

Original Article

Levosimendan ameliorates cardiomyocyte injury and mitochondrial dysfunction in an Nrf2-dependent manner in mice with sepsis-induced cardiomyopathy

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Abstract

Sepsis-induced cardiomyopathy (SIC) is a severe complication of sepsis and septic shock and is characterized by cardiac dysfunction. Levosimendan (LEVO), a calcium sensitizer, has shown therapeutic potential in SIC, although its underlying mechanism remains unclear. Nrf2, a pivotal regulator of antioxidant and anti-inflammatory responses, may represent a potential target for SIC treatment. In this study, we examine the effects of LEVO on SIC and explore the mechanistic role of Nrf2 in mediating its cardioprotective effects. A murine SIC model is established via cecal ligation and puncture (CLP), and cardiomyocyte injury is induced *in vitro* via lipopolysaccharide (LPS) exposure in HL-1 cells. The CLP procedure significantly elevates serum cTnI and IL-6 levels and reduces the survival rates of mice. Echocardiographic analysis reveals impaired cardiac structure and function, accompanied by mitochondrial morphological and functional damage, in SIC mice. Interestingly, these pathological changes in SIC are markedly attenuated by LEVO treatment. Similarly, LEVO administration restores proliferative capacity; increases mitochondrial ATP, mitochondrial membrane potential (MMP) and NADH levels; and reduces ROS production and intracellular calcium overload. Notably, the protective effects of LEVO on cardiomyocyte viability and mitochondrial function are significantly diminished following Nrf2 inhibition or Nrf2 knockout (KO). Collectively, these findings demonstrate that LEVO mitigates cardiomyocyte injury and mitochondrial dysfunction in SIC through an Nrf2-dependent mechanism.

Key words Sepsis-induced cardiomyopathy, Levosimendan, Cardiomyocyte injury, Mitochondrial dysfunction, Nrf2

Introduction

Sepsis is a life-threatening condition characterized by organ dysfunction resulting from a dysregulated host response to infection [1]. Among the affected organs, the heart is particularly vulnerable, and cardiac dysfunction caused by sepsis, commonly referred to as sepsis-induced cardiomyopathy (SIC), is a major contributor to sepsis-related morbidity and mortality [2,3]. The reported pre-

valence of SIC among septic patients varies widely, ranging from 10–70% [4]. Clinically, SIC is typically defined as reversible myocardial dysfunction occurring during the early stages of sepsis and is characterized by features such as a reduced left ventricular ejection fraction (LVEF ≤ 50%), left ventricular dilation, and subsequent restoration of cardiac function [5]. Multiple risk factors have been implicated in the pathogenesis of SIC, including cytokine storm,

oxidative stress, mitochondrial dysfunction, calcium dysregulation, cardiomyocyte apoptosis, and autonomic imbalance [2]. Accounting for approximately 30% of the cardiomyocyte volume, mitochondria are the primary sites of energy production in cardiac muscle. Dysfunction of these genes directly compromises myocardial energy metabolism, forming a critical pathological basis for SIC [6]. Recent studies have shown that disturbances in mitochondrial dynamics (fusion and fission), mitochondrial biogenesis and mitophagy can trigger or exacerbate various cardiac diseases, including SIC [7–9]. However, mitochondrion-targeted strategies remain underutilized in clinical settings. Moreover, the precise molecular mechanisms modulating mitochondrial dynamics and quality control in cardiomyocytes to improve SIC remain incompletely understood.

Levosimendan (LEVO), a calcium sensitizer agent, enhances myocardial contractility and vasodilation [10]. LEVO has been approved for the treatment of decompensated heart failure in many countries and is commonly employed in critical care and emergency medicine [11]. LEVO improves myocardial diastolic function by increasing the affinity of calcium for cardiac troponin C and opening ATP-dependent potassium channels on the cell surface without significantly increasing myocardial oxygen consumption [12]. Evidence has indicated that LEVO may have a therapeutic effect on SIC, although mechanistic studies are still in the early stage [11]. In addition to its positive inotropic effects, LEVO also has anti-inflammatory, antioxidant, and antiapoptotic properties [13]. In addition, it increases nitric oxide synthesis, protects vascular endothelial cells, and inhibits the expression of hypoxia-inducible factor-1 α (HIF-1 α) in cardiac tissue, making it superior to dobutamine and milrinone in SIC treatment [14,15]. Several studies have demonstrated that LEVO activates ATP-dependent potassium channels in the mitochondria of cardiomyocytes, thereby maintaining the cellular energy balance and protecting mitochondria from oxidative damage [16]. Moreover, LEVO plays a protective role in the mitochondria of cardiomyocytes and reverses cardiac malfunction and cardiomyocyte ferroptosis via connexin 43 signaling activation in mice [17]. While the exact mechanism remains unclear, LEVO may exert its therapeutic effects on SIC by modulating mitochondrial morphology and function.

Nuclear factor erythroid 2-related factor (Nrf2) is a transcription factor that plays an important role in cell defense against oxidative stress and inflammation [18]. In the cardiovascular system, Nrf2 not only regulates antioxidative genes but also alleviates mitochondrial damage to exert protective effects [19,20]. Compared with wild-type (WT) mice, *Nrf2*-knockout (KO) mice exhibit pathological cardiac hypertrophy, cardiac fibrosis and apoptosis, and subsequently, heart failure and increased mortality following transverse aortic constriction (TAC), which are reversed following the upregulation of Nrf2/heme oxygenase-1 (HO-1) [21]. Moreover, increased expression of Nrf2 improved cardiac remodeling and attenuated heart failure evoked by angiotensin II (Ang II) in a rat model [22]. Therefore, as a key regulator of redox balance, the modulation of Nrf2 could be a potential target for cardiovascular-related disease therapeutics in the future, and its role in cardioprotection should be further investigated. Overall, targeting the regulation of mitochondrial morphology and function represents a promising therapeutic strategy for SIC. LEVO may exert beneficial effects in this context by preserving mitochondrial integrity and improving cardiac performance. Moreover, Nrf2 has been shown to confer

protection against mitochondrial damage and cardiac injury in the heart [20]. Although its underlying mechanism is still elusive, it is possible that Nrf2 may be involved in mediating the benefits of LEVO in mitochondrial regulation. In this study, we utilized an *in vivo* SIC model established by cecal ligation and puncture (CLP), along with an *in vitro* model in which mouse cardiomyocytes were challenged with lipopolysaccharide (LPS), to investigate the relationship between Nrf2 signaling and mitochondrial function in mediating the cardioprotective effects of LEVO in the context of SIC.

Materials and Methods

Animals and treatments

This study was approved by the Tianjin Medical University Animal Care and Use Committee (approval number: SYXK-2019-0004) following the guidelines of the Chinese Academy of Sciences. Male C57BL/6 mice (8-week-old) were obtained from SPF Biotechnology Co., Ltd (Beijing, China) and maintained in a temperature-controlled environment with a 12:12 h light/dark cycle and free access to standard food and water. Thirty mice were randomly divided into 3 groups as follows: (1) Sham group, the mice underwent the same procedure without the CLP operation; (2) SIC group, the mice were subjected to the CLP operation while receiving saline; and (3) SIC + LEVO group, the mice were subjected to the CLP operation while receiving LEVO. *Nrf2*-KO mice were generated at the Model Animal Research Centre of Nanjing University (Nanjing, China). Twenty *Nrf2*-KO mice and twenty WT littermates were randomly assigned to 4 independent groups as follows: (1) WT (SIC) group: WT mice were subjected to the CLP operation while receiving saline; (2) WT (SIC + LEVO) group: WT mice were subjected to the CLP operation while receiving LEVO; (3) KO (SIC) group: *Nrf2*-KO mice were subjected to the CLP operation while receiving saline; and (4) KO (SIC + LEVO) group: *Nrf2*-KO mice were subjected to the CLP operation while receiving LEVO. The SIC model was established via the CLP procedure as described previously [23]. In brief, CLP features ligation below the ileocecal valve after midline laparotomy, followed by needle puncture of the cecum. Mice that died during surgery or were close to death during or within 18 h following surgery were excluded from the final analysis. Survival rates were monitored at 0, 2, 4, 6, and 8 days post-CLP. And 3 h after the CLP operation, LEVO (HY-14286; MCE, Shanghai, China) was immediately administered intraperitoneally at a dosage of 24 μ g/kg.

Cell culture and treatments

HL-1 cells (SCC065; Sigma-Aldrich, St Louis, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, 11965092; Gibco, Waltham, USA) supplemented with 10% fetal bovine serum (FBS, TMS-016; Sigma-Aldrich) and 1% penicillin/streptomycin-glutamine in a humidified atmosphere with 5% CO₂ at 37°C. For cardiomyocyte injury experiments, HL-1 cells were incubated with LPS (L6529, 1 or 10 μ g/mL; Sigma-Aldrich) for 24 or 48 h to determine the optimal exposure duration and concentration. For LEVO experiments, HL-1 cells were incubated with 0, 10, 50, or 100 μ M LEVO for 24 h to determine the optimal concentration. To downregulate Nrf2 expression, HL-1 cells were exposed to 20 μ M ML-385 (HY-100523; MCE) for 24 h. Subsequently, the LPS-treated HL-1 cells were incubated with LEVO in the absence or presence of ML-385. Cells exposed to LPS, LPS + LEVO and LPS + LEVO + ML-385 were classified into the control, LEVO and ML-385 groups,

respectively.

Enzyme-linked immunosorbent assay

Blood samples were obtained from the mice via terminal orbital puncture under deep anesthesia with 2% isoflurane. After confirming the absence of pedal reflexes, the mice were positioned laterally, and the orbital sinus was punctured using a sterile capillary tube. The volume collected did not exceed 5% of the body weight to adhere to ethical guidelines. Serum was isolated by centrifugation at 1000 *g* for 30 min at 4°C. Myocardial enzyme levels, including cardiac troponin I (cTnI) and interleukin 6 (IL-6) levels, were quantified at 0, 12, 24, 48, 72, and 120 h post-CLP via commercial enzyme-linked immunosorbent assay (ELISA) kits (ml001932, IC50325-1; mlibio, Shanghai, China) according to the manufacturer's instructions. All the experiments were performed in triplicate.

Echocardiography

At 24 h following the CLP procedure, the mice were anesthetized with 2% isoflurane and subjected to high resolution transthoracic echocardiography (Vevo 2100; VisualSonics, Toronto, Canada) as we previously reported [24]. The chest hair of each mouse was removed, and an ultrasonic coupling agent was used to acquire parasternal long-axis views. The main cardiac function parameters included left ventricular internal diameter diastolic (LVIDd), left ventricular internal diameter systolic (LVIDs), left ventricular volume in diastole (LVVd), left ventricular volume in systole (LVVs), LVEF and fractional shortening (FS).

Measurement of ATP content

ATP content of heart tissues was detected via an enhanced ATP assay kit (S0027; Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, cardiac tissues and cells were rinsed and lysed via ATP lysis buffer on ice. The samples were subsequently centrifuged at 10,000 *g* for 10 min at 4°C, after which the supernatants were collected for further determination. The ATP detection working dilutions were subsequently added, and the luminescence activity was immediately measured via a luminometer (GloMax 20/20; Promega, Madison, USA). The ATP contents were normalized to the protein contents in each sample. A standard curve of ATP was generated for each assay.

Measurement of the mitochondrial membrane potential (MMP)

For MMP (mt $\Delta\Psi$) analysis, a JC-1 kit (C2006; Beyotime) was used according to the manufacturer's manual. Briefly, for purified mitochondria from tissues, 0.9 mL of JC-1 working solution diluted 5 times was added to 0.1 mL of purified mitochondria with a total protein concentration of 10–100 μ g. For HL-1 cells, 1 mL of JC-1 working solution was added to a 6-well culture plate for 20 min. In cells with high mt $\Delta\Psi$, JC-1 dye forms J-aggregates that emit red fluorescence, whereas in cells with low mt $\Delta\Psi$, JC-1 remains in its monomeric form, emitting green fluorescence. The mt $\Delta\Psi$ was quantified by calculating the ratio of red-to-green fluorescence intensity. All the experiments were performed in triplicate to ensure reproducibility.

Electron microscopy

Mouse hearts were perfused with phosphate buffer (PB, 0.1 M)

supplemented with glutaraldehyde (2.5%) and PFA (2%). Samples were taken from the midventricular region and cut into approximately 1-mm³ blocks. The samples were sliced into 60- μ m coronal sections via a vibrating blade microtome (VT1000s; Leica, Wetzlar, Germany) as previously described [25]. Briefly, the samples were incubated in 0.1 M PB containing 1% OsO₄ for 60 min, followed by dehydration in an ascending ethanol series (50%, 70%, 80%, and 90%). The samples were subsequently embedded in epoxy resins through propylene oxide. The samples were subsequently placed on Formvar-coated 1-well Cu grids and sliced into ultrathin sections with an ultramicrotome (EM UC7; Leica, Wetzlar, Germany). The mitochondria were imaged via a transmission electron microscope (HT-7700; Hitachi, Tokyo, Japan) operating at 100 kV.

Cell proliferation assay

Cell viability was measured using a Cell Counting Kit-8 (CCK8, C0037; Beyotime) according to the manufacturer's instructions. In brief, HL-1 cells were seeded into a 96-well plate, and CCK-8 solution was added to each well and then incubated for 4 h. The optical density was determined at 450 nm with a microplate reader (Spark® Cyto; Tecan, Männedorf, Switzerland). The cell viability was subsequently calculated by normalizing the absorbance of the treated groups to that of the control group (set as 100% viability) via the following formula: Cell viability (Fold Change) = OD₄₅₀^{treated group} / OD₄₅₀^{control group}. All the experiments were conducted in triplicate.

Reactive oxygen species (ROS) assay

Intracellular ROS was detected via an ROS assay kit (S0033; Beyotime) according to the manufacturer's instructions. Briefly, HL-1 cells were incubated with aconitine for 4 h and then treated with 10 μ M DCFH-DA for 20 min at 37°C. The cells were washed with PBS three times prior to detection via a DM6B fluorescence microscope (Leica, Wetzlar, Germany). Mitochondrial ROS (mtROS) were measured via MitoSOX (M36008; Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Briefly, HL-1 cells were cultured in a 96-well plate overnight, after which 5 μ M MitoSOX was added to each well for 10 min. The cells were then incubated with 2 μ g/mL Hoechst33342 for 30 min and examined via the DM6B fluorescence microscope. The fluorescence intensities of MitoSOX were measured via ImageJ. All the experiments were performed in triplicate.

Fluo 8-AM staining

Fluo 8-AM (ab142773; Abcam, Cambridge, UK) was used to measure the level of intracellular Ca²⁺ according to the manufacturer's instructions. Briefly, the cells were incubated with 4 μ M Fluo 8-AM for 20 min at 37°C and then washed 3 times. Fluorescence images were acquired with the DM6B fluorescence microscope.

Measurement of nicotinamide adenine dinucleotide (NADH)

Cellular NADH was measured via an NADH-glo assay kit (G9072; Promega) according to a modified protocol described previously [26]. Briefly, the cells were rinsed and lysed in lysis buffer (a 1:1 mixture of PBS and bicarbonate base buffer with 1% DTAB) on ice. The cell lysate was subsequently transferred to 96-well plates for base treatment. The samples were incubated for 15 min at 60°C, after which 50 μ L of HCl/TrizmaR solution was added to each well.

Cellular NADH was calculated with a freshly prepared standard curve (NADH standards provided by the assay kit). The final NADH concentrations were then normalized to the total number of cells.

Western blot analysis

Cardiac tissues or cardiomyocytes were harvested in freshly prepared NP-40 lysis buffer containing protease and phosphatase inhibitor cocktail (HY-K0010; MCE) on ice. Equal amounts of protein were separated via 10% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (0.45 µm, ISEQ00010; Millipore, Darmstadt, Germany) at 300 mA for 2 h, and incubated overnight at 4°C with primary antibodies. The blots were then incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at 37°C. Specific band densitometry was analyzed via Quantity-one software. Antibodies against the following proteins were used: dynamic-related protein 1 (Drp1; A16661, 1:500; ABclonal, Wuhan, China), mitofusin 2 (Mfn2; 12186-1-AP, 1:1000; Proteintech, Wuhan, China), Parkin (14060-1-AP, 1:1000; Proteintech), PTEN-induced putative kinase 1 (Pink1; A7131, 1:1000; ABclonal), peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α; A12348, 1:1000; ABclonal), cytochrome c oxidase subunit 4 (COX4; A6564, 1:1000; ABclonal), β-actin (20536-1-AP, 1:10000; Proteintech), IL-6 (A0286, 1:1000; ABclonal), Nrf2 (A1244, 1:1000; ABclonal), HO-1 (ab13243, 1:1000; Abcam) and NAD(P)H quinone oxidoreductase 1 (NQO1; bs-2184R, 1:1000; Bioss, Woburn, USA).

Statistical analysis

Data are presented as the mean ± SEM. For comparisons involving a single independent variable, one-way ANOVA followed by Tukey's *post hoc* test was performed. For analyses involving multifactorial comparisons, two-way ANOVA with Tukey's *post hoc* test was used to assess interactions and main effects. Statistical comparisons were performed via SPSS statistics 22. A *P*-value <0.05 was considered to indicate statistical significance.

Results

Effect of LEVO on cardiac function in SIC mice

To evaluate the effects of LEVO on cardiac function, an SIC animal model was established via CLP in this study. Our results revealed that the serum levels of cTnI and IL-6 were significantly elevated starting at 12 h post-CLP, peaking at 72 h for cTnI (476 pg/mL) or 24 h for IL-6 (180 pg/mL) (Figure 1A,B). Additionally, the survival rate significantly decreased following the CLP operation (Figure 1C). Compared with sham mice, SIC mice presented significant increases in LVIDd (0.30-fold), LVIDs (0.51-fold), LVVd (0.22-fold), and LVVs (1.28-fold) (Figure 1D–H). In contrast, the LVEF and FS were significantly reduced by 51.95% and 58.13%, respectively, following the CLP procedure (Figure 1D,I,J). These findings indicate the successful establishment of the SIC animal model through CLP. Interestingly, LEVO treatment significantly attenuated SIC-induced increases in the serum cTnI and IL-6 levels and significantly increased the survival rate of SIC mice. Moreover, echocardiographic assessment further revealed that LEVO administration led to significant reductions in LVIDd, LVIDs, LVVd and LVVs in SIC mice (Figure 1D–H). However, LVEF and FS were significantly elevated following LEVO treatment (Figure 1D,I,J). These findings demonstrated that LEVO administration restored myocardial

geometry and contractile function in CLP mice.

Effect of LEVO on mitochondrial function in cardiomyocytes from SIC mice

To further investigate the effects of LEVO on mitochondria, cardiac tissues were harvested, and mitochondrial morphology and function were assessed following LEVO treatment. ATP production and MMP are key indicators of mitochondrial health and activity [27]. Our data revealed that ATP level and MMP were significantly lower in SIC mice than in Sham mice, and these effects were reversed by LEVO treatment (Figure 2A,B). Moreover, damaged mitochondria, characterized by vacuolization and reduced and disrupted mitochondrial cristae, were evident in cardiac tissues from SIC mice, which were notably improved following LEVO administration (Figure 2C). Additionally, SIC led to a significant increase in Drp1 (Figure 2D,E) and a significant decrease in several mitochondria-associated proteins, including Mfn2 (Figure 2D,F), Parkin (Figure 2D,G), Pink1 (Figure 2D,H) and PGC-1α (Figure 2D,I), in cardiac tissues. These findings indicate that sepsis induces an imbalance of mitochondrial fusion and fission, reduces mitochondrial biogenesis and inhibits mitophagy. Notably, these alterations were reversed by LEVO treatment. Our results suggest that LEVO treatment improves mitochondrial quality by regulating mitochondrial fusion and fission, activating mitochondrial autophagy and promoting mitochondrial biogenesis (Figure 2D–I). These findings are consistent with a recent report that LEVO can improve mitochondrial performance and exert a protective effect on cardiomyocytes [28].

Nrf2 is essential for the protective effects of LEVO against LPS-induced cardiomyocyte injury

To further explore the protective effects of LEVO on cardiomyocytes, HL-1 cells were incubated with LPS to establish an *in vitro* model of SIC. As shown in Figure 3A,B, the viability of HL-1 cells markedly decreased upon exposure to 10 µg/mL LPS for 48 h. Furthermore, the increase in IL-6 protein and ROS production in HL-1 cells subjected to 10 µg/mL LPS for 24 and 48 h further confirmed the validity of the concentrations and incubation periods of LPS employed to develop the SIC model *in vitro* (Supplementary Figure S1A,B). Additionally, the decrease in proliferative capacity induced by LPS was reversed when HL-1 cells were treated with 100 µM LEVO for 24 h (Figure 3C). Therefore, HL-1 cells subjected to 10 µg/mL LPS for 48 h and 100 µM LEVO for 24 h were utilized in the subsequent experiments. Recent evidence has highlighted a crucial role of Nrf2 in the progression of oxidative stress-associated cardiac remodeling, heart failure and sepsis [29–31]. In this study, SIC resulted in a significant decrease in Nrf2 protein expression in the myocardium, whereas this response was reversed by LEVO treatment (Supplementary Figure S1C). To investigate the role of Nrf2 in cardiac function and its potential regulatory relationship with LEVO, LPS-injured HL-1 cells were treated with LEVO and/or the Nrf2 inhibitor ML-385. Consistent with these findings *in vivo*, the LPS-induced reduction in Nrf2 was reversed by LEVO treatment (Supplementary Figure S1D). These results indicate that Nrf2 plays a critical role in SIC and may be involved in LEVO-mediated protection of the myocardium.

Subsequently, we observed that the proliferative capacity of HL-1 cells was significantly increased following LEVO treatment, while it was markedly reduced upon cotreatment with the Nrf2 inhibitor

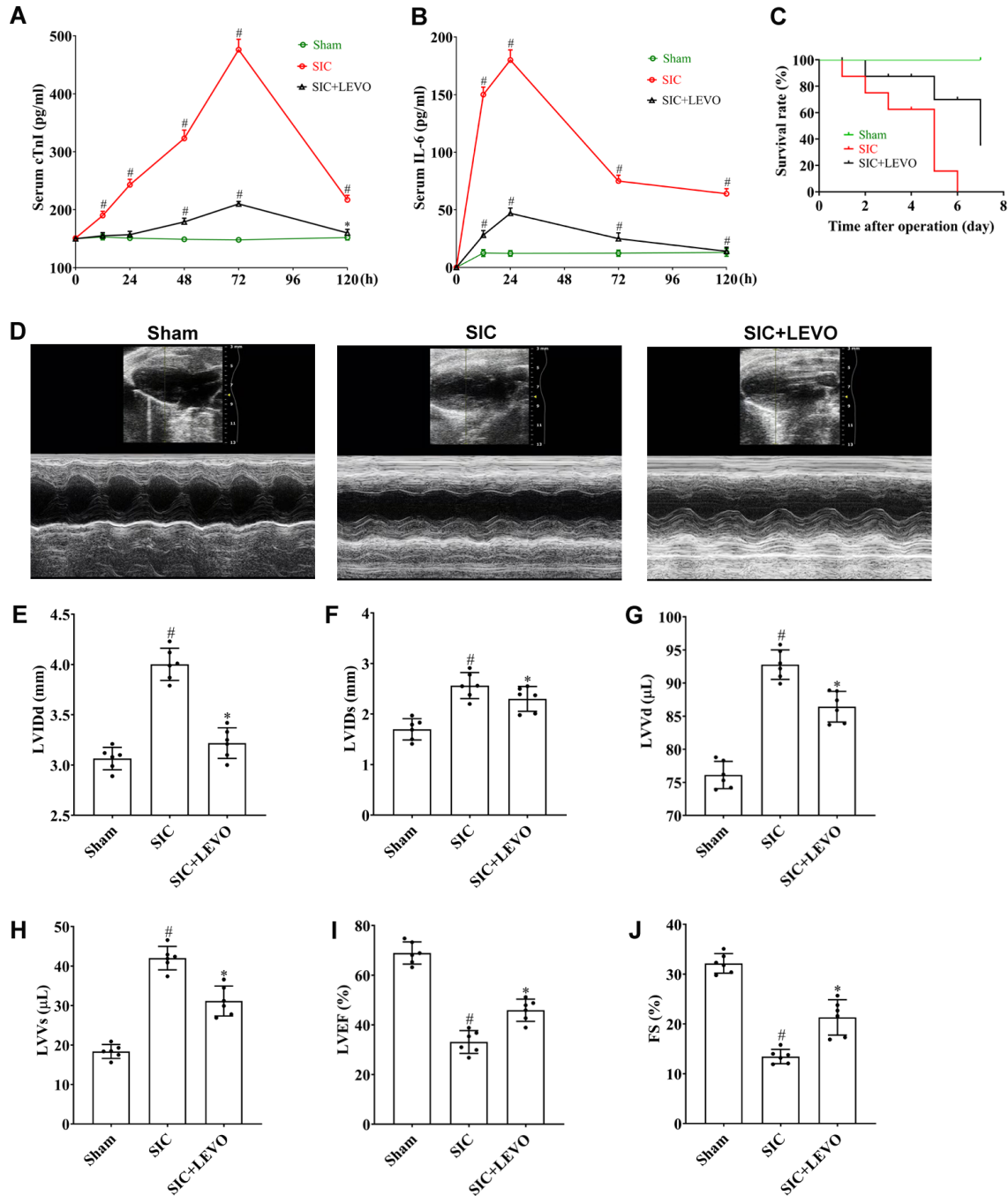


Figure 1. Effects of LEVO on cardiac function in SIC mice (A) Serum cTnI and (B) IL-6 levels were measured at 0, 12, 24, 48, 72, and 120 h post-CLP ($n = 6$). (C) Survival rates were monitored at 0, 2, 4, 6, and 8 days post-CLP ($n = 10$). (D) Cardiac function was evaluated by echocardiography following LEVO administration ($n = 6$). Echocardiographic parameters, including (E) LVIDd, (F) LVIDs, (G) LVVd, (H) LVVs, (I) LVEF, and (J) FS, were measured after LEVO administration ($n = 10$). The data are presented as the mean $\pm$ SEM. * $P < 0.05$; [#] $P < 0.01$.

ML-385 administration (Figure 3D). In addition, our data revealed that LEVO treatment led to a significant decrease in ROS production induced by LPS in HL-1 cells, whereas this trend was reversed after ML-385 administration (Figure 3E). As expected, we found that the levels of mitochondrial ATP, MMP, and NADH in HL-1 cells were significantly increased following LEVO treatment compared with those in control cells, whereas they were significantly decreased following Nrf2 inhibition (Figure 3F–H). These findings suggest that

LEVO partially reversed the LPS-induced reduction in ATP, MMP, and NADH in mitochondria, whereas the beneficial effects of LEVO were weakened following Nrf2 inhibition. Similar to ROS production, calcium ions were also significantly decreased after LEVO treatment and were significantly increased following Nrf2 inhibition in HL-1 cells (Figure 3I,J). These findings demonstrate that LEVO ameliorates cardiomyocyte injury and mitochondrial dysfunction induced by LPS in an Nrf2-dependent manner.

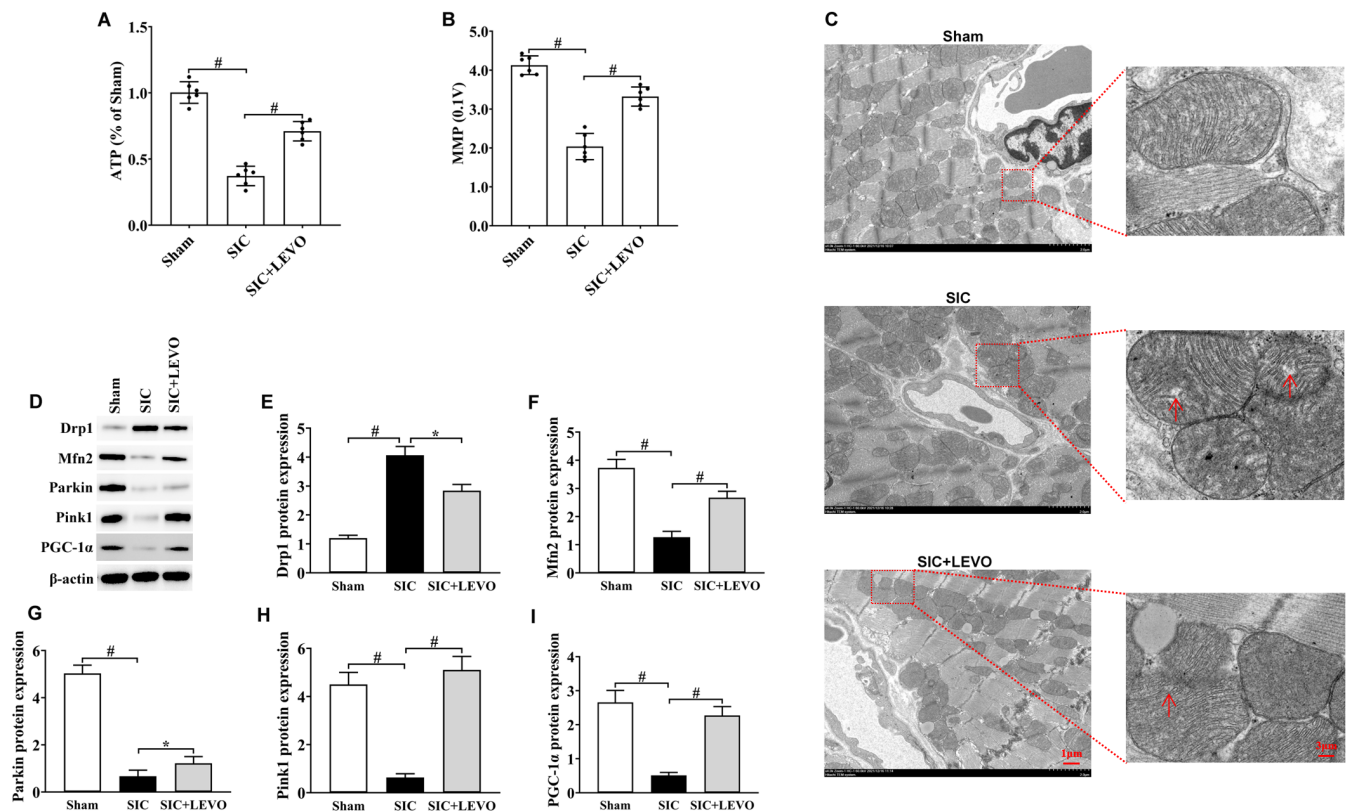


Figure 2. Effect of LEVO on mitochondrial function in cardiomyocytes from SIC mice (A) The levels of mitochondrial ATP and (B) MMP in cardiac tissue were examined after LEVO administration. (C) Representative images of the mitochondrial morphology of cardiomyocytes in mice from the Sham, SIC and SIC + LEVO groups (scale bar = 1 μ m), including high-magnification images of the areas indicated by red rectangles (scale bar = 3 μ m). The arrows indicate damaged mitochondria. (D) Blotting and quantitative analysis of mitochondrial-related proteins, including (E) Drp1, (F) Mfn2, (G) Parkin, (H) Pink1 and (I) PGC-1 α , in cardiac tissue after LEVO treatment. β -actin was used as the internal control. The results are presented as the mean $\pm$ SEM, $n = 6$. * $P < 0.05$; # $P < 0.01$.

Nrf2 protein is required for the protective effects of LEVO on mitochondrial function in LPS-injured cardiomyocytes

We further detected mitochondrial-related proteins in HL-1 cells challenged with LPS in the presence or absence of LEVO and/or Nrf2 inhibition. It was found that in LPS-challenged cells, the expression of the mitochondrial fission protein Drp1 was decreased following LEVO treatment (Figure 4A,B), whereas the expression of the mitochondrial fusion protein Mfn2 was concomitantly increased (Figure 4A,C). Additionally, the expression levels of the autophagy-related proteins Parkin (Figure 4A,D) and Pink1 (Figure 4A,E) and the biogenesis-related protein PGC-1 α (Figure 4A,F) were also upregulated following LEVO treatment. We further detected the expression of Nrf2 downstream targets, including HO-1 and NQO1, in the LPS-injured HL-1 cells. LEVO treatment led to a significant increase in HO-1 and NQO1 expressions in the LPS-injured HL-1 cells (Figure 4G–I), suggesting that LEVO could activate antioxidant defense such as HO-1 and NQO1. In addition, mtROS levels were also measured after LEVO treatment. Our data revealed that LEVO treatment led to a significant decrease in mtROS production in LPS-injured HL-1 cells (Figure 4J,K). However, these trends were reversed by the Nrf2 inhibitor ML-385, suggesting that the beneficial effects of LEVO are nullified following Nrf2 inhibition (Figure 4), thus confirming the obligatory role of Nrf2 in LEVO-related benefits in mitochondrial homeostasis by enhancing

mitochondrial biogenesis, reducing mitochondrial fission and activating mitophagy.

LEVO ameliorates cardiomyocyte injury and mitochondrial dysfunction in an Nrf2-dependent manner

To further explore the effects of Nrf2 on SIC and the precise molecular mechanism involving LEVO and Nrf2 in the process of SIC treatment, an SIC model was established in *Nrf2*-KO mice. Echocardiography revealed that knockout of *Nrf2* aggravated myocardial dysfunction in SIC mice and diminished the beneficial effects of LEVO on cardiac function (Figure 5A and Supplementary Figure S2A–F). Similarly, as shown in Figure 5B (high magnification) and Supplementary Figure S2G (low magnification), *Nrf2* knockout further damaged the mitochondrial structure of cardiomyocytes in SIC mice and reversed the protective effects of LEVO on mitochondrial morphology. In addition, compared with that of the WT (SIC) group, the protein expression of Drp1 was significantly increased in the cardiac tissue of *Nrf2*-KO mice, with concomitant decreases in the Mfn2, Parkin and Pink1 proteins (Figure 5C–G), demonstrating that *Nrf2* knockout disrupted mitochondrial fusion, fission and mitophagy. In WT mice, LEVO treatment led to a reduction in the Drp1 protein in cardiac tissue, accompanied by an increase in Mfn2, Parkin, Pink1 and PGC-1 α proteins (Figure 5C–H). However, these responses to LEVO were no longer observed in *Nrf2*-KO mice (Figure 5C–H). These findings reveal a vital role for

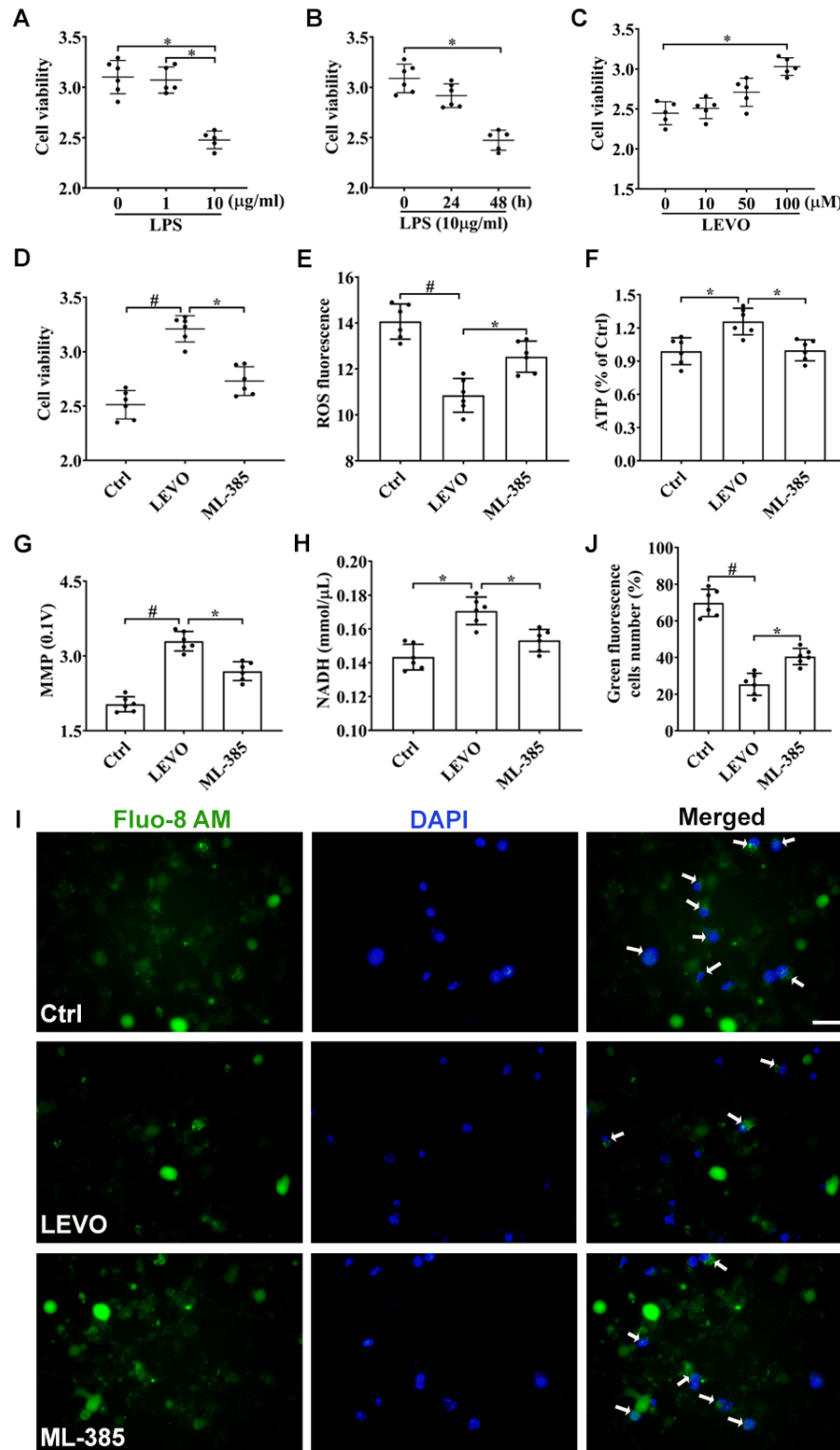


Figure 3. Nrf2 is essential for the protective effects of LEVO against LPS-induced cardiomyocyte injury (A) HL-1 cells were incubated with 0, 1 or 10 µg/mL LPS, and their proliferative capacity was evaluated via CCK-8 assay. (B) HL-1 cells were incubated with 10 µg/mL LPS for 0, 24 or 48 h, and their proliferative capacity was evaluated via CCK-8 assay. (C) The proliferative capacity of the LPS-treated HL-1 cells was evaluated via CCK-8 assay after treatment with 0, 10, 50 or 100 µM LEVO. (D) The proliferative capacity of HL-1 cells was evaluated via CCK-8 assay after LEVO treatment, with or without the Nrf2 inhibitor ML-385. (E) ROS production was detected in LPS-treated HL-1 cells following LEVO treatment, with or without ML-385. (F) Mitochondrial ATP levels and (G) MMP and (H) NADH levels in LPS-treated HL-1 cells were examined after LEVO treatment, with or without ML-385. (I) Representative images and (J) quantitative analysis (above the fluorescence images) of green fluorescent cells incubated with or without LEVO or ML-385 in LPS-treated HL-1 cells. Scale bar = 50 µm. The results are presented as the mean ± SEM, $n = 6$. * $P < 0.05$; # $P < 0.01$.

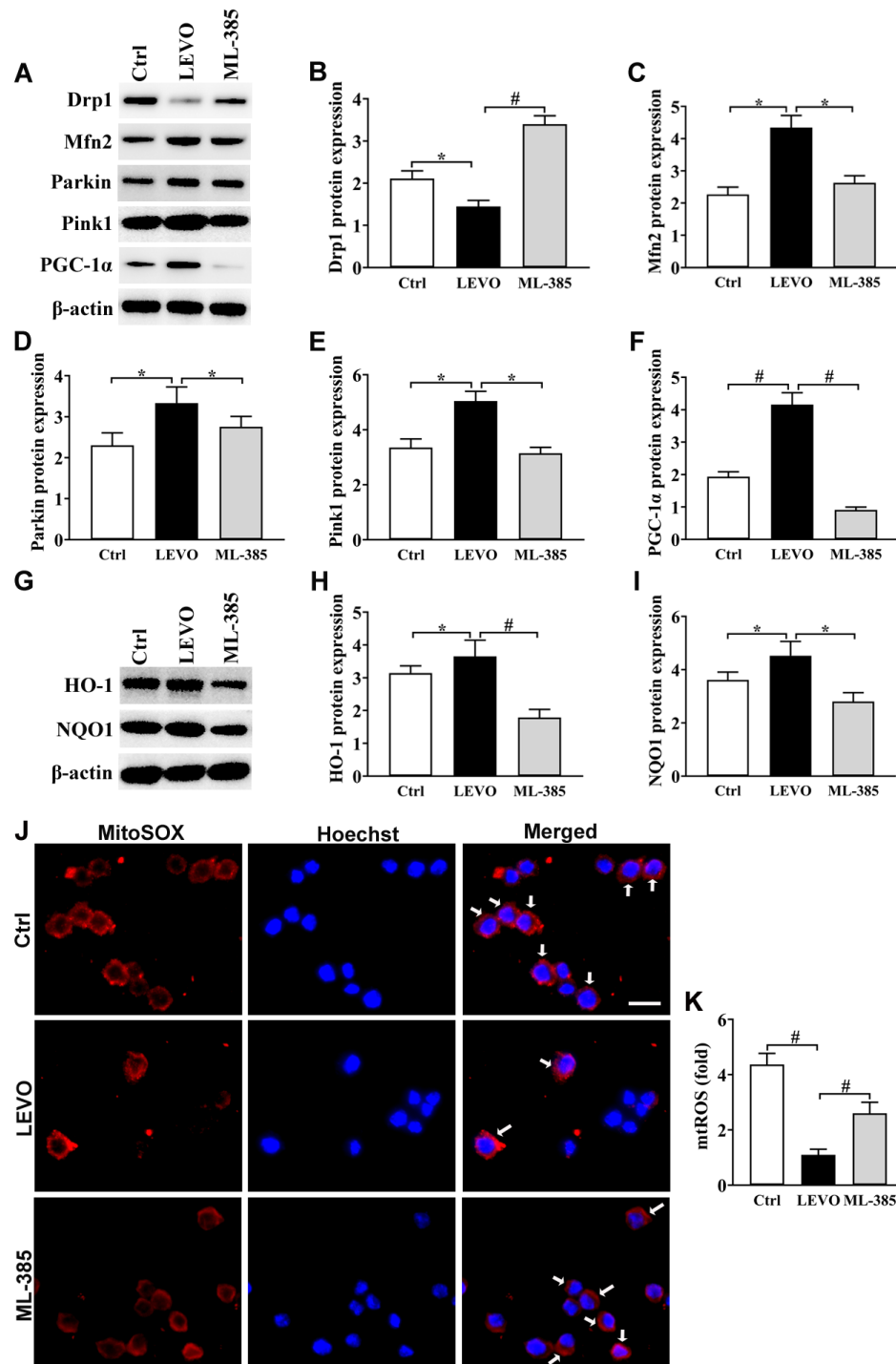


Figure 4. Nrf2 protein is required for the protective effects of LEVO on mitochondrial function in LPS-injured HL-1 cells (A) Western blot analysis and quantification of (B) Drp1, (C) Mfn2, (D) Parkin, (E) Pink1 and (F) PGC-1α proteins in the LPS-injured HL-1 cells following LEVO treatment, with or without ML-385. (G) Western blot analysis and quantification of (H) HO-1 and (I) NQO1 protein expression in the LPS-injured HL-1 cells following LEVO treatment, with or without ML-385. β-actin was used as an internal loading control. (J) Representative images and (K) quantitative analysis of mtROS in LPS-injured HL-1 cells incubated with or without LEVO or ML-385. Scale bar = 50 μm. The data are presented as the mean ± SEM, $n = 6$. * $P < 0.05$; # $P < 0.01$.

Nrf2 in maintaining myocardial and mitochondrial function, demonstrating that LEVO attenuates cardiomyocyte injury and mitochondrial dysfunction via an Nrf2-dependent mechanism in SIC mice.

Discussion

CLP in rodents has become the most widely utilized and validated model for experimental sepsis and is currently regarded as the gold standard in sepsis research [32]. cTnI, a regulatory protein

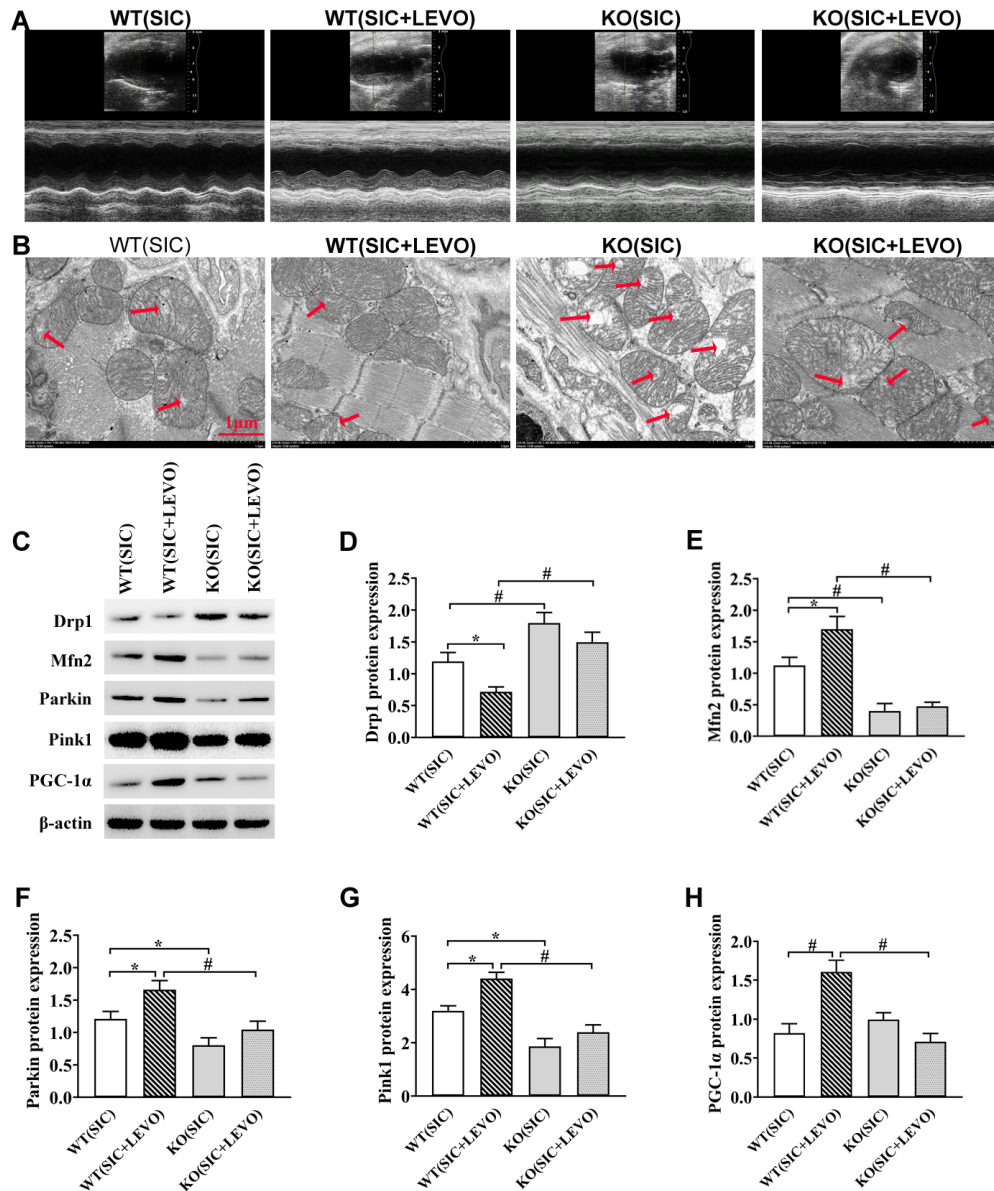


Figure 5. LEVO ameliorates cardiomyocyte injury and mitochondrial dysfunction in an Nrf2-dependent manner (A) Cardiac function was assessed by echocardiography after LEVO administration in WT and *Nrf2*-KO mice ($n = 6$). (B) Mitochondrial morphology (high magnification) of cardiomyocytes after LEVO administration in WT and *Nrf2*-KO mice ($n = 6$). The arrows indicate damaged mitochondria. Scale bar = 1 μ m. (C) Blotting and quantitative analysis of (D) Drp1, (E) Mfn2, (F) Parkin, (G) Pink1 and (H) PGC-1 α proteins in cardiac tissue from the WT (SIC), WT (SIC + LEVO), KO (SIC) and KO (SIC + LEVO) groups. β -actin was used as the internal housekeeping control. The results are presented as the mean $\pm$ SEM, $n = 6$ (C–H). * $P < 0.05$; # $P < 0.01$.

involved in cardiac muscle contraction, serves as a highly specific biochemical marker for myocardial injury and is widely used for risk stratification in clinical settings [33]. In the present study, the serum levels of cTnI and IL-6 were significantly elevated at 12 h post-CLP and peaked at 72 h and 24 h, respectively. In addition, the survival rate of the mice was markedly reduced following the CLP operation, confirming the successful establishment of the SIC model.

One of the intriguing findings from our study is that LEVO rescues against SIC, as evidenced by improved cardiac morphology and function in CLP-challenged mice. Preclinical and clinical studies have identified three interrelated pharmacological effects of LEVO:

vasodilation, inotropy, and cardioprotection. Unlike other inotropic agents, LEVO enhances myocardial contractility without increasing intracellular calcium levels or myocardial oxygen consumption [34]. Its vasodilatory properties reduce both preload and afterload, thereby improving the myocardial oxygen supply [35]. LEVO promotes energy-efficient myocardial function and exerts antiapoptotic effects, contributing to its cardioprotective role [36]. Multiple animal models and small-scale clinical trials have revealed that LEVO improves systemic hemodynamics, increases survival, and ameliorates SIC [37], positioning it as a promising therapeutic agent for sepsis-induced myocardial injury. In this study, LEVO administration significantly reduced serum cTnI and IL-6 levels in

SIC mice and improved survival rates. Echocardiographic assessments further demonstrated enhanced myocardial contractility and improved cardiac function in SIC mice following LEVO treatment, suggesting a pivotal role of LEVO in ameliorating SIC. Despite its therapeutic potential, LEVO has certain limitations. Its elimination half-life is approximately one hour, leading to rapid clearance from circulation upon cessation of infusion [38]. Moreover, common adverse events such as headaches and hypotension are frequently observed, likely due to their vasodilatory effects [38]. LEVO may be beneficial in preconditioning scenarios, such as perioperative cardiac surgery, where myocardial protection is critical. However, its utility in broader clinical contexts warrants further investigation. Importantly, LEVO is metabolized to OR-1896, an active metabolite that shares similar positive inotropic and vasodilatory effects [38,39]. Unlike LEVO, OR-1896 has a prolonged half-life of approximately 80 h and remains detectable in circulation for up to 2 weeks after treatment cessation [38], offering more sustained hemodynamic benefits and necessitating further studies to characterize its long-term pharmacological profile fully. In our study, we also observed significant mitochondrial dysfunction in SIC mice, characterized by reduced ATP production and MMP, as well as ultrastructural abnormalities, including mitochondrial vacuolization and disrupted cristae. ATP production and the MMP are key indicators of mitochondrial health and activity [27], and mitochondrial dysfunction is characterized by low ATP, abnormal MMP values and high ROS levels [40]. Additionally, damaged mitochondria, including vacuolization and reduced and disrupted mitochondrial cristae, were noted in cardiac tissues from SIC mice. Moreover, sepsis induced an imbalance in mitochondrial fusion and fission and suppressed mitochondrial biogenesis and mitophagy. These mitochondrial defects likely contribute to the pathogenesis of mitochondrial injury in SIC. Previous studies have linked mitochondrial dysfunction to a range of cardiac pathologies, including elevated oxidative stress, disrupted Ca^{2+} homeostasis, and arrhythmogenesis, particularly atrial fibrillation [41,42]. Therefore, improving the mitochondrial function of cardiomyocytes represents a potential therapeutic strategy for treating SIC. In this study, LEVO treatment restored mitochondrial ATP and MMP levels in CLP model murine cardiomyocytes. Moreover, LEVO treatment improved mitochondrial quality by enhancing mitochondrial biogenesis, regulating mitochondrial fusion and fission and activating mitochondrial autophagy, thereby ameliorating cardiac dysfunction and providing novel insights into sepsis treatment. Our findings are consistent with recent reports demonstrating LEVO's capacity to improve mitochondrial performance and protect cardiomyocytes from injury [28].

To further explore the molecular mechanisms underlying the protective effects of LEVO on SIC, HL-1 cells were incubated with LPS to mimic SIC *in vitro*. Previous studies have demonstrated that LPS induces cardiomyocyte injury *in vitro* [43,44], which is consistent with our findings. In this study, LPS exposure led to a marked reduction in cell proliferation, increased IL-6 protein levels, and elevated ROS in HL-1 cells, confirming successful model induction. Emerging evidence suggests that Nrf2 plays an important role in the progression of sepsis. A study by Lei and colleagues revealed that the overexpression of ubiquitin-conjugating enzyme 9 (Ubc9), which is essential for Nrf2 ubiquitination, counteracted Nrf2 inhibition or directly activated Nrf2 by dihydroquercetin (DHQ), a flavonoid compound with antioxidant properties, thereby

enhancing anti-inflammatory responses and improving survival in SIC mice [29]. Additional studies have shown that Nrf2 confers protection against murine endotoxemia by regulating downstream genes such as *HO-1* [30,31].

In our study, Nrf2 protein expression was significantly reduced in the myocardium of SIC mice, whereas LEVO treatment reversed this downregulation, suggesting that Nrf2 may mediate LEVO's protective effect. To further explore this hypothesis, HL-1 cells were treated with LPS in the presence or absence of LEVO and/or the Nrf2 inhibitor ML-385. Consistent with the *in vivo* observations, LEVO restored Nrf2 expression levels suppressed by LPS. Similar to Nrf2 protein, LEVO treatment also led to a significant increase in HO-1 and NQO1 expression in LPS-challenged HL-1 cells, suggesting that Nrf2 executes its protection through activating downstream antioxidant defense mechanisms, such as HO-1 and NQO1. Previous studies have suggested that HO-1 promotes the production of endogenous carbon monoxide (CO), which subsequently stimulates mitochondrial H_2O_2 generation and activates the Akt-GSK3 β -Nrf2 pathway in the heart [45]. In this study, LEVO treatment led to a significant increase in HO-1 expression in LPS-injured HL-1 cells, indicating that HO-1 may mediate LEVO-induced Nrf2 upregulation. Subsequently, our data showed that LEVO treatment significantly improved cell viability, decreased ROS and calcium overload in the realm of LPS exposure, while Nrf2 inhibitor diminished these beneficial effects of LEVO in HL-1 cells. The LPS-triggered increase in ROS accumulation is primarily mediated by NADPH oxidase in macrophages [46].

Calcium overload can lead to defective oxidative phosphorylation and a significant decrease in the mitochondrial MMP and ATP production; therefore, calcium overload may serve as the main cause of mitochondrial damage [47]. The redox states of NADH and NADPH modulate various biological processes, including energy metabolism, mitochondrial functions, antioxidation/generation of oxidative stress and cell death; moreover, NADH serves as a marker for the mitochondrial respiratory chain, and its content is an optimal parameter for evaluating mitochondrial function [48]. Therefore, we subsequently assessed the levels of ATP, MMP, and NADH in mitochondria to evaluate mitochondrial function. Our results showed that LEVO partially reversed the decreases in ATP, the membrane potential and NADH induced by LPS in mitochondria, and the beneficial effects of LEVO were weakened after Nrf2 inhibition. These findings demonstrate that LEVO ameliorates cardiomyocyte injury and mitochondrial dysfunction evoked by LPS in an Nrf2-dependent manner. This may explain why the Nrf2 protein is required for LEVO-mediated reductions in ROS and calcium overload in cardiomyocytes. While the antioxidative function of Nrf2 partially explains these outcomes, our data suggest that Nrf2 also modulates mitochondrial quality control. Under conditions of reduced ATP production, mitochondrial biogenesis is often activated as a compensatory mechanism to maintain mitochondrial homeostasis [49]. We further assessed mitochondria-related proteins in LPS-injured HL-1 cells with or without LEVO or Nrf2 inhibition, confirming that LEVO maintained mitochondrial homeostasis by increasing mitochondrial biogenesis, reducing mitochondrial fission and activating mitophagy in an Nrf2-dependent manner. Notably, however, the LEVO concentration used in our *in vitro* experiment was significantly higher than the clinically administered doses. This discrepancy may be attributed to the fact that clinical treatment involves continuous infusion (days to

weeks), whereas *in vitro* experiments are usually conducted over 24–72 h, necessitating higher drug concentrations to achieve comparable biological effects. Furthermore, *in vivo* factors such as hepatic metabolism (e.g., conversion to the active metabolite OR-1896) and renal clearance may also contribute to sustained pharmacological activity, processes that are absent in cell culture systems. Thus, high-dose bolus administration *in vitro* may be required to replicate *in vivo* pharmacodynamics.

To further explore the effects of Nrf2 on SIC and clarify the molecular mechanism between LEVO and Nrf2 in the context of SIC, we used *Nrf2*-KO mice to establish an SIC model, followed by the administration of LEVO. Our echocardiography results demonstrated that knockout of *Nrf2* aggravated myocardial dysfunction in SIC mice, which was accompanied by further damage to the mitochondrial structure of cardiomyocytes in SIC mice. We subsequently investigated the effects of *Nrf2* KO on mitochondria-related proteins in cardiomyocytes, and the results revealed that *Nrf2* KO led to imbalanced mitochondrial fusion and fission and impaired mitochondrial biogenesis and mitophagy. These results are consistent with those of a study reported by Yao and colleagues, which demonstrated that activation of Nrf2 can attenuate mitochondrial apoptosis and oxidative stress in myocardial cells, thereby playing a crucial role in the treatment of myocardial ischemia–reperfusion injury [50]. Consistent with the *in vitro* results, we found that Nrf2 deficiency also limited the beneficial effects of LEVO on myocardial and mitochondrial function. These findings support the hypothesis that LEVO ameliorates cardiomyocyte injury and mitochondrial dysfunction in an Nrf2-dependent manner *in vivo*. Currently, there are few reports on the regulatory relationship between LEVO and Nrf2, which have been documented in only the brain and kidney but not the heart [51,52]. LEVO enhanced the renoprotective effects of ischemic preconditioning against acute renal injury via the upregulation of the expression of the antioxidant genes *Nrf2*, *HO-1*, and *NOQ-1* in an ischemia/reperfusion rat model [51]. LEVO attenuated LPS-induced inflammation in microglia by inhibiting the NF- κ B pathway and Nod-like receptor protein 3 (NLRP3) inflammasome activation via Nrf2/HO-1 signaling [52]. In accordance with these previous findings, our results showed that LEVO not only upregulated Nrf2 expression but also led to a concomitant increase in the levels of HO-1 and NQO1 in cardiomyocytes. Thus, findings from our laboratory and others have revealed the beneficial effects of Nrf2-mediated LEVO, even in different experimental models. While the present study demonstrated an essential role of Nrf2 through genetic knockout models, future studies employing Nrf2 overexpression (especially in KO backgrounds) or cardiac-specific *Nrf2* knockout should be valuable to validate its sufficiency and downstream signaling hierarchy in LEVO effects.

In conclusion, we successfully established myocardial injury models in mice and HL-1 cells through CLP and LPS stimulation, respectively. Our findings demonstrate that LEVO alleviates myocardial injury in SIC mice and LPS-induced cardiomyocyte damage by enhancing the MMP and ATP production, preserving the mitochondrial ultrastructure, promoting mitochondrial biogenesis, suppressing excessive mitochondrial fission, and activating mitophagy. Notably, genetic deletion or pharmacological inhibition of Nrf2 exacerbated cardiomyocyte injury and attenuated the protective effects of LEVO both *in vivo* and *in vitro*, underscoring the essential role of Nrf2 in mediating LEVO-induced cardioprotection.

Supplementary Data

Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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