Levosimendan Attenuates Myocardial Injury Induced by Coronary Microembolization in Swine by Inhibiting Myocardial Inflammation and Apoptosis

Han Chen¹, Jiang-You Wang²
Departments of ¹Cardiac Surgery and ²Cardiology, Wuhan Asia Heart Hospital and Wuhan Asia General Hospital, Wuhan, China

Abstract

Aims: In addition to its cardiotonic effect, Levosimendan (Levo) has been thought to have multiple cardiovascular benefits, including anti-inflammatory and anti-apoptotic effects. The present study was undertaken to determine whether the Levo pretreatment could attenuate myocardial apoptosis and inflammation and improve cardiac function in a swine model of coronary microembolization (CME).

Materials and Methods: A total of 15 swine were randomly and equally divided into a sham-operated (control) group, CME group, and CME plus Levo group. Swine CME was induced by intracoronary injection of inertia plastic microspheres (42 µm diameter) into the left anterior descending (LAD) coronary artery, with or without pretreatment of Levo. Echocardiological measurements, a pathological examination, Terminal-deoxynucleoitidyl Transferase-Mediated dUTP Nick End-Labeling (TUNEL) staining, H and E staining, and Western blotting were performed to assess the functional, morphological, and molecular effects in CME. Results: The expression levels of caspase-3 and tumor necrosis factor-α (TNF-α) were increased in cardiomyocytes following CME. Downregulation of caspase-3 and TNF-α with Levo pretreatment was associated with improved cardiac troponin I (cTnI) and high sensitivity C-reactive protein. In addition, through Pearson correlation analysis, the left ventricular ejection fraction was negatively correlated with caspase-3, TNF-α, and cTnI.

Conclusion: This study demonstrated that Levo pretreatment could significantly inhibit CME-induced myocardial apoptosis and inflammation and improve cardiac function. The data generated from this study provide a rationale for the development of myocardial apoptosis and inflammation-based therapeutic strategies for CME-induced myocardial injury.

Keywords: Apoptosis, coronary microembolization, inflammation, Levosimendan

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Levosimendan (Levo) has been developed for the treatment of acute heart failure and other cardiac conditions where the use of an inodilator is considered as appropriate. The pharmacology of Levo includes positive inotropy with energy-sparing effects, positive effects on ventriculo-arterial coupling, peripheral vasodilation, and increasing tissue perfusion, anti-stunning effects, and anti-inflammatory and anti-apoptotic effects. Recent studies have revealed that the protective effects of Levo on ischemia/reperfusion injury are primarily related to the regulation of apoptosis and inflammation. In addition, our recent studies have also suggested that Levo could lower the extent of myocardial injury after CME and improve the cardiac function in swine primarily related to the regulation of cardiomyocytes apoptosis. The aims of the present study were to determine the role of Levo pretreatment in CME-induced myocardial inflammation and apoptosis. The data generated from this study provide a rationale for the development of myocardial apoptosis and inflammation-based therapeutic strategies for CME-induced myocardial injury.

**Materials and Methods**

**Animal preparation and experimental procedures**

Healthy swine (25–30 kg) were purchased from the Animal Center of the Agriculture College of Guangxi University (Nanning, People’s Republic of China). Throughout all experimental stages, the animals were maintained under controlled conditions of temperature (22°C–25°C), humidity (40%–60%), and light, with pig feed and water provided ad libitum. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85–23, revised 1996). The Clinical and Animal Research Ethics Committees of the Guangxi Medical University approved all procedures.

**Modeling and grouping**

A total of 15 miniature swine were randomly assigned into three groups, including a sham-operated (control) group, CME group, and CME plus Levo group, with five swine in each group. The Levo group was pretreated with Levo 24 h (0.05 µg/kg/min) and high loading dose of Levo (0.2 µg/kg/min) 60 min before microsphere injection. The CME model was induced by the manual unremitting injection of microspheres into the LAD artery. The swine were initially sedated through an IM injection of a combination of ketamine and atropine (10–15 mg/kg and 2 mg, respectively). After endotracheal intubation, anesthesia was maintained through an intravenous drip of diazepam into the ear vein. The right femoral artery was separated, was maintained through an intravenous drip of diazepam, and high loading dose of Levo (0.05 µg/kg/min) 60 min before microsphere injection. The CME model was induced by the manual unremitting injection of microspheres into the LAD artery. The swine were initially sedated through an IM injection of a combination of ketamine and atropine (10–15 mg/kg and 2 mg, respectively). After endotracheal intubation, anesthesia was maintained through an intravenous drip of diazepam into the ear vein. The right femoral artery was separated, and a 6F (Cordis, USA) vascular sheath was placed. Before the coronary cannulation, the animals were anticoagulated through intravenous injection of 200 U/kg heparin followed by 100 U/kg/h to maintain heparinization. A 6F JL 4.0 guiding catheter was used for coronary angiography. After the coronary angiography, a 1.8F infusion catheter (Cordis, Inc., USA) was placed into the LAD artery with the tip located between the second and third diagonal branches. Microspheres with a diameter of 42 µm (Dynospheres; Dyno Particles; Lillestrøm, Norway) at a mean dosage of 100,000 were selectively infused into the LAD within 40 min followed by a flush with 10 ml of saline. The sham-operated swine (sham group) were subjected to the same procedures, except that the injection was saline rather than microspheres. The systemic blood pressure and heart rate were continuously monitored during the procedure.

**Echocardiography**

The animals were sedated as previously described and then placed on the experimental platform in the right lateral position. One experienced investigator who was blinded to the study protocol captured the transthoracic echocardiogram using a GE VIVID 7 system and a 1.5–4.3 MHz transducer. Briefly, the 1.5–4.3 MHz transducer was placed on the left anterior chest wall to obtain the left ventricular end-systolic diameter, fractional shortening (FS), and cardiac output (CO), and the left ventricular ejection fraction (LVEF) was calculated using a cubic formula. All parameters were averaged from ≥3 consecutive cardiac cycles. After the functional measurements, the animals were sacrificed through the intravenous injection of 10 ml of 10% potassium chloride, and the hearts were fixed in 4% paraformaldehyde or quickly frozen at −80°C for further use.

**Coronary sinus levels of cTnI and high-sensitivity C-reactive protein**

Ethylendiaminetetraacetic acid-anticoagulated blood samples were collected from the coronary sinus. Immediately after collection, the blood samples were centrifuged at 4000 rpm for 15 min, and the serum samples were stored at −80°C until assay. The serum levels of cTnI and high-sensitivity C-reactive protein (hsCRP) were measured using commercially available electrochemical luminescence kits according to the manufacturer’s instructions (Roche, Inc., Switzerland). All measurements were performed in duplicates.

**Tissue sampling**

After blood sample collection, the hearts were arrested in diastole through an injection of 10 ml of 10% potassium chloride into the ear vein. Hearts were isolated and cleaned with normal saline immediately. Myocardial tissues were obtained from the anterior wall of the left ventricle dominated by the middle of the LAD artery. Part of the myocardial tissue was immediately frozen in liquid nitrogen and stored at −80°C for the Western blot analysis. The other was fixed with 4% paraformaldehyde for 12 h, embedded in paraffin, and serially sectioned into slices of 4-µm thickness for hematoxylin-basic fuchsin-picric acid (HBFP) staining and immunohistochemical staining, and terminal deoxynucleotidyl transferase-Mediated dUTP Nick End-Labeling (TUNEL) assay.

**Measuring the myocardial microinfarction area**

HBFP staining is an important method for the early diagnosis of myocardial ischemia. HBFP stains ischemic cardiac muscle, normal myocardial cytoplasm, and nuclei red, yellow, and blue, respectively. A DMR-Q550 pathological image...
pattern analysis instrument (Leica, Germany) was used to analyze the HBFP-stained slices. Briefly, five microscopic visual fields (original magnification, ×100) were randomly sampled from each slice for analysis using QWin analysis software (Leica, Germany), and the planar area method was used to measure the infarction zone, which was expressed as the average percentage of the area of infarction out of the total analyzed slice area.

TUNEL assay
Apoptotic cardiomyocytes were detected using the Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay kit (Roche, USA). TUNEL-positive signal was located in nuclei, and apoptotic nuclei were stained yellow brown, while the normal cell nuclei light blue. Meanwhile, morphological features of apoptosis (small, condensed nuclei, cell shrinkage, and nuclear fragmentation) were taken into consideration. In each section, ten random high-powered fields (×400) were observed to count TUNEL-positive cardiomyocyte nuclei, and the apoptotic index (%) was calculated as the percentage ratio of TUNEL-positive cell nuclei to the total nuclei.

Western blot analysis
Briefly, the protein concentrations were determined using a Bicinchoninic Acid Protein Assay Kit, and bovine serum albumin was used as the standard. Equal amounts of protein (100 μg) were fractionated through SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 2 h using 5% nonfat milk in tris-buffered saline containing Tween-20 and were then probed overnight at 4°C using one of the following primary antibodies: tumor necrosis factor-α (TNF-α) (1:1000 dilution, Abcam), caspase-3 (1:1000 dilution, Abcam), or anti-glyceraldehyde 3-phosphate dehydrogenase (1:1000 dilution, Abcam Biotechnology). The membranes were incubated with a secondary antibody (Abcam) for 1 h at room temperature. The membranes were probed and then exposed to X-ray film. The X-ray films were scanned, and the optical density was determined through Bio-Rad image analysis (Bio-Rad, Hercules, CA, USA).

Statistical analysis
All quantitative data are expressed as the means ± standard deviation and are analyzed using the SPSS 13.0 software, Statistical Product and Service Solutions. Two-tailed, unpaired Student’s t-tests, one-way ANOVA, and Pearson correlation analysis were used for statistical evaluation of the data. Differences were considered statistically significant when P < 0.05.

RESULTS
Animal groups
No significant differences in body weight, blood pressure, or heart rate were observed before or after the operation among the three groups. However, porcine arterial blood pressure decreased after intravenous Levo pretreatment [Table 1].

Levosimendan pretreatment improved cardiac function following CME
Results of echocardiographic examination [Table 2] showed that 12 h after CME modeling, the CME group exhibited significantly decreased cardiac systolic function as compared with the control group, as indicated by significantly reduced LVEF, FS, and CO as well as increased left ventricular end-diastolic diameter in the CME group (P < 0.05). In addition, Levo pretreatment was associated with improved cardiac function in the CME swine.

Levosimendan pretreatment attenuated myocardial injury marker following coronary microembolization
Myocardial injury following CME could be assessed by levels of cTnI in the blood obtained from the coronary sinus. At 12 h after CME modeling, the serum level of cTnI in swine from CME group was higher than control group (0.215 [0.056] ng/mL vs. 0.059 [0.012] ng/mL, P = 0.0232). Moreover, Levo pretreatment attenuated myocardial injury following CME, as reflected by the

| Table 1: Changes of heart rate and body weight before and after the procedure between the four groups |
|----------------------------------|----------------------------------|----------------------------------|
| Animal groups                  | Sham                             | CME                             | Levo                             |
| Body weight (kg, mean±SD)      | 27.23±7.25                       | 27.89±6.98                       | 28.56±9.63                       |
| Heart rate (bpm, mean±SD)      | 83.56±10.23                      | 85.69±9.35                       | 85.69±9.68                       |

CME: Coronary microembolization, Pre: Preprocedure, Post: Postprocedure, SD: Standard deviation, Levo: Levosimendan

| Table 2: Parameters of cardiac function in swine of each group 12 h after coronary microembolization modeling |
|-------------------------------------------------|----------------------------------|----------------------------------|
| Groups                                         | LVEF, %                          | FS, %                            | LVEDd, mm                        |
| Control                                        | 68.62±2.52                       | 43.32±2.26                       | 31.26±2.78                       |
| CME                                            | 49.82±5.25                       | 24.68±3.75                       | 40.62±2.68                       |
| Levo                                           | 61.86±3.25                       | 34.56±3.06                       | 35.62±2.86                       |

Data are presented as mean±SD. *P<0.05 compared with sham, **P<0.05 compared with CME (n=5). CME: Coronary microembolization, LVEF: Left ventricular ejection fraction, FS: Fractional shortening, LVEDd: Left ventricular end-diastolic diameter, LVESD: Left ventricular end-systolic diameter, CO: Cardiac output, LVEDV: Left ventricular end-diastolic volume, LVESV: Left ventricular end-systolic volume, Levo: Levosimendan, SD: Standard deviation

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reduced cTnI levels in the Levo group compared with CME group (0.112 [0.035] μg/L vs. 0.215 [0.056] ng/mL, \( P = 0.035 \)).

**Levosimendan pretreatment attenuated inflammation marker following coronary microembolization**

Myocardial inflammatory response following CME could be assessed by levels of hsCRP in the blood obtained from the coronary sinus. At 12 h after CME modeling, the serum level of hsCRP in swine from CME group was higher than control (6.35 [1.89] mg/L vs. 1.28 [0.85] mg/L, \( P = 0.019 \)). Moreover, Levo pretreatment attenuated myocardial inflammatory response following CME, as reflected by the reduced hsCRP levels in the Levo group compared with CME group (3.26 [1.12] mg/L vs. 6.35 [1.89] mg/L, \( P = 0.039 \)).

**Levosimendan pretreatment decreased myocardial infarct area following coronary microembolization**

As revealed by Mayer’s hematoxylin and eosin (H and E) [Figure 1a and b] and HBFP staining [Figure 2a-c]. The control animals exhibited subendocardial ischemia without infarction foci, whereas the CME animals exhibited multiple microinfarction foci. However, the administration of Levo reduced the microinfarct volume and inflammatory cell infiltration. HBFP staining revealed myocardial karyolysis or hypochromatosis based on the red cytoplasmic staining of the microinfarction foci. In addition, peripheral cardiac muscle edema and denaturation, peripheral inflammatory cell infiltration, and erythrocyte effusion were detected. For the all groups, the infarct area each group was sham 0.012 (0.008)%, CME 6.28 (3.25)%, and Levo 3.49 (2.82)%, \( P = 0.029 \) [Figure 2d].

**Levosimendan pretreatment decreased myocardial apoptosis following coronary microembolization**

Myocardial apoptosis was assessed using TUNEL staining. Compared with the control group [Figure 3a], more TUNEL-positive (brown) cardiomyocytes could be detected in swine from the CME groups [Figure 3b]. Interestingly, Levo treatment significantly decreased the relative proportion of apoptotic cells following CME [Figure 3c]. The percentages of myocardial apoptotic cells in the control, CME, and Levo were 0.56 (0.32), 9.56 (3.65), and 5.26 (2.13), \( P = 0.036 \) [Figure 3d].

**Levosimendan pretreatment inhibited myocardial tumor necrosis factor-α and caspase-3 in coronary microembolization swine**

Western blotting showed significant upregulation of caspase-3 [Figure 4a] and TNF-α [Figure 4b] proteins following CME modeling compared with those from the control group (\( P < 0.05 \)). However, Levo pretreatment was associated with reduced levels of TNF-α and caspase-3 proteins compared with CME group (\( P < 0.05 \)).

**Pearson correlation analysis**

Using Pearson correlation analysis, the LVEF negatively correlated with caspase-3 (\( r = -0.803, P < 0.001 \)), TNF-α (\( r = -0.812, P < 0.001 \)), and cTnI (\( r = -0.834, P < 0.001 \)).

**Discussion**

We demonstrated that the expression levels of caspase-3 and TNF-α increased in cardiomyocytes following CME, which is consistent with our previous research results. Downregulation of caspase-3 and TNF-α with Levo pretreatment was associated with improved cardiac function and attenuated cTnI and hs-CRP. These findings not only help us to understand the mechanisms by which myocardial apoptosis and inflammation mediate myocardial injury but also support our hypothesis that TNF-α and caspase-3 proteins may represent two potential intervention targets and that the data generated from this study provide a rationale for the development of myocardial apoptosis and inflammation-based therapeutic strategies for CME-induced myocardial injury.

CME is widely observed in acute coronary syndrome and is considered to be an iatrogenic complication following coronary interventions in clinical settings.\(^4\) CME, which is caused by the embolization of thrombotic material and debris or the rupture of an atherosclerotic plaque, is believed to generate a transient decrease in coronary blood flow, followed by reactive hyperemia and myocardial systolic dysfunction. Rioufol et al.\(^{22}\) demonstrated that the formation of atherosclerosis frequently presented with the rupture and repair of plaques. Therefore, our data further confirm the pathophysiological manifestations of CME.

Previous studies demonstrated that the aggregate amount of infarction involved a small area (<5%) of microembolized myocardium in pigs or dogs, as indicated by typical inflammatory responses, including increased TNF-α expression and leukocyte infiltration.\(^{21-27}\) In addition, our previous studies demonstrated that an intense inflammatory response triggered by CME increases the protein level of TNF-α in cardiomyocytes.\(^9\) Furthermore, our previous study revealed that cardiomyocyte apoptosis plays a significant role in CME-induced myocardial injury and that the expression of the pro-apoptotic protein caspase-3 in cardiomyocytes was elevated.\(^{10}\)
Levo has been developed for the treatment of acute heart failure and other cardiac conditions where the use of an inodilator is considered as appropriate. At least three major pharmacological actions have been identified, that is, (i) the selective binding to Ca2+-saturated cardiac troponin C, (ii) the opening of ATP-sensitive potassium (KATP) channels in the vasculature, and (iii) the opening of KATP channels in the mitochondria. In addition to its cardiotonic effect, Levo has been thought to have multiple cardiovascular benefits, including anti-inflammatory and anti-apoptotic. The pharmacology of Levo includes positive inotropy with energy-sparing effects, positive effects on ventriculo-arterial coupling, peripheral vasodilation, and increasing tissue perfusion, anti-stunning effects, and anti-inflammatory and anti-apoptotic effects. Levo has been shown to protect cardiomyocytes from undergoing apoptosis, which depends on ATP-sensitive K + channels. Levo has been shown to protect cardiomyocytes from undergoing apoptosis, which depends on ATP-sensitive K + channels.

Notably, in other tissues, Levo can also show proapoptotic effects. Experimental data focusing on Levo and apoptosis are not as abundant as in I/R injury. Pioneering in vitro work by Maytin et al. showed that Levo, even at very low concentrations, protected cardiomyocyte from hydrogen peroxide-induced apoptosis by activating mitochondrial ATP-dependent K + channels. This effect was counteracted by the K + channel inhibitor 5-hydroxydecanoid acid. Thus, a hypothesis of how Levo might influence I/R-induced cardiac apoptosis was provided. Highly interesting in this context, although investigated in a considerably different experimental setting, is the observation that Levo and dextrosimendan, another simendan, can induce caspase dependent apoptosis (assessed by DNA fragmentation). The molecular mechanism was mediated by c-Jun NH2-terminal kinase activation, but not by ATP-dependent K + channels. In addition, extracellular signal-regulated kinase and p38 mitogen-activated kinase signaling pathways seemed to play no role in its action. In a recently published study, 20 anesthetized (ketamine/pentobarbitone) and ventilated pigs underwent the left-sided thoracotomy. The second branch of the LAD was obstructed by a tourniquet resulting in about 50% reduced poststenotic myocardial systolic shortening. Hypoperfusion was maintained for 4 h. A bolus of 12 µg/kg Levo was administered over 15 min into the LAD proximal of the occluded branch. Levo prevented the downregulation of anti-apoptotic Bcl-2 and the release of cytochrome C from the mitochondria into the cytosol resulting in fewer fragmented nuclei in TUNEL staining. In addition to its cardiotonic effect, Levo has been thought to have cardiovascular benefits, including anti-inflammatory. In a recently published study, Levo displays anti-inflammatory effects and decreases...
myeloperoxidase bioavailability in patients with severe heart failure.\(^{[32]}\) Another study showed 5-year mortality in cardiac surgery patients with low CO syndrome treated with Levo, providing a prognostic evaluation of NT-proBNP and CRP.\(^{[32]}\)

At present Levo plays an important role in the process of cardiac ischemia-reperfusion injury, mainly by inhibiting myocardial apoptosis and inflammatory reaction. However, the pathologic mechanism of CME leading to cardiac function injury has been proved by a large number of previous studies to be achieved by inducing myocardial cell apoptosis and inflammatory reaction. Interestingly, the pathogenesis of CME and ischemia-reperfusion injury are similar, therefore, we have reason to suspect that whether can Levo in CME to play a role in the process of myocardial injury. In this study, we
found that pretreatment with Levo 24 h (0.05 µg/kg/min) and high loading dose of Levo (0.2 µg/kg/min) 60 min before the microsphere injection was associated with substantially reduced myocardial apoptosis and inflammation, and then reserved myocardial function. These results suggest that Levo can reduce CME-induced myocardial injury through inhibiting myocardial apoptosis and inflammation.

Taken together, in this study, we revealed an important role of cardiac-specific caspase 3 and TNF-α in the CME-induced myocardial apoptosis and inflammation. The results of this study highlighted that caspase 3 and TNF-α could be viewed as two potential interventional targets for the treatment of CME related myocardial apoptosis and inflammation. Moreover, the potential therapeutic role of Levo seemed to be related to its regulatory effects on the mitochondrial apoptotic pathway and inflammatory pathway. However, it should be noted that our was performed in a swine model and the findings may not be extrapolated directly to humans. Therefore, further research, especially the translational research in humans is needed to evaluate whether the potential regulatory effects of Levo on caspase 3 and TNF-α pathways could become a promising treatment strategy for CME-related cardiac dysfunction in clinical scenarios.

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Conflicts of interest
There are no conflicts of interest.

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