



Levosimendan protects from sepsis-inducing cardiac dysfunction by suppressing inflammation, oxidative stress and regulating cardiac mitophagy via the PINK-1-Parkin pathway in mice

Jian Shi¹, Yuhong Chen^{1,2}, Haijun Zhi³, Hui An⁴, Zhenjie Hu^{1,2}

¹Department of Intensive Care Unit, Hebei Medical University Fourth Affiliated Hospital and Hebei Provincial Tumor Hospital, Shijiazhuang, China; ²Hebei Key Laboratory of Critical Disease Mechanism and Intervention, Shijiazhuang, China; ³Emergency Department, Cangzhou Central Hospital, Cangzhou, China; ⁴Department of Intensive Care Unit, Baoding First Central Hospital, Baoding, China

Contributions: (I) Conception and design: Z Hu, Y Chen, J Shi; (II) Administrative support: Z Hu; (III) Provision of study materials or patients: Z Hu, Y Chen, J Shi, H Zhi; (IV) Collection and assembly of data: J Shi, H Zhi; (V) Data analysis and interpretation: J Shi; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Zhenjie Hu. Department of Intensive Care Unit, Hebei Medical University Fourth Affiliated Hospital and Hebei Provincial Tumor Hospital, 12 Jiankang Road, Shijiazhuang 050011, China. Email: syicuhzj@163.com.

Background: Sepsis is a leading cause of death in China, the mortality rate of which is elevated when cardiac dysfunction is induced. Levosimendan is used for the treatment of decompensated cardiac failure. In this study, we sought to investigate the role of levosimendan in the inflammation, oxidative stress, and mitophagic response of the septic heart.

Methods: A lipopolysaccharide (LPS)-induced septic myocardial dysfunction mouse model was established. To study the relationship between levosimendan and inflammation, oxidative stress, and mitophagy response, mice were pretreated with mdivi-1 (an inhibitor of mitophagy) prior to LPS administration. Levosimendan was given (24 µg/kg) via intraperitoneal injection 3 h after LPS had been administered. At 6 h after LPS injection, echocardiographic analysis, enzyme-linked immunosorbent assay (ELISA), oxidative stress index, myocardial pathological changes, transmission electron microscopy (TEM), immunofluorescence, and western blot were used to investigate the protective effects of levosimendan against LPS-induced myocardial dysfunction.

Results: In the sepsis model, levosimendan markedly ameliorated myocardial dysfunction, decreased the release of myocardial enzymes and inflammatory cytokines, improved oxidative stress index and myocardial pathological changes, reduced mitochondrial division, and activated mitophagy. To confirm whether the protection of levosimendan was mediated by mitophagy, a mitophagy inhibitor-mdivi-1 was used in this study. It significantly impaired the protective effects of levosimendan. In addition, our studies further confirmed the protection of levosimendan against LPS-induced myocardial injury and the mechanisms involving PINK-1-Parkin mediated mitophagy signaling.

Conclusions: Levosimendan was able to rescue the LPS-induced cardiac dysfunction mice, supporting its mechanism of action by suppressing inflammation, oxidative stress, and directly targeting the PINK-1-Parkin pathway.

Keywords: Levosimendan; lipopolysaccharide (LPS); septic cardiac dysfunction; mitochondrial dynamics; mitophagy

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Introduction

Sepsis is a leading cause of death in China, the mortality rate of which is elevated when cardiac dysfunction is induced. About 46% of patients with sepsis have myocardial injury or cardiac dysfunction (1); however, the mechanism of septic cardiac dysfunction is not completely understood. To date, mitochondrial dysfunction has been confirmed as playing a pivotal role in sepsis-induced cardiac dysfunction (2). *In vitro* and *in vivo* studies have indicated that mitochondria structure and function is regulated by sepsis in myocardial volume (3,4). The quality and quantity control of mitochondria is mainly the balance of mitochondrial dynamic function, that is, fission, fusion, mitophagy, and mitochondrial biological function (5).

Autophagy appears to be a double-edged sword, as under different degrees of stimulation the regulation of cell survival is inconsistent (6). Under normal physiological responses or mild stress, autophagy is appropriate and could promote survival. The process during which dysfunctional mitochondria is segregated and eliminated through autophagy is known as mitophagy (7). However, under severe stress, autophagy is inappropriate, resulting in massive self-degradation or accumulation of toxic substances; neither of these outcomes is adaptive. Evidence from a mouse LPS model showed that enhanced autophagy in severe sepsis protects the myocardium (8). The Pink-1/Parkin pathway induced by the phosphatase and PTEN, is the most representative signaling pathway in mitophagy. It was reported that inhibition of PINK1/Parkin pathway protein expression can reduce the level of mitophagy, and aggravate mitochondrial injury, whereas activation of PINK1/Parkin pathway can play a protective effect on heart (9).

Levosimendan is used for the treatment of decompensated cardiac failure as a calcium sensitizer and mitochondrial potassium-adenosine triphosphate (ATP) channel opening agent (10,11). But the application of levosimendan in sepsis-inducing cardiac dysfunction is still controversial. Some small clinical studies have confirmed Septic patients with myocardial dysfunction may partly benefit from levosimendan than dobutamine (12). Over the recent past, it has become evident that decompensated cardiac failure is associated with subclinical inflammation oxidative excess and inappropriate mitochondrial quality control (13,14). Some animal studies have showed that besides levosimendan's positive inotropic effect, it has pleiotropic actions including antioxidant, anti-inflammatory,

and anti-apoptosis effects (15-17). Grossini *et al.* indicated that levosimendan participates in autophagy during regional myocardial ischemia in pigs (10). Given that anti-inflammatory, antioxidant, and anti-apoptosis properties are involved in cardioprotection, we sought to determine the role of levosimendan in the mitophagic response of the septic heart, so as to determine whether levosimendan can improve cardiac function in sepsis-inducing cardiac dysfunction through other ways. Establishment of a mouse sepsis myocardial injury model via LPS intraperitoneal injection is simple, economical, and similar to the clinical reality. It is an important method to study sepsis myocardial injury. We evaluated (I) the effect of levosimendan in sepsis-induced cardiac dysfunction; (II) the underlying molecular mechanisms; and (III) we sought to clarify the role of levosimendan in quality control of cardiac mitochondria through mitophagy. We present the following article in accordance with the ARRIVE reporting checklist (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-483/rc>).

Methods

Animals and treatment

Experiments were performed using male healthy C57BL/6J mice aged 8–10 weeks and weighing 21 ± 3.5 g (Vital River Laboratories, Beijing, China). Mice were maintained under standard pathogen-free conditions at about 22 °C with a 12-h light-dark cycle with free access to a standard rodent diet and water. Experiments were performed under a project license (No. 2020002) granted by the Laboratory Animal Ethical Committee Fourth Hospital Hebei Medical University, in compliance with the National Institutes of Health (USA) Guidelines for the care and use of animals (NIH Publication No. 85-23, revised 1985: <http://grants1.nih.gov/grants/olaw/references/phspol.htm>). All experiments were performed in the Animal Experiment Center of the Fourth Hospital of Hebei Medical University.

The septic myocardial dysfunction model was established by intraperitoneal injection of LPS (10 mg/kg; Solarbio, Beijing, China) (18). The study comprised two parts. In the first part, mice were randomly assigned into control group (n=6), levosimendan (Levo) group (n=6), LPS group (n=6), and LPS + levo group (n=6) according to the random number method. Mice allocated to the LPS group and LPS + levo group underwent echocardiography (echo) at 3 h after LPS injection. If myocardial dysfunction was indicated

by echo, they entered the experimental process; if not, they were euthanized by cervical dislocation. Levosimendan (Qilu Pharmaceutical Co., Ltd, Shandong, China) was injected via an intraperitoneal injection at 24 µg/kg (dissolved in Glu 0.5%) according to previous research and preliminary experiment in 3 h after the LPS intervention (19). In the second part, mice were randomly assigned into a control group (n=6), LPS group (n=6), LPS + levosimendan group (n=6), and LPS + levo + Mdivi-1 group (n=6). Mice assigned into the LPS group, LPS + levo, and LPS + levo+Mdivi-1 group underwent echo at 3 h after LPS. If myocardial dysfunction was indicated by echo, they entered the experimental process; if not, they were euthanized by cervical dislocation. As an activity inhibitor of mitophagy, Mdivi-1 (dissolved in dimethyl sulfoxide (DMSO), 338967-87-6, MCE, Monmouth Junction, NJ, USA) was administrated 1 h prior to the LPS injection at a dose of 25 mg/kg intraperitoneally (20). Mice were euthanized by cervical dislocation after echocardiographic experiments, and the blood and myocardial tissues were collected. Blood samples were immediately centrifuged (3,000 rpm, 15 min) at 4 °C to isolate serum. The serum was then stored at -80 °C until used. The myocardial tissues were washed in phosphate-buffered saline (PBS), and stored at -80 °C.

Echo

When mice were anesthetized with isoflurane (2%), echocardiographic examinations were performed by an ultrasound system (Vevo 2100 imaging system, VisualSonics, Ontario, Canada) equipped with a 13-MHz linear transducer and professional ultrasound technicians who were blind to animal grouping. The left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were calculated from M-mode images of left ventricular (LV) dimensions.

Heart biological markers of myocardial injury and inflammation

Heart biological markers of myocardial injury cardiac troponin I (cTnI) (Cat No. E-EL-M1203c, Elabscience biotechnology, Wuhan, China), Creatine kinase-myocardial isoenzyme (CK-MB) (Cat No. E-EL-M0355c, Elabscience Biotechnology, Wuhan, China), and markers of inflammation tumor necrosis factor- α (TNF- α) (Cat No. KE10002, Proteintech, Wuhan, China), interleukin-1 β (IL-1 β) (Cat No. KE10003, Proteintech, Wuhan, China) levels

were detected using enzyme-linked immunosorbent assay (ELISA) kits.

Heart oxidative stress indicators

We collected the myocardial tissues of mice as described above. The CuZn-superoxide dismutase (CuZn-SOD), glutathione-peroxidase (GSH-Px), and malondialdehyde (MDA) levels were detected using biochemical analysis kits (Elabscience biotechnology, Wuhan, Hubei, China) according to the manufacturers' instructions.

Transmission electron microscopy (TEM)

Myocardial tissues of the LV wall were cut into 1 mm³ thick slices and fixed (buffer: 1% OsO₄ in 0.1 M PB, pH 7.4), embedded, and polymerized. Sections (60–80 nm) were cut, stained, and examined under a transmission electron microscopy (TEM; HT7800/HT7700, Hitachi Ltd., Tokyo, Japan). The autophagosome was observed and counted. We selected 5 random regions for each sample for quantification (21).

Histological analysis

Myocardial tissues were fixed in 4% paraformaldehyde at room temperature for 48 h, paraffin-embedded, sectioned, and then hematoxylin-eosin (H&E) stained for morphological analysis. Slides were observed under a light microscope (DM3000 LED, Leica, Wetzlar, Germany). The changes of myocardial histological staining were scored as previous study described (22). The sections were graded (0 to 4+) for cellular infiltration and myocardial necrosis by two experienced observers who were blind to the experimental treatments. The grades were as follows: 0, no myocardial lesion; 1, lesions involving less than 25% of the myocardium; 2, lesions involving 25–50% of the myocardium; 3, lesions involving 50–75% of the myocardium; and 4, lesions involving more than 75% of the myocardium. The score was calculated as the mean derived from two sections of each sample.

Immunofluorescent microscopy

After deparaffinization, the sections were incubated with a microtubule-associated protein light chain 3 (LC3) antibody (1:200, ab192890, Abcam, Cambridge, UK). We used LC3-conjugated goat polyclonal secondary antibody to rabbit

immunoglobulin G (IgG)—H&L as a secondary antibody (1:500, ab150081, Abcam, UK). These sections were then counterstained with Beyotime c1006 (Beyotime, Nanjing, china) and examined under the microscope (DM3000 LED, Leica, Germany).

Western blot analysis

Whole proteins were extracted from myocardial tissues in radioimmunoprecipitation assay (RIPA) buffer containing phosphatase inhibitor on ice, and quantified using a bicinchoninic acid (BCA) Kit (Cat No. E-BC-K318-M, Elabscience Biotechnology, Wuhan, China). After electrophoresis on 8% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, the proteins were blotted to polyvinylidene fluoride (PVDF) membranes (Millipore-Upstate, Beijing, China, 0.45 μ m). After blocking in 5% skim milk (Cat No. 1172GR500, Saiguoguo, Guangzhou, China) or albumin (Solarbio, Beijing, China) in Tris-buffered solution containing 0.1% Tween-20 (TBST) for 2 h at 37 °C, the membranes were incubated at 4 °C for at least 16 h with primary antibodies against uncoupling protein 2 (UCP2; 1:1,000, Cat No. 11081-1-AP, Proteintech, Wuhan, China), dynamin-related peptide 1 (Drp-1; 1:8000, Cat No. A2586, ABclonal, Wuhan, China), pDrp-1 (Ser616; 1:3,000, Cat No. #12749, Signalway Antibody (SAB), College Park, MD, USA), tensin homolog (PTEN)-induced putative kinase 1 (PINK1; 1:1,000, Cat No. ab186303, Abcam, UK), Parkin (1:2,000, Cat No. ab77924, Abcam, UK), LC3 (1:3,000, Cat No. 14600-1-AP, Proteintech, Wuhan, China), and beta-actin (1:50,000, catalog number AC026, ABclonal, Wuhan, China). After washing 3 times with TBST (15 min \times 3), the membranes were incubated with secondary antibodies (1:10,000, Cat No. SA00001-2, Proteintech, Wuhan, China) at room temperature for 2 h. The protein bands were developed using an ECL detection kit and visualized using a BLT GelView 6000Plus (Bolu, Guangzhou, China). The band intensities were quantified using the AlphaView software (ProteinSimple, San Jose, CA, USA).

Statistical analyses

Statistical analysis was performed with SPSS software version 26.0 (IBM Corp., Chicago, IL, USA). Western blotting and immunofluorescent microscopy analysis were completed by scanning and analyzing the intensity of signals

on film by using NIH Image J (<https://imagej.nih.gov/ij/>). Data were presented as the mean \pm standard deviation (SD). A one-way analysis of variance (ANOVA) following the least significant difference (LSD) or Student-Neuman-Keuls (SNK)-q test was carried out for comparisons of more than two groups. The *t*-test was used for comparisons between two groups. Statistical significance was defined as $P < 0.05$.

Results

Effects of levosimendan treatment on LPS-induced cardiac dysfunction mice

A total of 36 mice were obtained for this investigation, and 24 were included and completed. We excluded 12 mice because of no occurrence of myocardial dysfunction. Clinical signs of endotoxemia were observed in mice treated with LPS, including lethargy, mild closure of the eyes, ruffled fur, and diarrhea. No death was observed across all groups at 6 h after LPS injection and there was no significant difference in body weight among the groups. Compared to the control group, echo examinations revealed decreased LVEF and LVFS after LPS injection. Treatment of levosimendan could reverse cardiac dysfunction induced by LPS as indicated by a higher LVEF and LVFS (*Figure 1A-1C*). Furthermore, after LPS exposure, cTNI and CK-MB levels in serum expression were increased, and these effects were attenuated after levosimendan administration (*Figure 1D,1E*).

Effects of levosimendan treatment on cardiac inflammation, oxidative stress, and pathological changes of mice with LPS-induced cardiac dysfunction

The plasma TNF- α and IL-1 β levels were significantly higher in the LPS group than in the other three groups. Treatment with levosimendan significantly reduced TNF- α and IL-1 β levels (*Figure 2A,2B*).

Furthermore, compared to control mice, cardiac GSH-Px levels and Cu/ZnSOD activities were decreased, but those of MDA were increased in LPS-treated mice. Levosimendan improved cardiac GSH-Px levels and Cu/ZnSOD activities, and reduced cardiac MDA levels (*Figure 2C-2E*).

Additionally, levosimendan administration significantly ameliorated myocardial pathological changes caused by LPS (*Figure 2F-2H*).

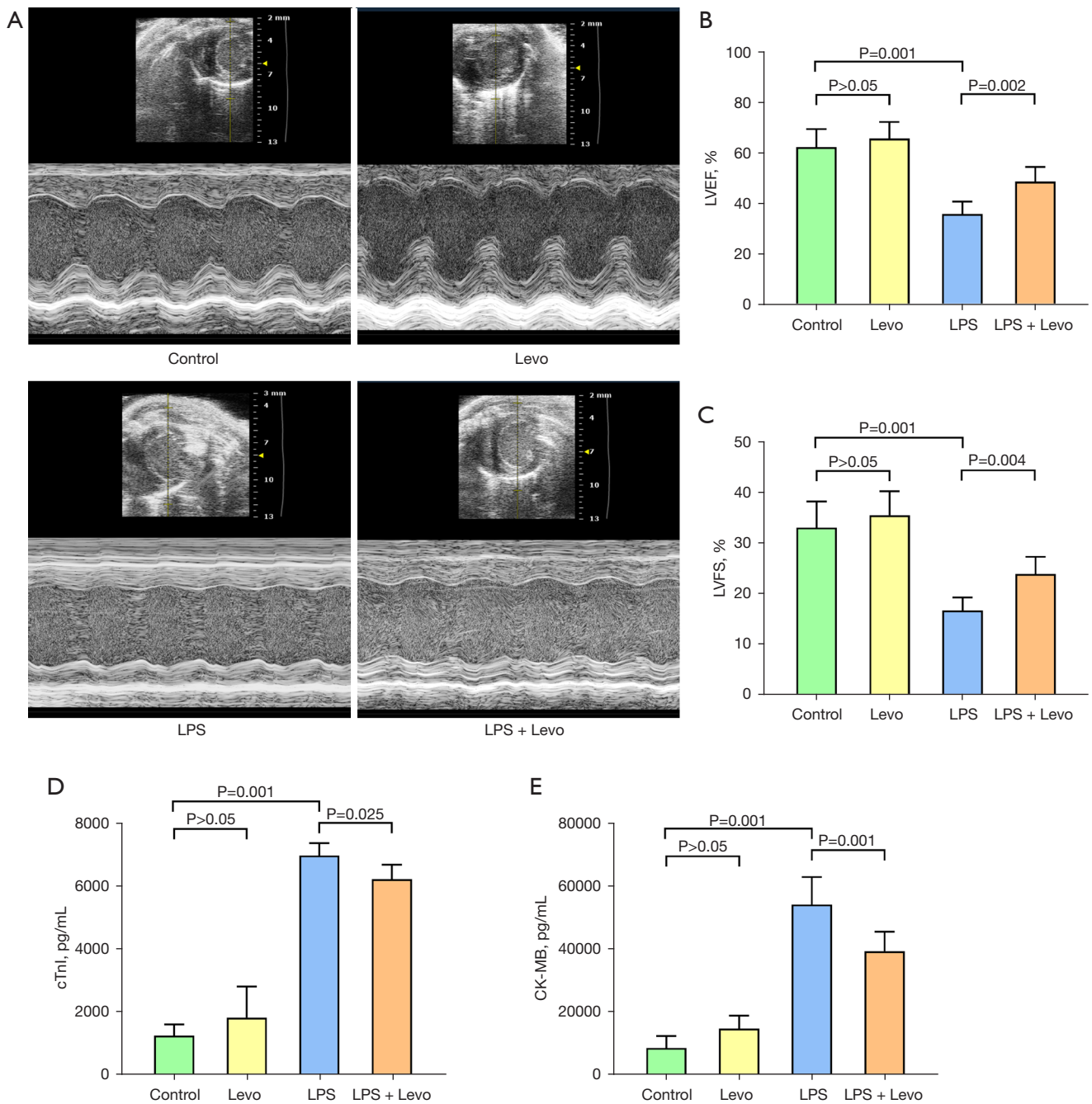


Figure 1 Levosimendan administration improves cardiac contractility and myocardial injury. (A) Representative M-mode images from mice in each group (n=6); (B,C) quantitative analysis of each group mice LVEF and LVFS (n=6); (D,E) evaluation of myocardial injury by cTnI and CK-MB in serum (n=6). LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; cTnI, cardiac troponin I; CK-MB, creatine kinase-myocardial isoenzyme; LPS, lipopolysaccharide; Levo, Levosimendan.

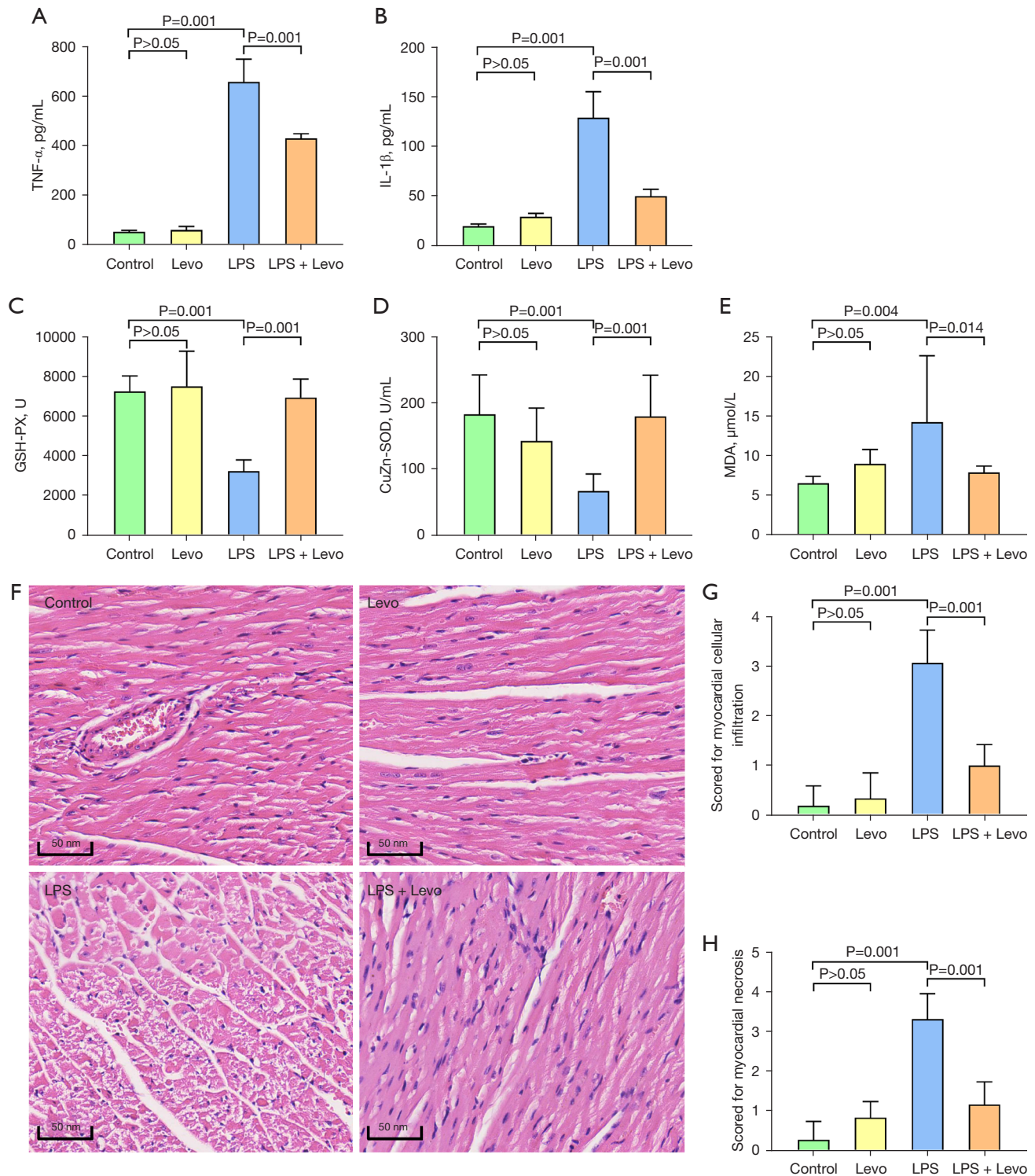


Figure 2 Levosimendan administration improves cardiac inflammation, oxidative stress and pathological changes. (A,B) Evaluation of cardiac inflammation by TNF- α and IL-1 β in serum (n=6); (C-E) evaluation of cardiac oxidative stress by GSH-Px, CuZn-SOD, and MDA in serum (n=6); (F) representative HE-stained cardiac sections (n=6); (G,H) myocardial cellular infiltration and necrosis score of 4 groups (n=6). TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; GSH-Px, glutathione-peroxidase; CuZn-SOD, CuZn-superoxide dismutase; MDA, malondialdehyde; HE, hematoxylin and eosin; LPS, lipopolysaccharide; Levo, Levosimendan.

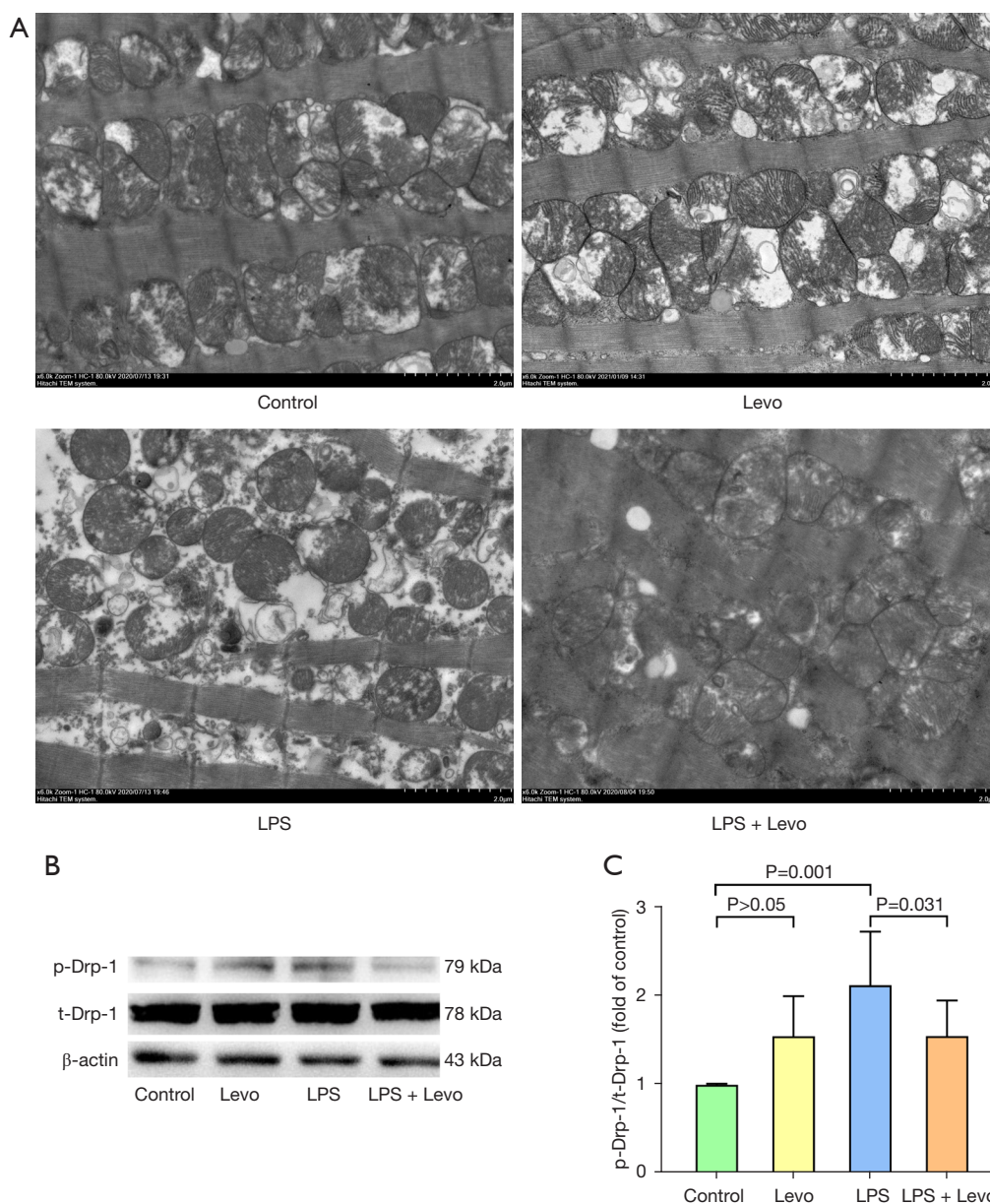


Figure 3 Levosimendan administration improves mitochondrial ultrastructure induced by LPS. (A) Representative images of mitochondrial morphology (n=6); (B,C) representative bands of p-Drp-1 and Drp-1, and quantitative analysis of p-Drp-1 and Drp-1 expressions (n=6). LPS, lipopolysaccharide; Levo, Levosimendan.

Effects of levosimendan on cardiac mitochondrial injury and fragmentation of mice with LPS-induced cardiac dysfunction

Examination with TEM revealed normal mitochondrial structure in control group, including intact double membrane, dense matrix granules, and tightly packed cristae. The most obvious alterations induced by LPS

were loss of internal cristae matrix granules and electron-dense matrix and shift toward smaller cardiac mitochondria distribution. Levosimendan could prevent LPS-induced mitochondrial injury (*Figure 3A*).

In the LPS group, mitochondrial fragmentation was associated with phosphorylated Drp1 (phospho ser 616 Drp1) expression elevation, and levosimendan could

prevented Drp1 phosphorylation (Figure 3B,3C).

Effects of levosimendan on cardiac mitophagy of mice with LPS-induced cardiac dysfunction

Conversion of the soluble form of LC3 (LC3-I) to autophagosome-associated and lipidated form (LC3-II) expression were used to assess mitophagy. The significant elevation in LC3-II to LC3-I ratio was observed in the LPS group, and levosimendan reinforced the LC3-II to LC3-I ratio in LPS group on LC3-positive punctate staining assessed by immunofluorescence (Figure 4A,4B) and western blot (Figure 4C,4D).

We found that LPS insult slightly increased PINK-1 and Parkin expression, which is a key pathway involved in the regulation of mitophagy, and levosimendan treatment further significantly increased PINK-1 and Parkin expression (Figure 4E,4F).

Mitophagy plays an important role in myocardial protection of levosimendan

A total of 42 mice were utilized for this component and 24 were included and completed. We had to exclude 18 mice due to no occurrence of myocardial dysfunction. No death was observed across all groups at 6 h after LPS injection and there was no significant difference in body weight among the groups.

The critical role of mitophagy was further validated through PINK1-Parkin pathway in LPS-induced cardiac dysfunction. We investigated the effects of mitophagy inhibition by mdivi-1. Compared to the LPS + Levo group, administration of mdivi-1 significantly reduced LVEF and LVFS (Figure 5A-5C). In addition, mdivi-1 increased the levels of cTNI, CK-MB, TNF- α , and IL-1 β (Figure 5D-5G). Meanwhile, mdivi-1 was shown to reverse the improvement of oxidative stress index and myocardial pathological changes by levosimendan (Figure 5H-5M).

Detection with TEM revealed that autophagosomes in the myocardium in the LPS group were significantly increased, compared to the Control group. Moreover, compared to the LPS group, levosimendan administration further increased the number of autophagosomes. Compared to the LPS + Levo group, the administration of mdivi-1 significantly decreased the number of autophagosomes (Figure 6A,6B). Immunofluorescence and western blot analysis showed that mdivi-1 significantly reduced LC3B-II/LC3B-I ratio (Figures 6C,6D,7A,7B). In

addition, mdivi-1 could reduce the expression of PINK-1 and Parkin but not affect the expression of p-DRP-1 (Figure 7C-7E).

Discussion

Sepsis is the leading cause of death in the intensive care unit (ICU), and sepsis-induced myocardial dysfunction is a major global healthcare problem. Sepsis induces myocardial mitochondrial structure damage and loss of mitochondrial function (23). In this study, we found that levosimendan significantly inhibited mitochondrial fission and strengthened myocardial mitophagy to reduce mitochondrial damage and improve cardiac function. Moreover, levosimendan treatment significantly activated PINK-1 and Parkin, and thus increased the autophagic LC3-II/LC3-I protein ratio. These data indicate that levosimendan regulates mitophagy activation and mitochondrial integrality preservation to improve cardiac function via PINK-1-Parkin mediated mechanisms.

Cardiac dysfunction is a critical component of multi-organ dysfunction in sepsis. It is manifested by the inhibition of systolic and diastolic cardiac function, elevation myocardial injury markers, and destruction of myocardial mechanisms (24). Our results suggested that LPS caused significant myocardial injury and cardiac function reduction represented by higher cTNI and CK-MB and lower EF and FS. Levosimendan is widely used as a cardioprotective positive inotropic drug, and its administration significantly improved EF, FS, cTNI, and CK-MB levels. This is consistent with previous studies of levosimendan in myocardial protection (25).

Inflammation, oxidative stress disorder, and mitochondrial dysfunction, play an important role in the pathogenesis of sepsis-induced cardiac dysfunction. Septic cardiac dysfunction is closely associated with the excessive production of pro-inflammatory cytokines such as IL-1 β and TNF- α (26,27). Levosimendan has been shown to have an anti-inflammatory effect (17). In our study, serum TNF- α and IL-1 β elevation caused by LPS were significantly attenuated by levosimendan administration. Thus, levosimendan may have a protective effect on septic myocardial injury through anti-inflammatory mechanisms. While being good markers of oxidative stress, GSH, SOD, and MDA can act as a cofactor for a variety of enzymes, helping to repair DNA, remove H₂O₂, HO, and lipid peroxides, and regenerate other antioxidants, such as ascorbic acid and tocopherols. In our study, sepsis

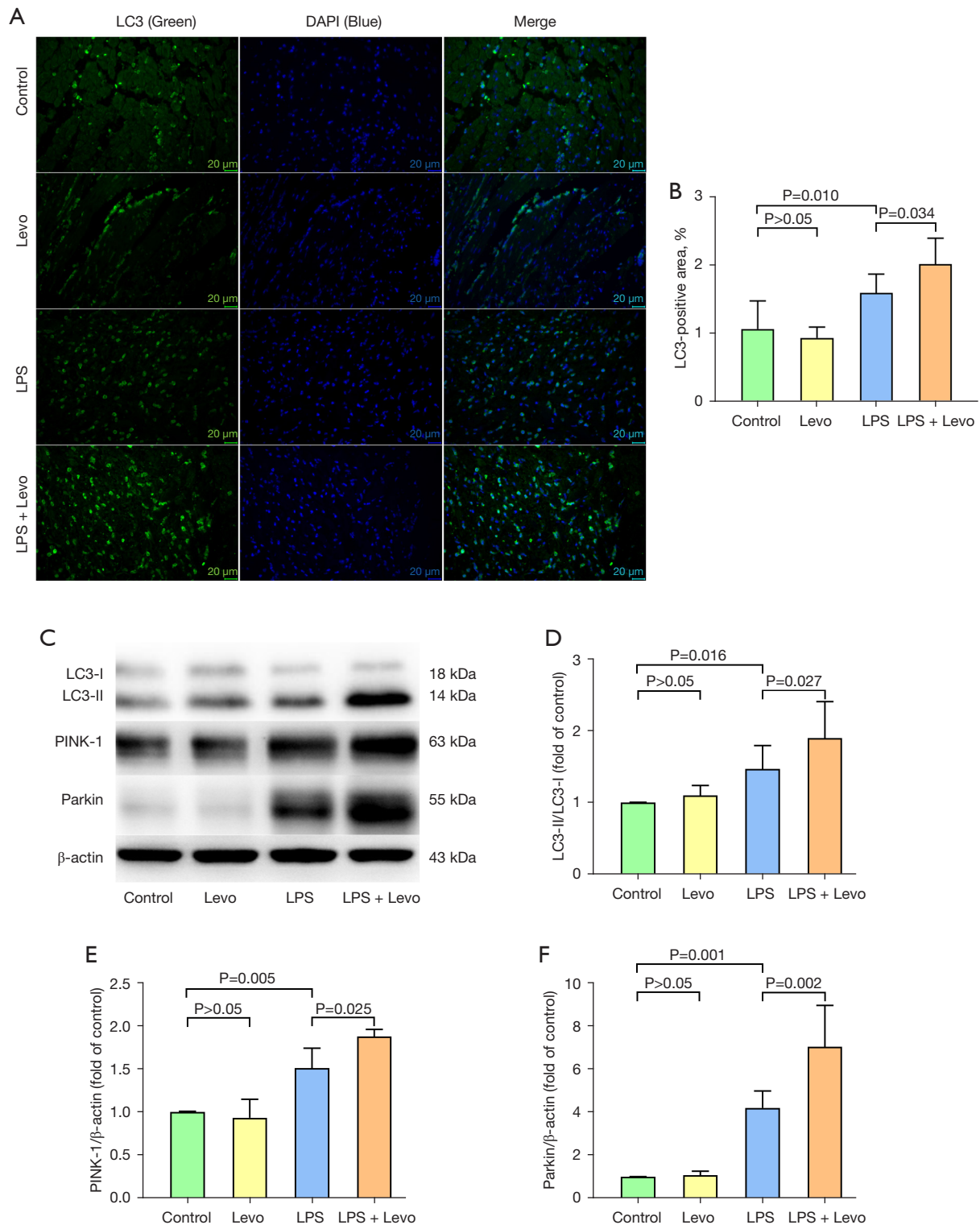
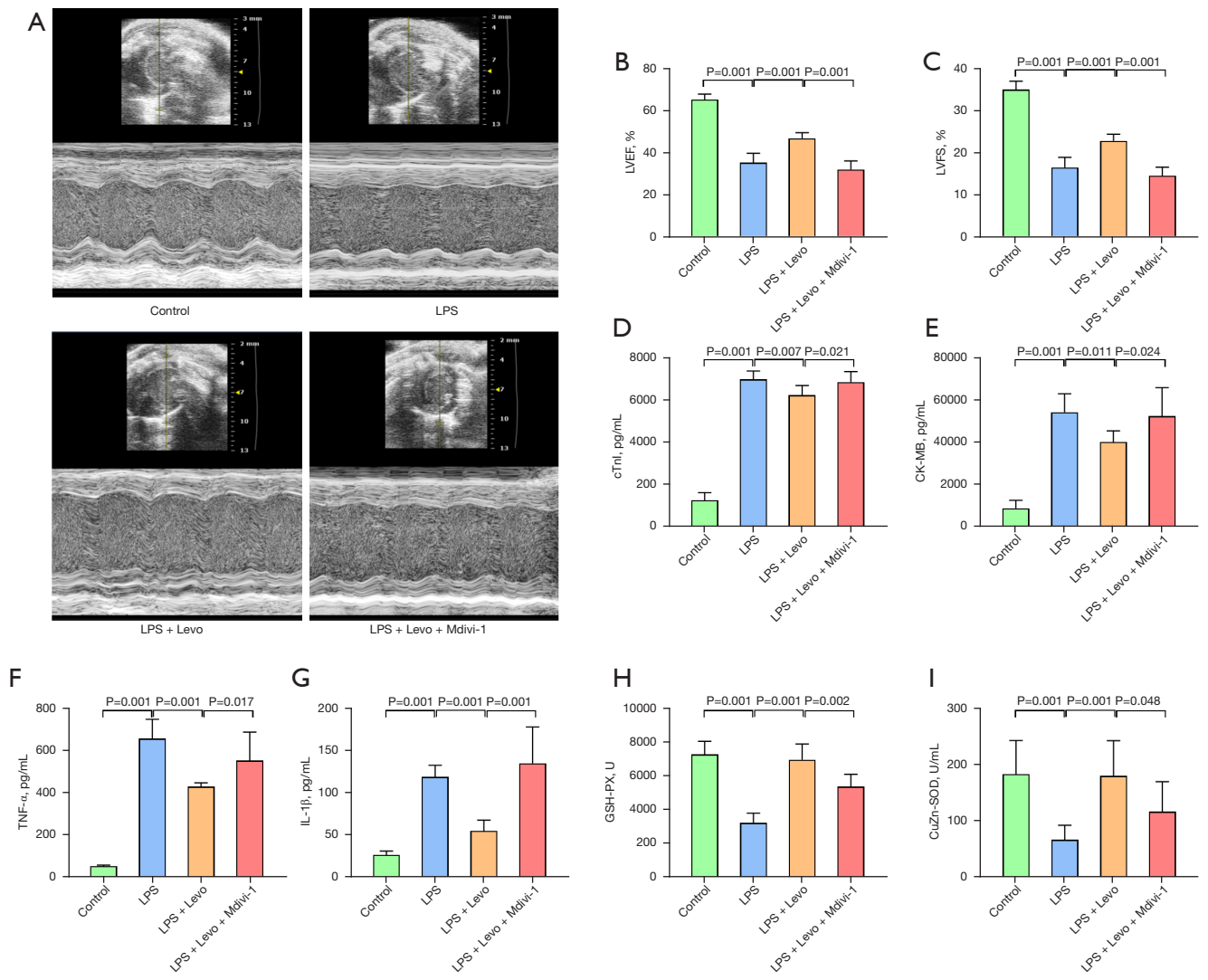


Figure 4 Levosimendan administration on myocardial mitophagy, mitophagy pathway after LPS injury. (A) Representative images LC3 dots LV tissue sections from mice in each group (n=6); Nucleus is blue by labeling with DAPI, positive cells are green. (B) Quantitative analysis of LC3 in LV tissue sections from mice in each group (n=6); (C-F) representative bands of western blot and quantitative analysis of LC3B-II/LC3B-I ratio, PINK-1 expression and Parkin expression (n=6). LPS, lipopolysaccharide; Levo, Levosimendan.



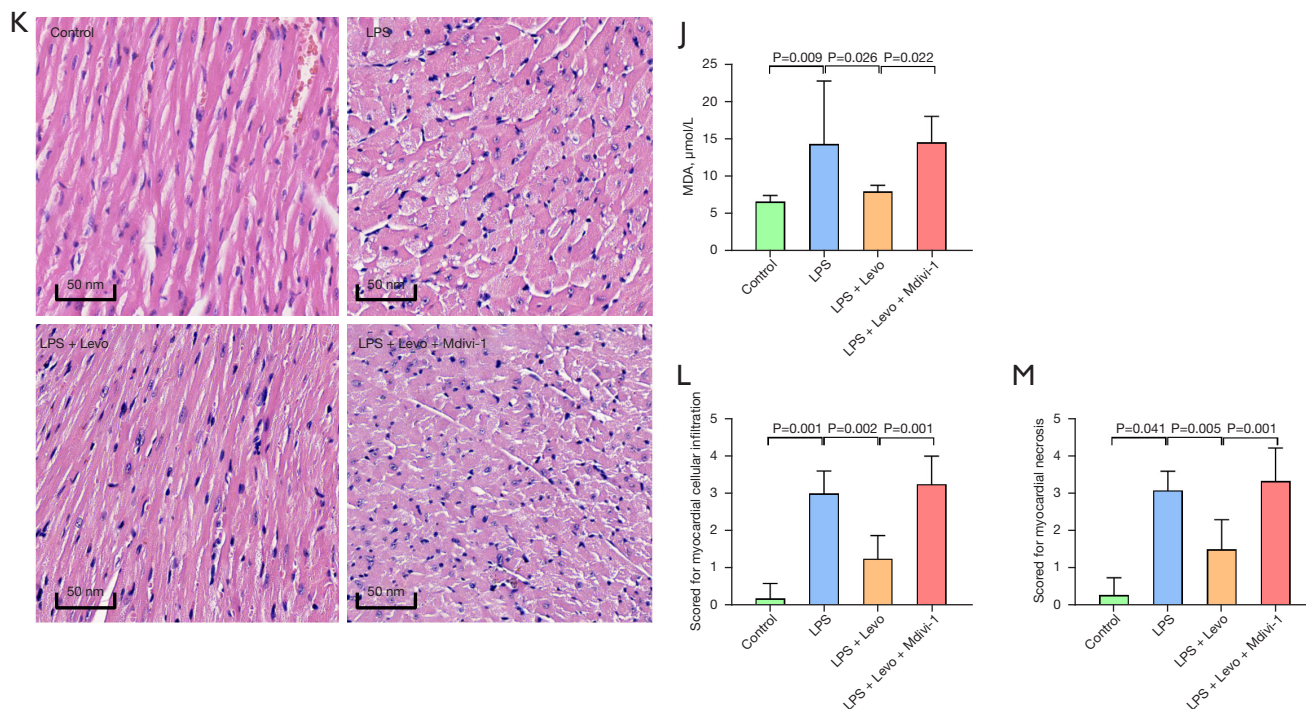


Figure 5 Mdivi-1 treatment exacerbated LPS-induced injury and impaired levosimendan-induced cardiac protection. (A-C) Representative M-mode images from mice in each group and quantitative analysis of mice LVEF and LVFS (n=6); (D,E) evaluation of myocardial injury by cTnI and CK-MB in serum (n=6); (F,G) evaluation of cardiac inflammation by TNF- α and IL-1 β in serum (n=6); (H-J) evaluation of cardiac oxidative stress by GSH-Px, CuZn-SOD, and MDA in serum (n=6); (K) representative HE-stained cardiac sections (n=6); (L,M) myocardial cellular infiltration and necrosis score of 4 groups (n=6). LPS, lipopolysaccharide; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; cTnI, cardiac troponin I; CK-MB, creatine kinase-myocardial isoenzyme; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; GSH-Px, glutathione-peroxidase; CuZn-SOD, CuZn-superoxide dismutase; MDA, malondialdehyde; Levo, Levosimendan.

reduced GSH-Px and CuZn-SOD activities and resulted in MDA elevation, leading to iliac crest loss, mitochondrial swelling, and vacuole formation, and levosimendan exerted antioxidant activity in the myocardium. Previous research has demonstrated that mitochondria limit the availability of inflammasome activators and/or components, and reduce mitochondrial DAMPs via mitophagy, and then control inflammation (28,29). Furthermore, mitochondria are both the sources and targets of oxidants, and play a critical role in redox dysregulation (30). Therefore, we can speculate that levosimendan may have the effect of anti-inflammatory and antioxidant stress by protecting mitochondria.

In animal experiments and clinical studies on sepsis, cardiomyocytes manifested as mitochondrial fragmentation and mitochondrial ultrastructural damage (31). Mitochondrial fragmentation, on the other hand, relies on the activity of a cytosolic molecule, dynamic-

related protein-1 (Drp-1), which is the main mediator of mitochondrial fission and encoded by Dnm1l in mice and DNMI1 in humans (32). We observed that LPS-treated mice exhibited cardiac characteristics of mitochondrial fragmentation/fission, such as small-size mitochondria and phosphorylation Drp-1 (p-Drp-1), and levosimendan prevented both Drp-1 phosphorylation and mitochondrial fragmentation. Mitochondrial fission inhibition suppressed the formation of autophagosome, leading to the accumulation of damaged mitochondria and promoted the systolic dysfunction of cardiomyocytes. However, fission is not an isolated phenomenon, as it is associated with processes such as the regulation of mitophagy. In order to further investigate the mechanisms of mitochondrial dysfunction, we focused on mitophagy. In our study, LPS exposure caused rapid initiation of mitophagy, which was demonstrated by autophagosome formation and LC3II

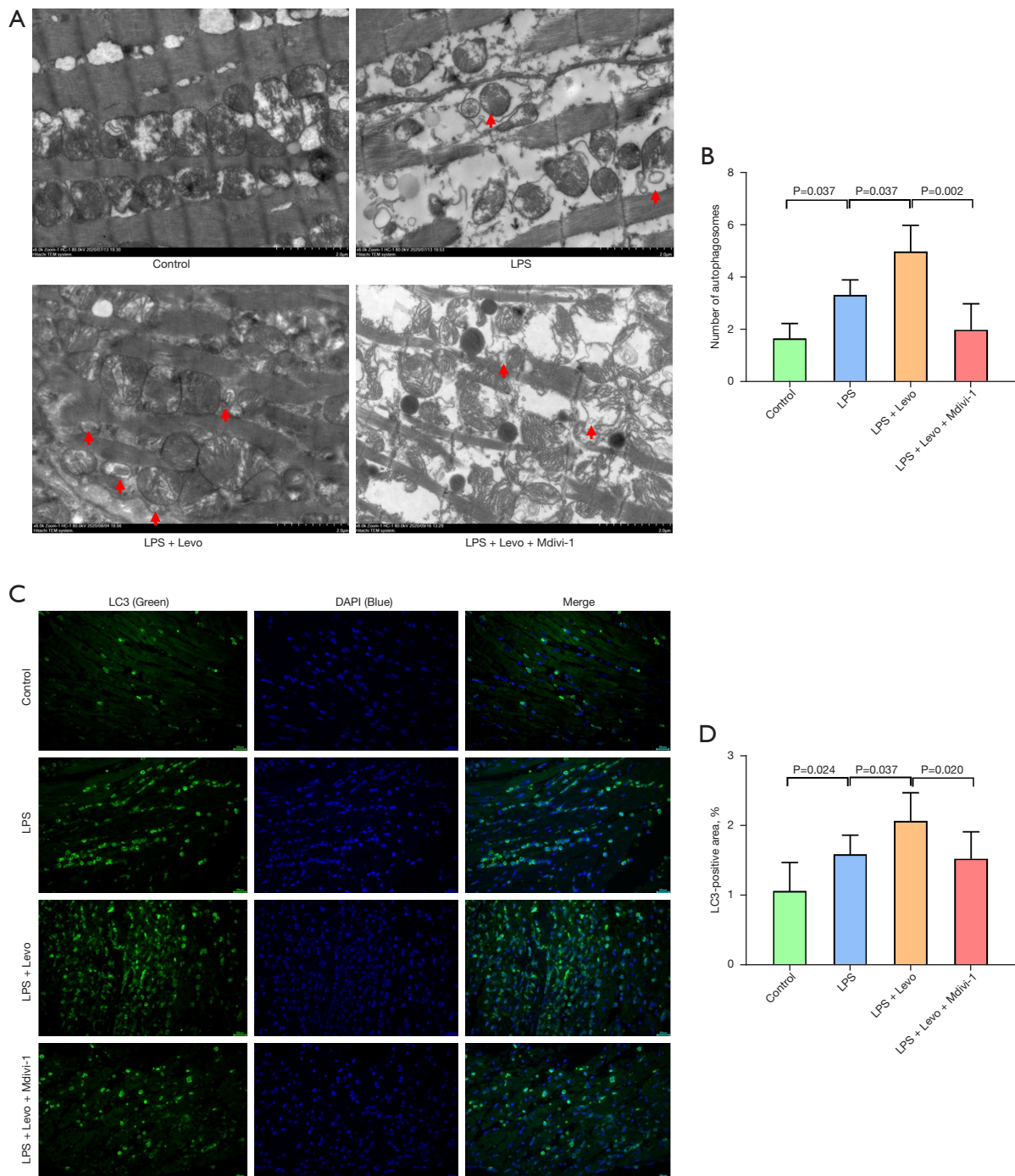


Figure 6 Mdivi-1 reversed levosimendan-induced cardiac protection via inhibiting autophagy activation. (A) Representative images of mitochondrial morphology and autophagosomes in the myocardium (n=6), the red arrow indicates an autophagosome; (B) quantitative analysis the number of autophagosomes in cardiomyocytes (n=6); (C) representative images of LC3 dots LV tissue sections from mice in each group (n=6), Nucleus is blue by labeling with DAPI, Positive cells are green; (D) quantitative analysis of LC3 in LV tissue sections from mice in each group (n=6). LPS, lipopolysaccharide; Levo, Levosimendan.

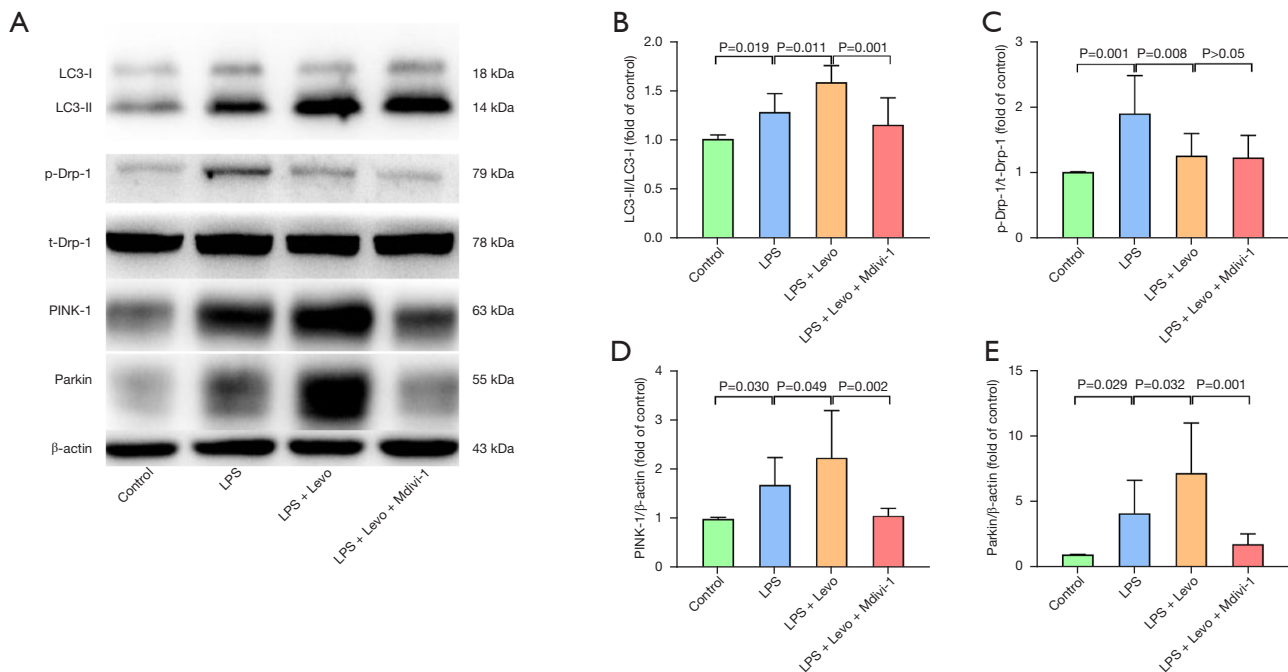


Figure 7 Mdivi-1 reversed levosimendan-induced cardiac protection via inhibiting mitophagy pathway after LPS injury. (A-E) Representative bands of western blot and quantitative analysis of p-Drp-1 phosphorylation levels, LC3B-II/LC3B-I ratio, PINK-1 expression and Parkin expression (n=6); LPS, lipopolysaccharide; Levo, Levosimendan.

conversion, and levosimendan further promoted this process. This is in consistent with previous findings in LPS and bacterial sepsis models. However, insufficient mitophagy led to mitochondrial dysfunction and adverse consequences, whereas complete induction of autophagy was essential for cardiac protection (33).

Mitochondria constantly adapt to their morphology by fusion and fission. Under the influence of a variety of stressors, mitochondria fragment into smaller entities through DRP1-mediated fission. Mitochondrial membrane potential reduction leads to stabilization of PINK1, which in turn recruits and phosphorylates the E3-ligase Parkin. Poly ubiquitinated proteins serve as a “tag” for autophagy receptors that interact with the autophagosome-resident protein LC3 (14). The central role of the PINK1-Parkin pathway in the regulation of mitophagy has been demonstrated in several studies (34). Therefore, we further detected the expression of PINK-1 and Parkin, which revealed that LPS could increase the expression of PINK-1 and Parkin, and levosimendan could further enhance them. We inferred that levosimendan attenuated LPS-induced myocardial dysfunction may function through activating PINK-1-Parkin pathway.

However, it remains unclear whether the PINK-1-Parkin pathway is involved in the protective effects of levosimendan against sepsis-induced myocardial dysfunction. Our study used mdivi-1, which is a potent inhibitor of mitochondrial division dynamin-related GTPase and a specific inhibitor of the PINK-1-Parkin pathway, to verify this hypothesis and found that mdivi-1 could prevent the improvement of cardiac function such as LVEF and LVFS, myocardial enzyme markers such as cTNI and CK-MB, inflammatory cytokines levels, oxidative stress index, and myocardial pathological changes by levosimendan administration in LPS-induced cardiac dysfunction mice. Furthermore mdivi-1 markedly reduced Drp-1 phosphorylation, mitochondrial fragmentation, autophagosome formation and LC3II conversion. Defects of autophagosome formation in LPS mice treated by mdivi-1 were correlated with lysosome vacuoles increases within the myocardium, which suggest that autophagic vacuoles fail to trap mitochondria cargo (35).

In the present study, levosimendan exerted its protective effects against LPS-induced cardiac dysfunction through mitophagy and PINK-1-Parkin signaling. However, whether the other signaling pathway induces mitophagy

has not been investigated in our article, and whether levosimendan has a protective effect on cardiac dysfunction in patients with sepsis requires further clinical trials. In addition, this model had the same limitations for example, vital signs such as hemodynamic monitoring are difficult, and there are differences between mouse models and human diseases as the typical characteristic of human sepsis infection is local infection followed by systemic infection, and it is multi bacterial infection, including gram-negative bacteria and gram-positive bacteria. In this study, there was a certain incidence rate of myocardial dysfunction due to sepsis. In order to reduce the number of mice, 6 mice were eventually analyzed by statistical analysis, and the sample size was small, which may have led to statistical deviation.

Taken together, we consider that the improved status of the mitochondria in the hearts of LPS mice is likely the result of multiple signaling regulations such as inflammation and oxidative stress. The induction of mitophagy by levosimendan preserved cardiac function and suppressed the increase in circulating cytokines following LPS challenge. Levosimendan was able to rescue the LPS-induced cardiac dysfunction mice, supporting its mechanism of action by directly targeting the PINK-1-Parkin pathway. This provides evidence for the use of levosimendan in sepsis patients with cardiac dysfunction. We will investigate these aspects in future studies to fully understand the other therapies in sepsis.

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Footnote

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