, Olympus, Japan). Cellular TUNEL staining was done by TUNEL kit (Vazyme, Cat# A113-01, China). Specifically, cardiomyocytes were inoculated on cell crawls, treated with drugs, fixed using paraformaldehyde, and stained and sealed with DAPI (ThermoFisher) for cell nuclei after membrane breaking and labeling staining. Images were acquired using a fluorescence microscope (BX53, Olympus, Japan).

RNA isolation and qPCR

Total RNA was extracted from tissues and cells using RNAiso plus (Takara, Japan) using heart tissue and RAW264.7 cells and H9c2 cells according to the reagent instructions. 1 μg RNA was reverse transcribed using HiScript®II Q RT SuperMix kit (Vazyme, China). qPCR was performed with AceQ qPCR SYBR Green Master Mix (Low ROX PreMixed) (Vazyme). amplification reactions were performed on the StepOne™ Real-Time Polymerase Chain Reaction System, and the specific primers used are described in Exhibit I. Amplification was quantified using the 2-ΔΔCT method and then normalized to the mean of the control (shown as a multiple of the control mean on the Y-axis). The sequences of primers were available in the Supplemental table 2.

Western blot

RAW264.7 cells and H9c2 cells were lysed in RIPA buffer (Beyotime) with protease inhibitor cocktail and phosphatase inhibitor (Roche, Switzerland). All extracted proteins were quantified with Piells BCA protein analysis kit (ThermoFisher), loading buffer was boiled and different molecular weight proteins were separated using SDSPAGE. The following antibodies were used: rabbit anti-NLRP3 (1:1000, abcam, Cat#ab263899), mouse anti-ASC (1:100, santa, Cat#514414), rabbit anti-tubulin (1:2000, abcam, Cat#ab6160), rabbit anti-pro caspase-1 (1:1000, abcam, Cat#ab1872), rabbit anti-pro IL-1β (1:1000, abcam, Cat#ab234437), rabbit anti-Bcl2 (1:1000, proteintech, Cat#26593-1-AP), rabbit anti-Bax (1:1000,
proteintech, Cat# 50599-2-lg), rabbit anti-cleaved-caspase 3 (1:1000, biorbyt, Cat# Orb106556). Immunoblot primary antibody was diluted with 5% bovine serum albumin (BSA, Sigma Aldrich). Band intensities were quantified using Image J software (National Institutes of Health, USA).

**Measurement of serum hydrogen sulfide concentration**

The concentration of hydrogen sulfide in human serum was determined by the methylene blue spectrophotometric method. Specifically, 5% zinc acetate solution, 0.2% N,N-dimethyl-p-phenylenediamine hydrochloride solution, 10% ferric sulfate solution and 20% trichloroacetic acid solution were prepared, and the absorbance of each sample was measured at 670 nm. The concentration of hydrogen sulfide in each sample was calculated according to the standard curve of NaHS.

The concentration of hydrogen sulfide in mouse serum was determined by high performance liquid chromatography method[19]. In detail, isocratic elution, with a mobile phase consisting of a mixture of acetonitrile and water in the ratio of 60:40 (v/v), was used. Temperature was set at 25uC, the flowrate 1 mL/min and the detector wavelength 375 nm. Identification of peaks was based on comparison of retention times and diode-array spectra, taken at time of analysis, with corresponding sets of data obtained for authentic compounds.

**Caspase-1 activity assay**

Caspase-1 activity in mouse myocardial tissue and RAW264.7 cells was determined using a kit (Hypertime, China). Caspase-1 activity was measured in each group of samples according to the kit instructions and results are presented as a multiple of the control group activity.

**Cytokine assay**

Cytokines IL-1α, IL-1β, IL-6, MCP-1 in mouse serum as well as in cell culture supernatants were determined by ELISA kits (Multi sciences, China) and all operations were performed according to the kit instructions.

**Statistical analysis**

Data are expressed as mean ± SD. All samples were independent, including those measured over time in the experimental samples. And statistical analysis was performed using GraphPad Prism V 8.0.2 (GraphPad, La Jolla, CA, USA). Data normality was tested using the Shapiro-Wilk test and equal variance was tested using the Brown-Forsythe test. For normally distributed data, unpaired t-tests were used to compare two groups when the data showed equal variance; otherwise, t-tests assuming uneven variance were performed. One-way analysis of variance (ANOVA) and Tukey's multiple comparison test were used as appropriate for comparison of more than two groups, and Welch's ANOVA and Tamhane's T2 methods were used for post hoc group analysis, and one-way ANOVA and Tukey's multiple comparison test. Special post hoc tests were performed only when the F (or equivalent variance) in the ANOVA reached statistical significance below 0.05 and there was no significant variance inhomogeneity. Probability
values of $P < 0.05$ were considered significant. No data points were excluded from the analysis in any experiment. All possible outliers were included in the statistical analysis and tabulations.

**Results**

**H$_2$S levels are associated with the severity of sepsis in clinics**

To explore whether circulating H$_2$S is associated with sepsis in clinics or not, we measured the H$_2$S levels in serum from patients with sepsis and non-sepsis control, showing the reduction of H$_2$S in serum from septic patients compared with un-septic control (Supplemental table 1). BNP and EF values are considered as typical indexes for heart failure. Comparison of the relationship between NT-pro BNP or EF and the concentration of hydrogen sulfide in serum samples of all collected patients, there was a negative correlation between NT-pro BNP and H$_2$S (spearman R = -0.505, $p < 0.001$), and there was no obvious linear relationship between EF and H$_2$S concentration (spearman R = -0.104, $p = 0.2127$). Herein, the septic patients were subgrouped according to BNP and EF values[20], respectively. Among the patients suffered with sepsis, H$_2$S levels was lower in serum from the cohort with the low values of BNP and EF, compared with those from the high levels of BNP and EF values, respectively (Figure 1C and 1D). What's more, H$_2$S levels were detected in serum from mouse model of LPS induction as one typical model of sepsis. Less H$_2$S was in serum after LPS induction (Supplemental Figure 1). In LPS-induced sepsis mouse model, the $Bnp$ mRNA level and EF values were increased, showing the cardiac dysfunction after sepsis. And the levels of H$_2$S were negatively with the levels of $Bnp$ mRNA and EF values (Supplemental Figure 1). These data indicated that the levels of serum H$_2$S is negatively associated with cardiac function in response to sepsis.

**GYY4137 alleviates cardiac dysfunction in response to sepsis**

To identify whether H$_2$S plays a protective role in response to sepsis or not, GYY4137 as the H$_2$S donor was administrated in the sepsis mouse model of cecum ligation and puncture (CLP). It was consistent with previous results that, in another sepsis model of CLP, H$_2$S levels were declined in serum from CLP mice (Figure 2A). Additionally, CLP caused cardiac dysfunction, reflecting from the reduction of the values of EF and FS (Figure 2B and 2C), accompanying with the increase for $Bnp$ mRNA levels in heart tissues (Figure 2D). After GYY4137 administration, the reduced levels of serum H$_2$S in CLP mice were recovered (Figure 2A), and the reduction for EF and FS was increased, while the increase of $Bnp$ mRNA levels was suppressed. It indicated that the cardiac function was negatively associated with serum H$_2$S levels after GYY4137 administration. What's more, TUNEL staining in heart tissues showed that GYY4137 prevented cardiomyocytic apoptosis in heart tissues from CLP mice (Figure 2E). These results showed that exogenous H$_2$S alleviates cardiac dysfunction in CLP-caused sepsis.

**GYY4137 prevents cardiac inflammatory response in sepsis**
To identify the mechanism underlying how GYY4137 alleviates cardiac function in sepsis, an unbiased RNA sequencing assay was performed using heart tissues. GO enrichment analysis revealed, in sepsis condition, GYY4137 mainly regulated pathways of inflammatory response. To further confirm the results from RNAseq assay, we performed RT-PCR assay using heart tissues, verifying that GYY4137 suppressed sepsis-caused increase at mRNA levels of inflammatory cytokines, such as \( \text{Il-1} \alpha, \text{Il-1}\beta, \text{Il-6} \) and \( \text{Mcp-1} \). The activity of Caspase-1, typical downstream for Nlrp3-mediated inflammatory pathway, was also detected in hearts, showing that GYY4137 repressed the increased levels of Caspase-1 activities. And H&E staining with these hearts showed that CLP caused nucleated dense inflammatory cell infiltrates in the hearts, and GYY4137 prevented cell infiltration. Furthermore, we identified that GYY4137 mainly prevented macrophage infiltration, not neutrophils infiltration is mainly occurred by macrophages, not T lymphocytes, B lymphocytes or neutrophils.

Considered that it is possible that sepsis caused the systemic inflammatory response, and then caused cardiac injury, we detected the expression levels of circulating inflammatory cytokines. GYY4137 prevented sepsis-caused increase for IL-1\( \beta \), IL-6 and MCP-1. The above results indicated that GYY4137 suppressed sepsis-caused cardiac inflammatory response with the reduction of macrophage infiltration.

**GYY4137 represses inflammasome activity in macrophages in response to LPS**

Because GYY4137 prevented macrophage infiltration and suppressed the increased levels of proinflammatory cytokines, we determined GYY4137-caused anti-inflammatory response in lipopolysaccharide (LPS)-treated macrophage *in vitro*. The expression levels of Nlrp3 and pro-caspase-1 were increased in a dose-dependent way of LPS (Figure 5A). And also the activities of IL1b were up-regulated in dose-dependent way of LPS. GYY4137 repressed LPS-induced increase for Nlrp3 and pro-IL-1b in macrophage. The anti-inflammatory response of GYY4137 was abolished after treatment with MCC950 an inhibitor of NLRP3 in LPS-incubated macrophages, reflecting from the declined expression and activities of pro-IL1b. Additionally GYY4137 alleviated LPS-induced increase for *Nlrp3* and *Asc* mRNA levels. Thus, it indicated that GYY4137 can act as an inhibitor of inflammatory response by inhibiting the expression of NLRP3.

**GYY4137 represses the crosstalk between LPS-incubated macrophages and cardiomyocytes via down-regulation of inflammation**

Considered the elevated levels of circulating pro-inflammatory cytokines and the activation of macrophage after CLP surgery, we suspected that once the activation of macrophages was abolished by GYY4137, the crosstalk between macrophages and myocytes caused cardiomyocyte apoptosis was prevented. The supernatant of LPS-incubated macrophages was added for myocyte culture (Figure 5A). The supernatant from LPS-incubated macrophages induced myocytic apoptosis, showing the evaluated levels of Bax and cleaved-caspase-3 and the decrease for Bcl-2. Once the supernatant was inactivated after heating, there was no change for Bax, Bcl-2, or cleaved caspase-3, further confirming that the crosstalk between LPS-induced macrophages and myocytes caused myocytic apoptosis. Once GYY4137
was added in LPS-incubated macrophages, supernatant-induced myocytic apoptosis was abolished. The results from TUNEL staining were consistent with the previous data.

Additionally, the supernatant from LPS-incubated macrophages decreased the mRNA levels of antioxidant genes, such as \textit{Ho-1}, \textit{Sod-1}, \textit{Sod-2}, and \textit{Nqo-1}. Consistently, GYY4137 abolished the decrease for these antioxidant gene expressions. These data showed that GYY4137 regulated the secretory factors in macrophages, and then affects cardiomyocytes.

\textbf{GYY4137 exerts myocardial protection through inhibition of NLRP3 pathway}

To verify whether GYY4137 regulates macrophages via Nlrp3 pathway in vivo, we generated SICM mode in Nlrp3 knockout mice. GYY4137 did not affect the cardiac function in \textit{Nlrp3}^-/- mouse of SCIM model, reflecting from the values of EF and FS. And in \textit{Nlrp3}^-/- deficient condition, BNP levels were not changing after GYY4137 administration, and the same results of IL-1\(\beta\), IL-6 and MCP-1, showing the protective role of GYY4137 was abolished by \textit{Nlrp3}^-/- deficiency. And GYY4137 did not affect macrophage infiltration in \textit{Nlrp3}^-/- deficient condition. The ROS levels and myocyte apoptosis were also not changed after GYY4137 administration in \textit{Nlrp3}^-/- deficient condition. These results showed that the protective role of GYY4137 was abolished by \textit{Nlrp3}^-/- deficiency.

\textbf{Discussion}

In our study, we demonstrate that GYY4137, as a donor of hydrogen sulfide, abolishes that the crosstalk between macrophages and cardiomyocytes, alleviates sepsis-induced cardiac dysfunction. During the process of SICM, the macrophages are infiltrated in the heats, and NRLP3-mediated inflammatory response is occurred in these macrophages, releasing multiple proinflammatory factors, and causing the accumulation of ROS production in cardiomyocytes, resulting in cardiac dysfunction. GYY4137 can repress the activation of NRLP3-mediated inflammasomes, rescuing the cardiac function in sepsis condition.

During the process of sepsis, it is easy to observe the macrophage activation and responses in macrophages. In our study, we found the activation of NRLP3-mediated inflammasome in macrophages, leading to impair the cardiac function in the development of SICM. Sepsis is a complicated symptoms that occur in response to systemic inflammation caused by infection, mainly resulting in hypotension, organ failure, and even death. Although multiple mechanisms of injury are involved in sepsis, the widely accepted one is considered as a complex immune/inflammatory interaction. However, the detailed mechanisms under SICM remain unclear and are emergence to be further investigated[21]. In such complex immune response, macrophages play a pivotal role, involving with multiple signaling pathways and different stages of the process of sepsis[22]. Hui Zhang et al. found TMEM173 could control calcium release from the endoplasmic reticulum in macrophages and monocytes, thus participating in blood coagulation during the coagulation period of sepsis[23]. Daniele Carvalho Nascimento et al. elucidated that expanded CD39 cell populations can suppress the antimicrobial activity of macrophages, leading to
immunosuppression in the later stages of sepsis[24]. In our study, during the development of SICM, macrophage infiltration was occurred in myocardial tissue, affect the cardiac function and fibrosis. Excepted the direct effect of endotoxin on cardiomyocytes, inflammatory factors secreted from macrophages can induced the accumulation of ROS in cardiomyocytes and lead to abnormal heart function. Meanwhile, we found that GYY4137, as a donor of hydrogen sulfide, could inhibit the activation of NLRP3 inflammatory vesicles and reduce intracellular ROS in cardiomyocytes.

Crosstalk between different types of cells plays an important role in the pathophysiologic condition, especially in the development of disease. Numbers of studies have shown that infiltration either of macrophages or neutrophils is an vital step for pathological progression. In non-infectious diseases, crosstalk between macrophages and mesenchymal stem cells is involved in fracture healing[25]. Similarly, in the endocrine system, the crosstalk between macrophages and endocrine cells is an important therapeutic target[26]. In infectious diseases such as ARDS, the signaling crosstalk between AMP-activated protein kinase in macrophages and High-mobility group box-1 in neutrophils has been shown to be an important cause of alveolar function in inflammatory lung disease[27]. Herein, the crosstalk between infiltrating macrophages and cardiomyocytes was observed, accompanying with the process of SICM, leading to cardiac dysfunction. In detail, the inflammatory response is characterized by the activation of NLRP3 inflammasome in macrophages with the secretory protein IL-1β, which has effect on the accumulation of ROS in cardiomyocytes, leading to abnormal myocardial function. GYY4137, as a slow-release H₂S donor, can reduce the inflammatory factor IL-1β secreted by macrophages by inhibiting NLRP3, and can also act directly on cardiomyocytes to reduce ROS in cardiomyocytes and decrease the level of oxidative stress in cardiomyocytes. ultimately exhibiting a myocardial protective function.

Although H₂S, a small molecule that is traditionally conceptualized as toxic, can exhibit neurotoxicity and reduced cell survival at higher concentrations [28], it was reported that in microglia (Microglial), high concentrations of H₂S can increase cellular ROS[29]. The high concentrations of NaHS in HepG2 cells counteracts the anti-inflammatory effect at low concentrations In HepG2 cells, high concentrations of NaHS counteracted the anti-inflammatory effect at low concentrations of NaHS [30]. And at lower concentration condition, H₂S showed positive effects on different diseases, mainly reflecting from inflammatory responses[30–32]. Considered the results of the previous in vitro experiments, we took GYY4137 as the donor of H₂S, which showed a greater inhibitory effect on inflammation in macrophages and downstream signaling pathways in cardiomyocytes.

This study has some limitations. First, we mainly used the CLP-induced sepsis model to explore the specific protective mechanism of hydrogen sulfide, because this is the most similar to the clinical pathophysiology of the disease model[33]. However, the establishment of septic models by intraperitoneal injection of LPS or bacteria is also widely used, and whether hydrogen sulfide still has a good protective effect in these models remains to be determined. However, according to the pathogenic mechanism of each model and the specific mechanism of heart injury induced, we have full confidence in the protective effect of hydrogen sulfide. Secondary, in this study, the cells we used are mainly
commercial cell lines, and the results achieved using cell lines may be slightly different from those gained using primary cells of homologous origin. Last one, our studies have shown that hydrogen sulfide can reduce oxidative stress in the heart and play a protective role. However, we are not clear about the specific targets, such as the role of a specific protein, RNA or mitochondria in cardiomyocytes, which need to be further clarified.

**Conclusion**

In the development of SICM, the macrophages are infiltrated in the heats, and NRLP3-mediated inflammatory response is occurred in these macrophages, releasing multiple proinflammatory factors, and causing the accumulation of ROS production in cardiomyocytes, resulting in cardiac dysfunction. GYY4137 can repress the activation of NRLP3-mediated inflammasomes, and block the crosstalk between macrophages and cardiomyocytes, alleviating cardiac dysfunction in sepsis (Fig. 7). Our results indicate that GYY4137 could be considered as a potential therapeutical drug for sepsis treatment in clinics.

**Appendix**

Supplemental materials figures;

Supplemental materials table.

**Abbreviations**

NLRP3: NOD-like receptor protein 3; SICM: sepsis-induced cardiomyopathy; CLP: cecum ligation and puncture; PAMPs: pathogen-associated molecular patterns; DAMPs: damage-associated molecular patterns; CSE: cystathionine γ-lyase; CBS: cystathionine β-synthase; Nrf2: Nuclear factor E2-related factor 2; LPS: lipopolysaccharide; EF: ejection fraction; FS: short-axis shortening; H&E staining: hematoxylin-eosin staining; ROS: reactive oxygen species.

**Declarations**

**Ethical approval and informed consent**

We have received ethical approval (2020-SR-055) from the institutional review boards (IRBs) at the First Affiliated Hospital of Nanjing Medical University. Since this study does not contain protected health information and all data were anonymously used, a waiver of the requirement for informed consent was approved by the IRBs.

All animal husbandry and experiments were approved by the Animal Welfare Ethics Committee of Nanjing Medical University (Number ID: NJMU-2012006), and efforts have been made to minimize unnecessary use and discomfort of animals.
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Author’s contributions

TZ and HQ perform animal and cellular experiments with data collection and collation. NZ Collection of patient information and determination of plasma hydrogen sulfide concentrations. TZ analyzed and interpreted the data, then constructed the manuscript. QL and YH conducted the design of the project and reviewed the manuscript.

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Figure 1: Hydrogen sulfide levels in patients with sepsis-induced cardiomyopathy. (A) Negative correlation between serum H₂S and NT-pro BNP levels in patients. Spearman’s correlation analysis was performed (n=335). (B) No linear correlation was found between serum hydrogen sulfide and EF values. EF values determined by cardiac doppler. Spearman’s correlation analysis was performed. (n=145). (C) Among the sepsis-infected patients, differences in H₂S concentrations corresponding to different pro-BNP value subgroups (95 vs 80). (D) Among the sepsis-infected patients, the levels of hydrogen sulfide were showed as indicated between normal cardiac function and heart failure patients. Patients with EF less than 50% were considered as heart failure (n=53), and others were as normal (n=25). Data are median and interquartile range (Using the Mann-Whitney U test to compare the differences between the two groups of data).

Figure 1

See image above for figure legend.
Figure 2: GYY4137 alleviates cardiac dysfunction in response to sepsis. The mouse model of sepsis was generated by CLP surgery. Partial mice were administrated with GYY4137. (A) The hydrogen sulfide levels in serum were detected from these four groups (n = 6, each group). (B and C) The echocardiography was performed with this mice at the end point of the in vivo experiments. The values for EF (B) and FS (C) were presented. (D) The mRNA levels of Bnp expression were analyzed using RT-PCR assay in heart tissues from mice. (E) TUNEL staining of heart tissue. The left panel is the representative image for apoptosis, and the right panel is the quantization of the positive signal in the left images. Scale bars, 50 μm. Data are mean ± SD (one-way ANOVA with Tukey’s post-hoc test or Kruskal–Wallis ANOVA with post-hoc Dunn’s multiple comparison tests).

Figure 2

See image above for figure legend.
Figure 3: GYY4137 prevents cardiac inflammatory response in sepsis. The mouse model of sepsis was generated by CLP surgery. Partial mice were administrated with GYY4137. (A) GO enrichment analysis after RNA sequencing using heart tissues from CLP mice and CLP/GYY4137 mice. (B) Expression levels of inflammatory-related genes in heart tissues. (C) H&E staining of heart samples. Black arrows mark inflammatory cells infiltrating between cardiomyocytes. Scale bars, 20 μm. (D) Measurement of caspase-1 activity in cardiac tissue (n=6). (E) Immunofluorescence staining using heart tissues with antibody against CD68 was performed. Scale bars, 20 μm. (F) The levels of inflammatory factors (IL-1β, IL-6, MCP-1) in serum were measured by ELISA. Data are mean ± SD (one-way ANOVA with Tukey’s post-hoc test or Kruskal–Wallis ANOVA with post-hoc Dunn’s multiple comparison tests).

Figure 3
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Figure 4: GYY4137 represses inflammasome activity in macrophages in response to LPS.  

(A) The activities of Caspase-1 were detected in RAW 264.7 treated with different dose of GYY4137 (n=3 each group).  

(B) The protein levels of NLRP3 was detected by Western blot analysis in RAW 264.7 treated with different dose of GYY4137.  

(C and D) NLRP3 inhibitor MCC950 was cocultured with GYY4137 in LSP-pretreated RAW 264.7. (C) The protein levels for NLRP3, pro IL-1β and IL-1β were measured using Western blot analysis. (D) Concentration of inflammatory factor IL-1β in supernatant of RAW 264.7 measured by ELISA.  

(E) The mRNA levels of Nlrp3 and Asc were detected using RT-PCR assay in LPS-pretreated RAW 264.7 after GYY4137 treatment. Data are mean ± SD (one-way ANOVA with Tukey’s post-hoc test or Kruskal–Wallis ANOVA with post-hoc Dunn’s multiple comparison tests).

Figure 4

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Figure 5: GYY4137 represses the crosstalk between LPS-incubated macrophages and cardiomyocytes via down-regulation of inflammation. (A) Pattern diagram of cellular experiment. After stimulation of cultured macrophages with LPS for 24 hours, take the culture medium supernatant, inactivated it by 65°C water bath for 30 minutes and added it to the cardiomyocyte medium. (B) H9c2 cells were treated according to the method of panel A, and then separately given GYY4137. The protein levels for Bcl2, Bax and Cleaved caspase-3 in H9c2 were measured using Western blot analysis. (C) The mRNA levels of Ho-1, Sod2, Sod1 and Nqo-1 in H9c2. (D) TUNEL staining of H9c2, the right panel is the quantization of the positive signal in the left images. Scale bars, 50 μm. (E) DHE staining of H9c2, the left panel is the quantization of the positive signal in the left images. Scale bars, 100 μm. Data are mean ± SD (one-way ANOVA with Tukey's post-hoc test or Kruskal–Wallis ANOVA with post-hoc Dunn's multiple comparison tests).
Figure 6: GYY4137 exerts myocardial protection through inhibition of NLRP3 pathway. (A) The mRNA expression of Bnp in mouse heart (n=6, each group). (B) The echocardiography was performed with this mice at the end point of the in vivo experiments. The values for EF and FS were presented. (C) Concentration of inflammatory factors (IL-1β, IL-6, MCP-1) in serum measured by ELISA. (D) Immunofluorescence staining using heart tissues with antibody against CD68 was performed, the right panel is the quantization of the positive signal in the left images. Scale bars, 100 μm. (E) DHE staining of H9c2, the right panel is the quantization of the positive signal in the left images. Scale bars, 100 μm. (F) TUNEL staining of heart tissue. The left panel is the representative image for apoptosis, and the right panel is the quantization of the positive signal in the left images. Scale bars, 50 μm. Data are mean ± SD (one-way ANOVA with Tukey's post-hoc test or Kruskal–Wallis ANOVA with post-hoc Dunn's multiple comparison test).

Figure 6

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Figure 7: GYY4137 impaired cardiac insufficiency in SICM mice via the NLRP3 pathway.

Figure 7
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Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalMaterials.pdf
- Supplementaltable1Characteristics.docx
- Supplementaltable2primer.docx