**ABSTRACT:** Background and purpose: Sepsis is a severe infection-induced disease with multiple organ failure, and sepsis-induced cardiomyopathy is a fatal condition. Inflammatory response and oxidative stress are reported to be involved in the development of sepsis-induced cardiomyopathy. Dulaglutide is a novel antidiabetic agent that is currently reported to exert an anti-inflammatory effect. The present study aims to explore the potential protective property of dulaglutide on lipopolysaccharide (LPS)-induced injury on cardiomyocytes. Methods: LPS was used to induce an in vitro injury model on cardiomyocytes. The mitochondrial reactive oxygen species (ROS) level was measured using MitoSOX red, and reduced glutathione (GSH) was measured to evaluate the status of oxidative stress in H9c2 myocardial cells. The expressions of NADPH oxidase-1 (NOX-1) and inducible nitric oxidesynthase (iNOS) were determined using real-time PCR and western blot analysis. Real-time PCR and enzyme-linked immunosorbent assay (ELISA) were both used to detect the expressions and concentrations of tumor necrosis factor-α, interleukin-1β, interleukin-17, matrix metalloproteinase-2, and matrix metalloproteinase-9 in H9c2 myocardial cells, respectively. The production of nitric oxide (NO) was measured using the Griess reagent. The levels of creatine kinase isoenzyme-MB (CK-MB) and cardiac troponin I (cTnI) were detected using ELISA. Western blot was utilized to determine the expressions of toll-like receptor 4 (TLR4), myeloid differentiation factor 88 (MyD88), and p-NF-κB p65 in H9c2 myocardial cells in the nucleus. Results: First, dulaglutide ameliorated LPS-induced oxidative stress by suppressing the production of mitochondrial ROS and elevating the level of reduced GSH, as well as downregulating NOX-1. Second, the LPS-induced cardiomyocyte injury was alleviated by dulaglutide through downregulating CK-MB and cTnI, accompanied by inhibiting iNOS expression and NO production. Lastly, the production of inflammatory factors and upregulation of MMPs induced by LPS were both significantly reversed by dulaglutide through suppressing the TLR4/Myd88/NF-κB signaling pathway. Conclusions: Dulaglutide alleviated LPS-induced injury in cardiomyocytes by inhibiting inflammation and oxidative stress.

**INTRODUCTION**

Sepsis is defined as fatal organic dysfunction induced by the dysregulated host response to infection, the diagnostic standard of which occurs when infection combined with sequential organ failure assessment is higher than 2. According to an epidemiologic study, approximately 19 million patients have been diagnosed with sepsis annually, with roughly 32.6% morbidity. Approximately 25–50% of sepsis patients are reported to be diagnosed with a myocardial injury or cardiac dysfunction, regarded as important pathological signs for the poor prognosis of sepsis patients. Sepsis-induced cardiomyopathy was first described in the 1980s and defined as sepsis accompanied by decreased left ventricular ejection fraction (LVEF) and ventricular dilatation. The main clinical characteristics of sepsis-induced cardiomyopathy include systolic dysfunction, such as impaired ventricular contractility and decreased LVEF and diastolic dysfunction, such as ventricular dilatation and reversible cardiac dysfunction. Several molecular pathways have been claimed for the pathogenesis of sepsis-induced cardiomyopathy, among which pathogen-associated molecular pattern is well accepted. There are a bunch of causes of septic cardiomyopathy that have been reported in previous studies, including pathogen-associated molecular patterns, such as lipopolysaccharide (LPS). LPS is a component of the outer membrane of Gram-negative bacteria. LPS belongs to the endotoxin family and is able to induce immune cells and generate numerous proinflammatory cytokines, subsequently damaging various organs, including heart tissues. Toll-like receptors (TLRs) are a group of pattern-recognition receptors expressed in immune cells and myocardial cells and play an important role in the process of innate and acquired immunity. After the hosts are infected with microorganisms or pathogens, TLRs are activated by binding with such molecular patterns as lipopolysaccharide (LPS), and thereafter, the NF-κB pathway is activated, further contributing to the excessive production of proinflammatory factors, such as cytokines, antimicrobial peptides, and chem-
It is reported that LVEF is suppressed and the left ventricular end-diastolic volume is increased after injecting the healthy volunteers with LPS, which indicates that LPS is a key element for the pathogenesis of sepsis-induced cardiomyopathy. In addition, in the mouse sepsis model induced with LPS, inhibiting the activated toll-like receptor 4 (TLR4) signaling pathway by knocking down myeloid differentiation factor 88 (MyD88) significantly decreases the systemic inflammatory reaction and suppresses the release of proinflammatory factors, such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and IL-6, from the myocardial cells, which alleviates the prognosis and cardiomyopathy of sepsis mice. Inhibiting the function of inflammatory factors has been found to be effective in treating sepsis-induced cardiomyopathy. Conrad reported that the left ventricular cardiac dysfunction could be alleviated by treating the sepsis animals with TNF-\(\alpha\) antibodies. The myocardial depression in the sepsis mice was significantly ameliorated by propofol by suppressing the production of TNF-\(\alpha\). Therefore, the inflammatory reaction induced by the LPS/TLR4 signaling pathway might be a promising target for the treatment of sepsis-induced cardiomyopathy.

Dulaglutide is a novel agonist of the glucagon-like peptide-1 (GLP-1) receptor, approved for the treatment of adult type II diabetes (T2D) by the American Food and Drug Administration (FDA) in September 2014. As a fusion protein of GLP-1 and Fc, dulaglutide binds to the GLP-1 receptor. Long-term treatment with dulaglutide induces the secretion of blood glucose-dependent insulin, decreases fasting and fed blood-glucose, and alleviates the function of islet B cells. Recently, a prominent anti-inflammatory effect of dulaglutide has been reported in fibroblast-like synoviocytes and disuse muscle atrophy in mice. In the present study, we investigated the anti-inflammatory effect of dulaglutide on LPS-induced cardiomyocytes to explore the potential therapeutic property of dulaglutide in the treatment of sepsis-induced cardiomyopathy.

**RESULTS**

**Dulaglutide Ameliorated LPS-Induced Oxidative Stress in H9c2 Myocardial Cells.** To investigate the cytotoxicity of dulaglutide on H9c2 myocardial cells, cells were stimulated with dulaglutide at the concentrations of 5, 10, 50, 100, 500, and 1000 nM for 24 h. The MTT assay results indicate that dulaglutide did not affect the cell viability of H9c2 myocardial cells. However, 500 and 1000 nM dulaglutide reduced cell viability of H9c2 cells to 89 and 80% respectively, compared to the control group (Figure 1). Therefore, 100 and 50 nM dulaglutide were used in the subsequent study.

To evaluate the effect of dulaglutide on oxidative stress in H9c2 myocardial cells induced by LPS, cells were incubated with LPS (1 \(\mu\)g/mL) with or without dulaglutide (50 and 100 nM) for 24 h. As shown in Figure 2A, the production of mitochondrial reactive oxygen species (ROS) was significantly elevated by stimulation with LPS but greatly suppressed by treatment with dulaglutide in a dose-dependent manner. In addition, the decreased concentration of reduced glutathione (GSH) (Figure 2B) in H9c2 myocardial cells induced by LPS was dramatically promoted by the introduction of dulaglutide in a dose-dependent manner. These data indicate that the processing of oxidative stress in H9c2 myocardial cells was ameliorated by dulaglutide.

**Dulaglutide Reduced LPS-Induced Expression of NOX-1 in H9c2 Myocardial Cells.** We further investigated the expression of NADPH oxidase-1 (NOX-1), an important oxidase in the process of oxidative stress, following the different treatments. As shown in Figure 3, NOX-1 was significantly upregulated by stimulation with LPS but dramatically downregulated by the introduction of dulaglutide, indicating an inhibitory effect of dulaglutide on LPS-induced expression of NOX-1 in H9c2 myocardial cells.

**Dulaglutide Suppressed LPS-Induced Expressions and Secretions of TNF-\(\alpha\), IL-1\(\beta\), and IL-17 in H9c2 Myocardial Cells.** To investigate the effect of dulaglutide on the inflammation induced by LPS, the expressions of related inflammatory factors were evaluated. As shown in Figure 4A, the elevated gene expressions of TNF-\(\alpha\), interleukin-1\(\beta\) (IL-
β-1), and interleukin-17 (IL-17) in H9c2 myocardial cells induced by LPS were significantly inhibited by treatment with dulaglutide. As shown in Figure 4B, compared to control, the concentration of TNF-α was elevated from 113.6 ± 14.5 to 2056.9 ± 234.6 pg/mL by stimulation with LPS but greatly decreased to 1426.7 ± 162.7 and 1055.2 ± 124.2 pg/mL by the introduction of 50 and 100 nM dulaglutide, respectively. The concentrations of IL-1β in the control, LPS, LPS + 50 nM dulaglutide, and LPS + 100 nM dulaglutide group were 75.2 ± 7.9, 1378.8 ± 151.6, 963.4 ± 98.8, and 753.9 ± 78.3 pg/mL, respectively. Lastly, compared to control, the secretion of IL-17 in H9c2 myocardial cells was promoted from 83.3 ± 72.3 to 653.7 ± 67.6 pg/mL by stimulation with LPS but dramatically suppressed to 457.2 ± 47.9 and 346.6 ± 37.4 pg/mL by treatment with 50 and 100 nM dulaglutide, respectively. These data indicate that the severe inflammation induced by LPS was greatly alleviated by treatment with dulaglutide.

Dulaglutide Alleviated LPS-Induced Expression of MMP-2 and MMP-9 in H9c2 Myocardial Cells. We further checked the expression of matrix metalloproteinases in the treated H9c2 myocardial cells. As shown in Figures 5A,B, the elevated gene expressions of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) induced by LPS were pronouncedly inhibited by the introduction of dulaglutide. Compared to the control, the production of MMP-2 (Figure 5C) was significantly increased from 36.8 ± 3.8 to 213.7 ± 24.5 pg/mL by stimulation with LPS but dramatically decreased to 142.6 ± 16.8 and 105.2 ± 12.5 pg/mL by treatment with 50 and 100 nM dulaglutide, respectively. As shown in Figure 5D, the concentrations of MMP-2 and MMP-9 in the control, LPS, LPS + 50 nM dulaglutide, and LPS + 100 nM dulaglutide groups were 55.9 ± 5.8, 365.8 ± 39.1, 263.7 ± 28.7, and 193.5 ± 21.4 pg/mL, respectively. These data indicate that the upregulation of MMP-2 and MMP-9 in H9c2 myocardial cells induced by LPS was greatly suppressed by dulaglutide.

Dulaglutide Alleviated LPS-Induced Expression of iNOS and the Production of Nitric Oxide in H9c2 Myocardial Cells. The expression of inducible nitric oxide synthase (iNOS) and production of nitric oxide (NO) are important representatives for the cellular oxidation state. As shown in Figure 6A,B, we found that iNOS was significantly upregulated by stimulation with LPS but greatly downregulated by treatment with dulaglutide. The elevated production of NO induced by LPS was dramatically inhibited by the introduction of dulaglutide, indicating an obvious inhibitory effect of dulaglutide on the activated oxidation state induced by LPS.

Dulaglutide Mitigated LPS-Induced Cardiomyocyte Injury in H9c2 Myocardial Cells. We further evaluated the injury state of H9c2 myocardial cells following different treatments. As shown in Figure 7, the expressions of two important myocardial dysfunctional indicators, creatine kinase isoenzyme-MB (CK-MB) and cardiac troponin I (cTnI), were dramatically elevated by stimulation with LPS but pronouncedly inhibited by treatment with dulaglutide in a dose-dependent manner, indicating that the injured myocardial function triggered by LPS was greatly mitigated by dulaglutide.
Dulaglutide Mitigated LPS-Induced Activation of the TLR4/Myd88/NF-κB Pathway. The activation of the inflammatory signaling pathway was further investigated. As shown in Figure 8A, the expressions of TLR4 and Myd88 in H9c2 myocardial cells were significantly promoted by the introduction of LPS but greatly suppressed by treatment with dulaglutide in a dose-dependent manner. Importantly, our results demonstrate that the levels of both p-NF-κB p65 and NF-κB p65 in the nuclear fraction were increased by LPS stimulation, and remarkably inhibited by dulaglutide (Figure 8B). These findings suggest that the activated TLR4/Myd88/NF-κB pathway induced by LPS was inhibited by dulaglutide.

DISCUSSION

The activation of inflammatory factors can be induced by sepsis, which stimulates the excessive production of reactive nitrogen species (RNS) and ROS. The produced RNS and ROS trigger mitochondria damage and induce mitochondrial apoptosis by activating the cyclic adenosine monophosphate/protein kinase A and the cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG) signaling pathways. Oxidative stress is mainly induced by the excessive production of ROS and is reported to be involved in the myocardial damage induced by sepsis. In the present study, we found that oxidative stress was significantly induced in H9c2 myocardial cells by stimulation with LPS and verified by the elevated production of NO and the decreased concentration of reduced GSH. Through treatment with dulaglutide, the oxidative stress state was found to be alleviated, indicating a protective effect of dulaglutide against oxidative stress injury by LPS on myocardial cells. CK-MB and cTnI are two important indicators for the dysfunction of myocardial cells and are widely used in the diagnosis of acute myocardial infarction. We found that the expressions of CK-MB and cTnI in H9c2 myocardial cells were significantly elevated by stimulation with LPS, indicating that the dysfunction of myocardial cells was induced by LPS. The LPS-induced dysfunction of myocardial cells was dramatically alleviated by dulaglutide, indicating a promising protective effect of dulaglutide against sepsis-induced myocardial dysfunction. However, more evidence will be provided by treating the sepsis animal model with dulaglutide to further verify the protective property of dulaglutide against sepsis-induced myocardial dysfunction in our future work.

Currently, three subtypes of NOS have been identified in the myocardial cells, neuronal NOS (nNOS), iNOS, and endothelial NOS (eNOS). Under a physiological state, sustaining slight production of NO is triggered by nNOS and eNOS to maintain systemic vasodilation and regulate the load of the heart. However, iNOS is only expressed under the mediation of cytokines, contributing to the excessive production of NO, expansion of peripheral blood vessels, and impaired myocardial contractility. Xu reported that the upregulation of iNOS plays an important role in the development and processing of sepsis-induced cardiomyopathy. The synthesis of NO is activated by iNOS in sepsis patients, and peroxynitrite is produced by the reaction between excessively secreted NO and superoxide radicals, which further inhibits myocardial function, changes the heart load, downregulates the β-adrenergic receptor, suppresses the function of type I calcium channel, and attenuates the activity of the mitochondrial electron transport chain complex in cardiomyocytes. In the present study, we found that the elevated expression of iNOS and promoted production of NO-induced with LPS were significantly reversed by treatment with dulaglutide, indicating an inhibitory effect of dulaglutide on inflammatory factor-mediated iNOS activation. Further investigation will be conducted in our future work to verify the inhibitory effect of dulaglutide on iNOS by introducing an iNOS agonist into the experimental system.

Activation of inflammation and excessive production of inflammatory factors are the direct indications of sepsis on
myocardial cells. When TLR4 is activated by pathogen-associated molecular patterns, such as LPS, the TLR4/Myd88 signaling pathway is activated, inducing the phosphorylation of I\textsuperscript{κ}B, an important natural inhibitor of NF-κB. NF-κB is disassociated from the complex composed of NF-κB and I\textsuperscript{κ}B by the phosphorylation of I\textsuperscript{κ}B, which further transfers into the nucleus to activate the transcription of inflammatory factors.\textsuperscript{24,25} In the present study, the TLR4/Myd88/NF-κB signaling pathway was found to be significantly activated in H9c2 myocardial cells by stimulation with LPS, accompanied by the elevated production of inflammatory factors.\textsuperscript{26} By treatment with dulaglutide, the activated TLR4/Myd88/NF-κB signaling pathway and activated inflammation were dramatically alleviated, indicating a pronounced inhibitory effect of dulaglutide against inflammation in myocardial cells induced by LPS. Further investigations will be performed to confirm the anti-inflammatory effect of dulaglutide in a sepsis animal model in our future work. In addition, the specific target protein of dulaglutide against the TLR4/Myd88/NF-κB signaling pathway will also be further explored to better understand the protective property of dulaglutide against sepsis-induced cardiomyopathy.

The association of T2D with cardiovascular diseases has been reported before. The risk of developing cardiovascular diseases has been obviously increased in T2D patients.\textsuperscript{26} GLP-1R has been found to be located in cardiac and vascular tissues isolated from both human and animal models.\textsuperscript{27} Therefore, the beneficial effects of GLP-1R agonists in cardiovascular diseases have been widely investigated. For example, it has been recently reported that administration of the GLP-1R agonist exenatide or other glucose-lowering therapies might reduce the incidence of major adverse cardiovascular and cerebrovascular events by 19 and 12% in cardiovascular hospitalizations.\textsuperscript{28} Importantly, dulaglutide has been proven to be safe for the management of glycemic control in T2D patients with either previous cardiovascular disease or cardiovascular risk factors.\textsuperscript{29} In the current study, we used 50 and 100 nM dulaglutide to treat H9c2 myocardial cells, which is consistent with the concentrations used in previous studies.\textsuperscript{30,31} Importantly, the doses of dulaglutide used in this study are comparable with the doses of dulaglutide used in clinics as it has been shown that the efficacy and safety of 1.5 and 0.75 mg dulaglutide have been reported in the pooled and individual study data.\textsuperscript{32} Taken together, our data indicate that dulaglutide alleviated LPS-induced injury in cardiomyocytes by inhibiting inflammation and oxidative stress.
**MATERIALS AND METHODS**

**Cell Culture and Treatment.** The rat myocardial cell line, H9c2, was purchased from the American Type Culture Collection (ATCC, Manassas, USA) and cultured in complete Dulbecco’s modified Eagle medium (Thermo Fisher Scientific, USA) containing 5% fetal bovine serum at 37 °C and 5% CO₂. Cells were incubated with LPS (1 μg/mL) (Sigma-Aldrich, USA) with or without dulaglutide (50 and 100 nM) (Eli Lilly, USA) for 24 h.

**Real-Time PCR Analysis.** Total RNA was extracted from the treated H9c2 myocardial cells using the TRI Reagent RNA isolation kit (Sigma-Aldrich, USA) and transformed to cDNA with the First-Strand cDNA Synthesis kit (Pharmacia LKB, Uppsala, Sweden). In the present study, the polymerase chain reaction was performed using the TaqMan system (Thermo Fisher Scientific, USA), and the PCR amplification and product detection were carried out using an ABI PRISM 7300 sequence detection system (Thermo Fisher Scientific, USA). The relative expression of target genes was calculated using the 2^[-△△Ct] method with GAPDH taken as the negative control to normalize the relative expression. The following primers were used in this study:

- **TNF-α** (F: 5′-AAAGTCACCTCCCTCTCTGC-3′, R: 5′-GGACTCCGCAAGTCTAAGT-3′);
- **IL-1β** (F: 5′-CTTTTCGTAATGACACAGC-3′, R: 5′-GAGAAAACACAGGCTCTCT-3′);
- **IL-17** (F: 5′-ACCGAATAAGACCCCTGAT-3′, R: 5′-TCCCTCGCATGACA-3′);
- **iNOS** (F: 5′-GGGAGCAGGGCCAACCTCATATGTT-3′, R: 5′-GAGCTCTGTCCTTGCCCTCC-3′);
- **NOX-1** (F: 5′-ATAGCTACTGCCCACCGCAACGT-3′, R: 5′-TTGATACCACGGCACAGCA-3′);
- **MMP-2** (F: 5′-CAGCTAGACCGGCCC-3′, R: 5′-GAAGCCTAGCCGAGATTGT-3′);
- **MMP-9** (F: 5′-ATGTGCCTGTTCCGGGGCAGC-3′, R: 5′-TACATGAGGCTTCCGGGAC-3′);
- **GAPDH** (F: 5′-ATGACATCTCAAGAAGGTGTG-3′, R: 5′-TGTCATAACCAGGAATAGGAC-3′).

**Western Blot Assay.** Total proteins were isolated from the treated H9c2 myocardial cells using the lysis buffer (Beyotime, Shanghai, China). Nuclear fractions were prepared using a commercial nuclear/cytosol extraction kit (Thermo Fisher Scientific, USA). Protein concentration was quantified with a BCA kit (Beyotime, Shanghai, China). Then, approximately 50 μg of the protein for each sample was loaded and separated using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by being transferred to the polyvinylidene fluoride membrane (Thermo Fisher Scientific, USA); 5% BSA was used for incubation with the membrane to remove the nonspecific binding proteins, followed by being washed 3 times and incubation with the primary antibodies against NOX-1 (Cell Signaling Technology, 1:1000, USA), iNOS (Cell Signaling Technology, 1:1000, USA), TLR4 (Cell Signaling Technology, 1:1000, USA), Myd88 (Cell Signaling Technology, 1:1000, USA), p-NF-κB p65 (Cell Signaling Technology, 1:1000, USA), and GAPDH (Cell Signaling Technology, 1:1000, USA), respectively, overnight at 4 °C. Following being washed 3 times, the membrane was incubated with secondary antibodies at room temperature for 2 h. Finally, the ECL solution was added and the membrane was exposed to the Tanon 5200 (Tanon, Shanghai, China). ImageJ software (National Institutes of Health, USA) was used to analyze the bands.

**MitoSOX Red Staining.** The level of mitochondrial ROS in the treated H9c2 myocardial cells was evaluated using the MitoSOX red staining assay. In brief, the cells were loaded with 5 μM MitoSOX Red (Yeasen Technology, Shanghai, China) for 10 min at 37 °C. The cells were live-imaged using a laser scanning confocal microscope (Olympus, Tokyo, Japan).

**Measurement of Reduced GSH.** The concentration of reduced GSH in cellular lysis was detected using the GSH assay kit (Beyotime, Shanghai, China). In brief, the treated H9c2 myocardial cells were added with 5,5′-dithiobis (2-nitrobenzoic acid) to produce GSSG and 5′-thio-2-nitrobenzoic acid (TNB), the absorbance of which was measured at 405 nm. Subsequently, the concentration of reduced GSH was calculated according to the concentration of TNB, which was expressed as micromoles per gram protein.

**ELISA Assay.** The concentrations of TNF-α, IL-1β, IL-17, MMP-2, MMP-9, CK-MB, and cTnI in the treated H9c2 myocardial cells were detected using enzyme-linked immunosorbent assay (ELISA) assay with commercial kits (R&D Systems, Minneapolis, USA).
Systems, USA). In brief, the cellular samples were incubated with 5% BSA solution to remove the nonspecific binding proteins. Subsequently, the antibodies against TNF-α, IL-1β, IL-17, MMP-2, MMP-9, CK-MB, or cTnI were immobilized onto the 96-well plates and further added with the pretreated cellular samples for half an hour. Then, the HRP-conjugated antimouse immunoglobulin was added into the wells for 10 min incubation, followed by being washed and added with a TMB substrate solution for 30 min to terminate the reaction. Lastly, a spectrophotometer (Thermo Fisher Scientific, USA) was used to detect the absorbance at 450 nm.

**Measurement of NO.** The detection of NO production was conducted according to the instruction described previously. In brief, the 96-well plate was filled with 100 μL of samples and 100 μL of Griess reagent (mix of 2% sulfanilamide in 5% phosphoric acid and 0.2% N-(1-naphthyl) ethylenediamine hydrochloride-NEED) and there- after mixed and incubated for approximately 30 min. Subsequently, the absorbance at 550 nm was measured utilizing the spectrophotometer (Thermo Fisher Scientific, USA) to determine the concentration of NO in the samples.

**MTT Assay.** Cell viability of H9c2 myocardial cells was measured using a commercial MTT assay kit (#ab211091, Abcam, USA). After necessary treatment, media were discarded from the cell cultures. A total of 50 μL of serum-free media and 50 μL of MTT solution were then added into each well. After incubation at 37 °C for 3 h, 150 μL of MTT solvent was added to fully dissolve MTT formazan. Absorbance was read at 590 nm to index cell viability.

**Statistical Analysis.** Data are shown as the mean ± standard error. One-way analysis of variance (ANOVA), followed by Tukey’s test were both used for all pair comparisons. A value of \( P < 0.05 \) was considered statistically significant. Data were analyzed with the Statistical Package for Social Sciences (SPSS, Chicago, IL, USA).

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