의학석사 학위논문

Cardioprotection by a Novel Necrosis Inhibitor in a Rat Model of Myocardial Ischemia-Reperfusion Injury

백서 심근허혈-재관류 모델에서 새로운 괴사억제제의 심장보호효과에 대한 연구

2013년 2월

서울대학교 대학원

의학과 내과학 전공

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A thesis of the Master's degree

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February 2013

Seoul National University

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Abstract

Introduction: NecroX-7, a novel necrosis inhibitor, blocks the opening of mitochondrial permeability transition pore, consequently inhibits necrotic cell death which is the main pathophysiology of ischemia-reperfusion (I/R) injury. We investigated the cardioprotective effect of NecroX-7 and the minimal effective dose (MED) *in vivo* I/R injury model.

Methods: Rat I/R injury model was obtained by ligation of left anterior descending coronary artery for 45 minutes followed by reperfusion. 5% dextrose (vehicle) or multiple dosages of NecroX-7 (0.03, 0.1, 0.3, 1.0, and 3.0 mg/kg) was injected intravenously 5 minutes before reperfusion. Necrosis area was measured 12 hours after I/R injury, using anti-myosin antibody (n=5 per group). Echocardiograms were performed at baseline, 3rd, 7th, and 14th days, and fibrosis area was measured at 14th day (n=5 per group).

Results: Necrosis area was smaller in rats treated with 0.3 mg/kg of NecroX-7 compared to vehicle-treated group (17.0±1.2 [0.3 mg/kg of NecroX-7] versus 39.3±3.3% [vehicle], P=0.004). Dosages below 0.3 mg/kg were not effective. Left ventricular ejection fraction (LVEF) at 14th day was 57.8±1.9% in NecroX-7 group and 42.6±3.8% in vehicle group (P=0.016). LV end-systolic and end-diastolic dimensions (LVESD and LVEDD) were significantly smaller in NecroX-7 group (LVESD, 4.6±0.2 [0.3 mg/kg of NecroX-7] versus 6.4±0.4 mm [vehicle], P=0.016; LVEDD, 7.2±0.2 [0.3 mg/kg of NecroX-7] versus 8.4±0.3 mm [vehicle], P=0.008). Fibrosis area was significantly smaller in NecroX-7-treated rats when the dosages were equal or higher than 0.3 mg/kg. Serum concentrations of inflammatory

cytokines confirmed the beneficial effect and the MED of NecroX-7.

Conclusions: Pretreatment with NecroX-7 reduces myocardial necrosis and preserves cardiac function and geometry in rat I/R injury model. The MED of NecroX-7 was 0.3 mg/kg. NecroX-7 is a potent candidate as a cardioprotective agent against I/R injury.

Keywords: ischemia-reperfusion injury, necrosis inhibitor, NecroX-7, cardioprotection

Student Number: 2011-21872

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Introduction

Restoration of coronary blood flow is the mainstay of treatment of myocardial infarction (1, 2), however, revascularization might cause unintended detrimental results including the generation of oxidative stress, increase in intracellular calcium ion (Ca^{2+}) and rapid correction of acidosis (3-5). These processes elicit the opening of mitochondrial permeability transition pore (mPTP) and render mitochondria to become uncoupled and capable of hydrolyzing rather than synthesizing adenosine triphosphate (ATP), which finally lead to the loss of ionic homeostasis and necrotic cell death, collectively called “reperfusion injury” (6-9). Although there have been numerous approaches to find a cardioprotective agent against the deleterious effects of ischemia-reperfusion (I/R) injury, the clinical benefits are still insufficient or inconclusive (10).

NecroX series is a novel class of chemical agent developed as an antagonist of high-motility group box-1 (HMGB1), which is integral to oxidative stress and downstream cell death or survival as well as plays a major role in the early event of I/R injury (11, 12). Several studies showed that NecroX series block the non-apoptotic cell death against mitochondrial oxidative stresses (13-17). NecroX-7, a derivative of NecroX series, exhibited cytoprotective effect against harmful stresses including pro-oxidant (tertiary-butylhydroperoxide), doxorubidin, CCl_4, and hypoxic injury in *in-vitro* H9C2

cells and *in vivo* hepatotoxicity models (13, 14).

Since the underlying pathophysiology of myocardial I/R injury involves mPTP opening by reactive oxygen species (ROS) (8, 18, 19), we hypothesized that NecroX-7 would preserve cardiac function and prevent ventricular remodeling from I/R injury through inhibition of necrosis. The aims of this study were to investigate the cardioprotective effect of NecroX-7 against I/R injury and to determine the minimal effective dose (MED) of NecroX-7.

Materials and Methods

We used female Sprague-Dawley rats aged 8 weeks and weighing 290-330 g. Rats were fed and housed, according to institutional rules, and the experimental protocol was approved by the Seoul National University Hospital Biomedical Research Institute, in compliance with the guide for the card and use of laboratory animals of Institutional Animal Care and Use Committee (IACUC No. 11-0230, 12-0289).

Materials: NecroX

NecroX-7 (compound of LC28-126 and LC25-153; LG Life Sciences, Daejeon, Korea) is a novel necrosis inhibitor, developed and manufactured by LG Life Science. The study drug in a powder-form was delivered to the laboratory and mixed with 0.5 mL of 5% dextrose. Dosages of NecroX-7 used in this study were 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, 1.0 mg/kg, and 3.0 mg/kg. Total volume of the mixture of NecroX-7 and 5% dextrose was 0.5 mL for all study animals. As a placebo, 0.5 mL of 5% dextrose was used.

Experimental protocol

The experiment in this study was consisted of two protocols; protocol #1 for quantification of necrosis 12 hours after IR injury (**Figure 1-A**) and protocol #2 for hemodynamic assessment until 2 weeks after the injury with

measurement of fibrosis (**Figure 1-B**). Various dosages of NecroX-7 were used for both protocols. As a placebo, 5% dextrose of same volume was used. Additionally, sham operation was performed which included thoracotomy but neither LAD ligation nor NecroX-7 injection.

Rats were randomly allocated to each protocol. Study animals were anesthetized by intraperitoneal (IP) injection of mix ketamine hydrochloride (100 mg/kg, Yuhan Corp., Seoul, Korea) and 10 mg/kg of xylazine (10 mg/kg, Bayer, Shawnee Mission, KS, USA). After anesthesia, the study animals were intubated and ventilated with a mixture of 100% oxygen and room air (Harvard Apparatus Inc., Holliston, MA, USA). Myocardial ischemia was induced by ligation of left anterior descending artery (LAD) next to the left atrium, with an 8-0 Ethilon suture and a section of polyethylene tubing placed over. Ischemic condition was maintained for 45 minutes and then reperfusion was done by untying the knot. The mixture of NecroX-7 or placebo was administered via right internal jugular vein, using 24 gauge needles.

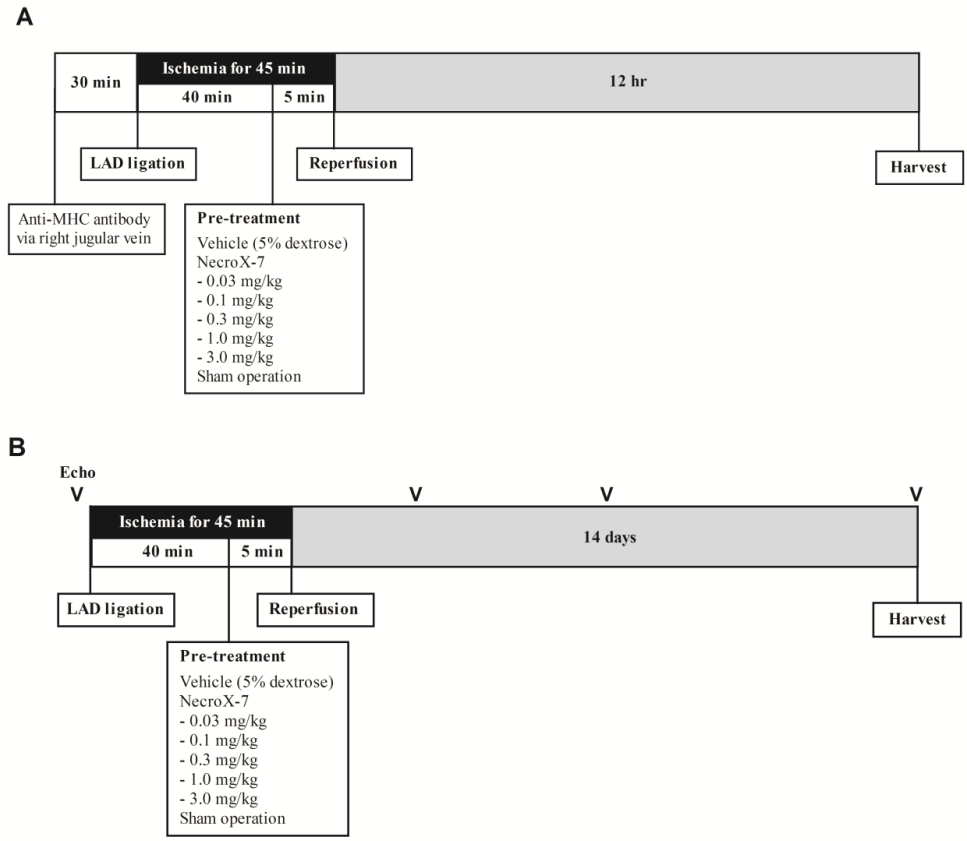

Figure 1. Experimental protocols. (A) Protocol #1 for quantification of necrosis in myocardium after ischemia-reperfusion (I/R) injury. **(B)** Protocol #2 for quantification of fibrosis and echocardiographic examination. In total, 70 rats were used (n=5 per group).

Protocol #1: quantification of necrosis

For quantification of necrosis, 5 rats per each group were used, totally 35 rats; 5 rats per each dosage of NecroX-7, vehicle and sham operation. Anti-myosin heavy chain (MHC) antibody was injected into the study animal 30 minutes before ischemic injury (20). The injected anti-MHC antibody would bind to the damaged myocardial sarcolemmal structures, thus indicating the necrosis area (21, 22). LAD ligation was maintained for 45 minutes and then reperfusion was followed by tying off the suture of LAD. Study drug of each dosage (0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, 1.0 mg/kg, and 3.0 mg/kg of NecroX-7 mixed in 0.5 mL of 5% dextrose) or placebo (0.5 mL of 5% dextrose) were injected 5 minutes before reperfusion. After 12 hour of IR injury, the study animal was sacrificed for harvest of cardiac tissue and blood sampling. The heart was embedded with OCT and underwent microscopic evaluations for quantification of necrosis area.

Blood samples were obtained at 12 hours after IR injury for assessment of serum concentration of interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α), using Bio-Plex Pro multiplex protein assay system (Bio-Rad Laboratories, Inc., CA, USA).

Protocol #2: hemodynamic assessment and measurement of fibrosis

For hemodynamic assessment and measurement of fibrosis, total 35 rats were used; 5 rats per group. Study animals were followed up with echocardiographic evaluation until 2 weeks after IR injury. LAD ligation was

performed and maintained for 45 minutes. Study drug or placebo was injected 5 minutes before reperfusion. Echocardiographic evaluation was performed at baseline (before LAD ligation), postoperative 3rd day, 7th day, and 14th day, under controlled anesthesia, using current ultrasound technology with a 9-MHz transducer (Nemio, Toshiba Co, Tokyo, Japan) and electrocardiographic monitoring. M-mode and 2-dimensional (2D) echocardiographic images were obtained in the parasternal short-axis views. The thickness of the interventricular septum and posterior wall, and left ventricular (LV) dimensions were determined at the tips of the papillary muscle, simulating the guideline of American Society of Echocardiography (23). To assess LV systolic function, we measured ejection fraction (EF) and fractional shortening (FS) for each rats, applying the following formula for human beings; FS = 100 × (LVEDD-LVESD)/LVEDD and EF = 100 × (LVEDD^3−LVESD^3)/LVEDD^3 (24). At postoperative 14th day, the study animals were sacrificed and the hearts were harvested and fixed with formalin.

Histological analysis: necrosis and fibrosis area measurement

For quantification of necrosis (protocol #1; **Figure 1-A**), rats pretreated with anti-MHC antibody were euthanized with carbon dioxide 12 hours after I/R injury. The rat hearts were harvested and cryopreserved in optical cutting temperature (OCT) compound (Tissue-Teck, Sakura, Torrance, CA, USA). Representative LV sections were cut into 4-μm slices from apex to base in a plane parallel to the atrioventricular groove and two slices were obtained at

midventricular level. For detection of the bindings of anti-MHC antibody to the injured sarcolemmal structure, the tissue sections were stained with secondary antibody (anti-mouse IgG HRP conjugated antibody) diluted in a blocking solution (1% BSA and 0.05% Tween 20). And then, the sections were incubated in room temperature for 90 minutes. For development of color reaction, AEC+ high sensitivity substrate chromogen (Cytomation, K3461; Dako) was used with hematoxylin (Chemmate, Code S2020, Dako) as counterstaining. Area of anti-MHC positive cells, which indicate necrosis, were quantitatively assessed by image analysis system (ImagePro version 4.5, MediaCybernetics, Bethesda, MD, USA) and provided as the ratio of necrosis area to total LV area.

Fibrosis areas were measured at 14 days after I/R injury (protocol #2; **Figure 1-B**). After euthanization, the rat hearts were embedded in paraffin and representative LV sections were cut into 4-μm slices. On Masson's trichrome-stained slides, myocardial fibrosis was quantitatively measured by image analysis software (ImagePro version 4.5, MediaCybernetics) and provided as the absolute area, length, and the ratio of fibrosis area to total LV area.

Statistical analysis

Data were averaged and shown as means ± standard error of means (SEM). For group comparisons, Kruskal-Wallis test or Mann-Whitney test were used. Multiple comparisons were performed by analysis of variance (ANOVA). All

statistical analyses were performed with software SPSS 18.0 (SPSS Inc; Chicago, IL, USA), and a P value <0.05 was considered statistically significant.

Results

Quantification of necrosis

In cardiac tissues harvested 12 hours after I/R injury, necrosis area was measured and provided as a ratio of necrotic myocardium to the total LV area (%). Percentages of necrosis area was the largest in vehicle group (39.3 ± 7.4%), whereas the smallest in NecroX-7 0.3 mg/kg group (17.0 ± 3.0%) (**Figure 2, Table 1**). Among the various dosages, the MED of NecroX-7 was 0.3 mg/kg. Higher concentrations, 1.0 mg/kg and 3.0 mg/kg, significantly reduced necrotic area compared with vehicle group. There were no difference between the groups of 0.3 mg/kg, 1.0 mg/kg, and 3.0 mg/kg of NecroX-7. Concentrations of NecroX-7 below 0.3 mg/kg slightly reduced necrotic area, but no significant difference was observed when compared with vehicle group.

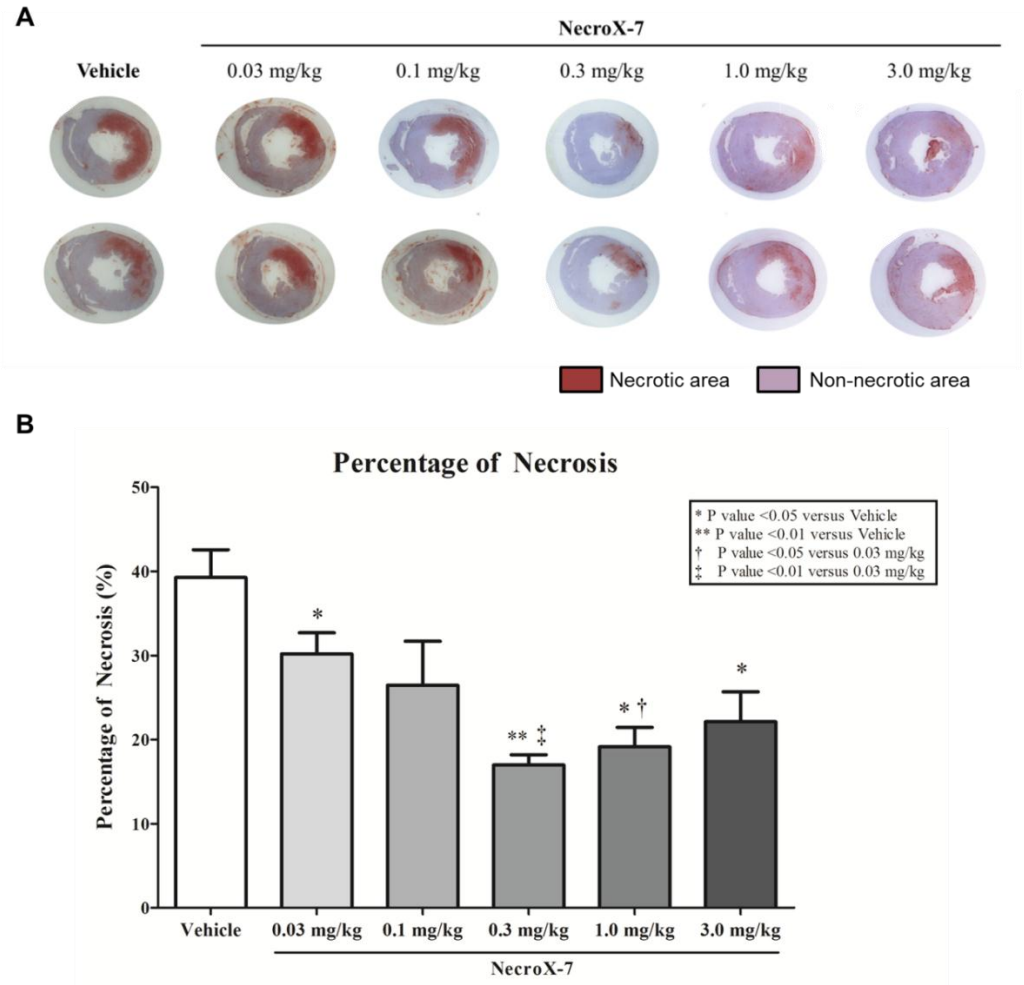

Figure 2. Myocardial necrosis area. Cardioprotective effect of NecroX-7 12 hours after ischemia-reperfusion injury were analyzed. **(A)** Representative figures of cardiac tissue sections, showing necrotic (dark brown) and non-necrotic zones (pinkish). **(B)** Comparisons of necrosis area, which was provided as the ratio of anti-myosin heavy chain (MHC) antibody positive cells to the total left ventricular (LV) area (%).

* P value <0.05 versus Vehicle, ** P value <0.01 versus Vehicle, † P value <0.05 versus 0.03 mg/kg, and ‡ P value <0.01 versus 0.03 mg/kg.

Inflammatory cytokines and cardiac enzymes

In accordance with the necrosis area measurements, inflammatory cytokines were lower in NecroX-7 treated groups of concentrations equal to 0.3 mg/kg or higher (**Table 2, Figure 3**). Concentrations lower than 0.3 mg/kg did not protect myocardium against I/R injury, showing similar results to vehicle treated group.

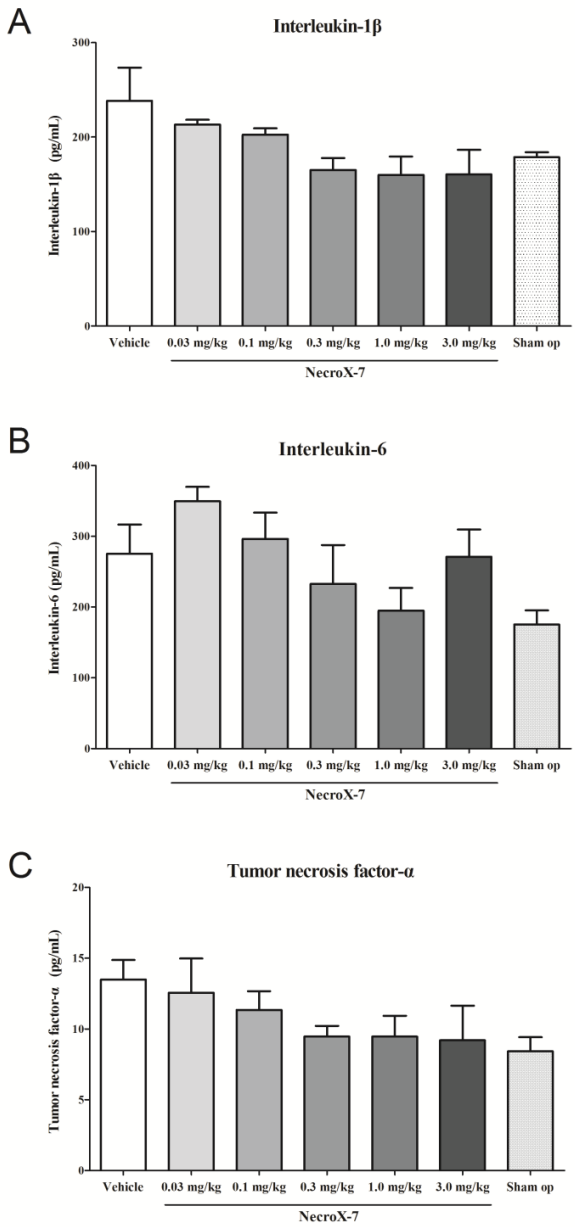

Figure 3. Inflammatory cytokines. Serum concentrations of **(A)** IL-1β, **(B)** IL-6 and **(C)** TNF-α were lower in NecroX-7 treated groups of concentrations equal to 0.3 mg/kg or higher.

Measurement of fibrosis

In study animals of protocol #2, myocardial fibrosis at 14th day was measured as the absolute area, length, and percentage of fibrotic myocardium to total LV area (**Figure 4, Table 3)**. Percentages of myocardial fibrosis were significantly smaller in NecroX-7 treated groups; 22.4±5.3% in vehicle group versus 6.8 ± 1.9% in NecroX-7 0.3 mg/kg group (P=0.028), 7.5 ± 2.0% in 1.0 mg/kg group (P=0.028), and 11.2 ± 1.0% in 3.0 mg/kg group (P=0.075). The absolute area and length of fibrosis were smaller in NecroX-7 treated groups but statistical significances were not coherent. Similar to other results, NecroX-7 0.03 mg/kg and 0.1 mg/kg were not effective in reduction of myocardial fibrosis after I/R injury.

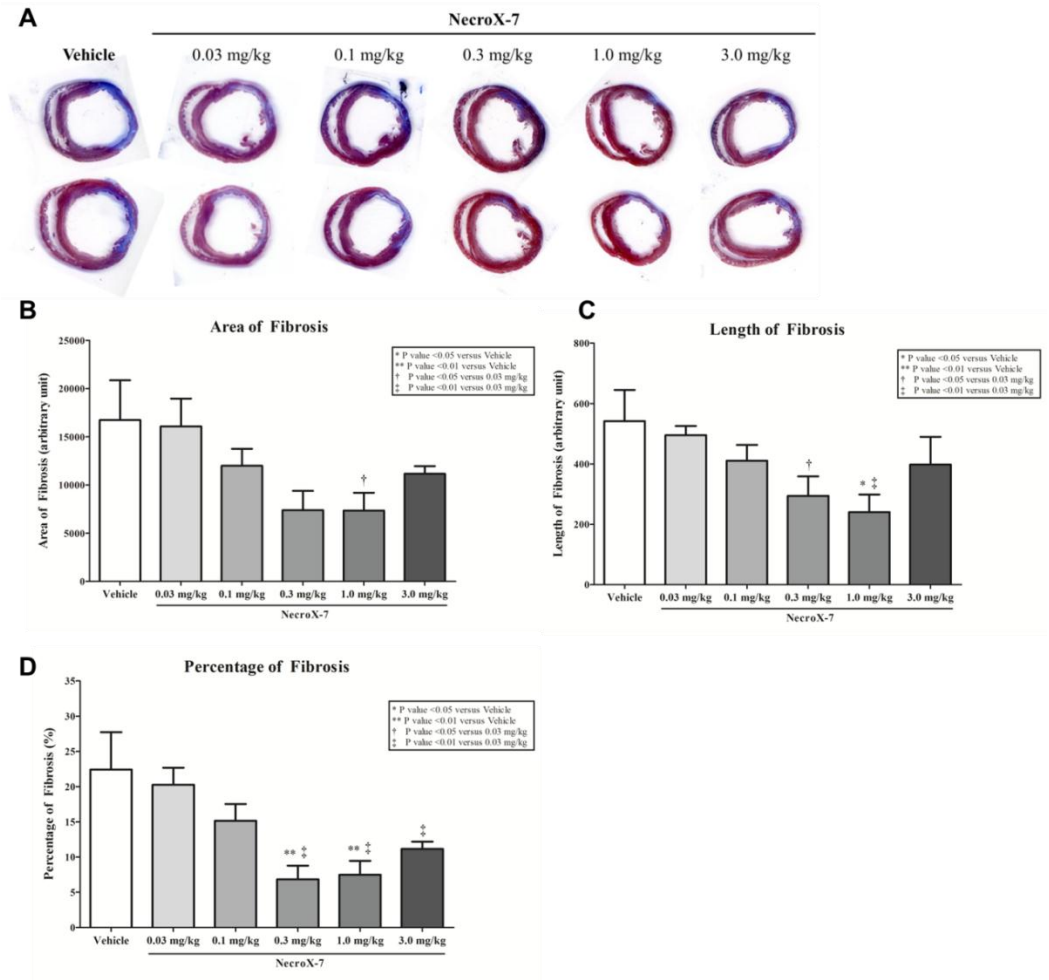

Figure 4. Myocardial fibrosis area. (A) Representative figures of Masson's trichrome-stained slides of cardiac tissue sections. **(B)** The absolute areas, **(C)** the lengths, and **(D)** the ratio of fibrosis area to the total left ventricular (LV) area (%) was provided.

* P value <0.05 versus Vehicle, ** P value <0.01 versus Vehicle, † P value <0.05 versus 0.03 mg/kg, and ‡ P value <0.01 versus 0.03 mg/kg.

Echocardiographic measurement

Echocardiograms were performed at baseline, postoperative 3rd day, 7th day, and 14th day, measuring LVEF(%), LVFS (%), LVESD (mm), and LVEDD (mm) (**Figure 5, Table 4**). Compared with control group, animals that received NecroX-7 showed better LV systolic function and remodeling profiles, except those received 0.03 mg/kg and 0.1 mg/kg of NecroX-7. At postoperative 3rd day, echocardiographic parameters of study groups were similar, whereas LV systolic function was impaired in placebo group. After postoperative 3rd day, echocardiographic parameters showed significant differences between the groups. LV systolic function, assessed in LVEF and LVFS, were preserved in subjects those received NecroX-7 0.3 mg/kg, 1.0 mg/kg, and 3.0 mg/kg. LVEF and LVFS of these groups were significantly higher than those in NecroX-7 0.03 mg/kg group. Preventive effect on LV remodeling was also significant in these groups. In subjects received 0.03 mg/kg or 0.1 mg/kg of NecroX-7, LVESD and LVEDD were not different compared with vehicle group at every time point. NecroX-7, in concentrations of 0.3 mg/kg or more, reduced LV cavity dimensions, showing significant differences at postoperative 7th and 14th days. Pretreatment with NecroX-7 preserved LV systolic function and attenuated LV remodeling, to a similar degree of sham operation group.

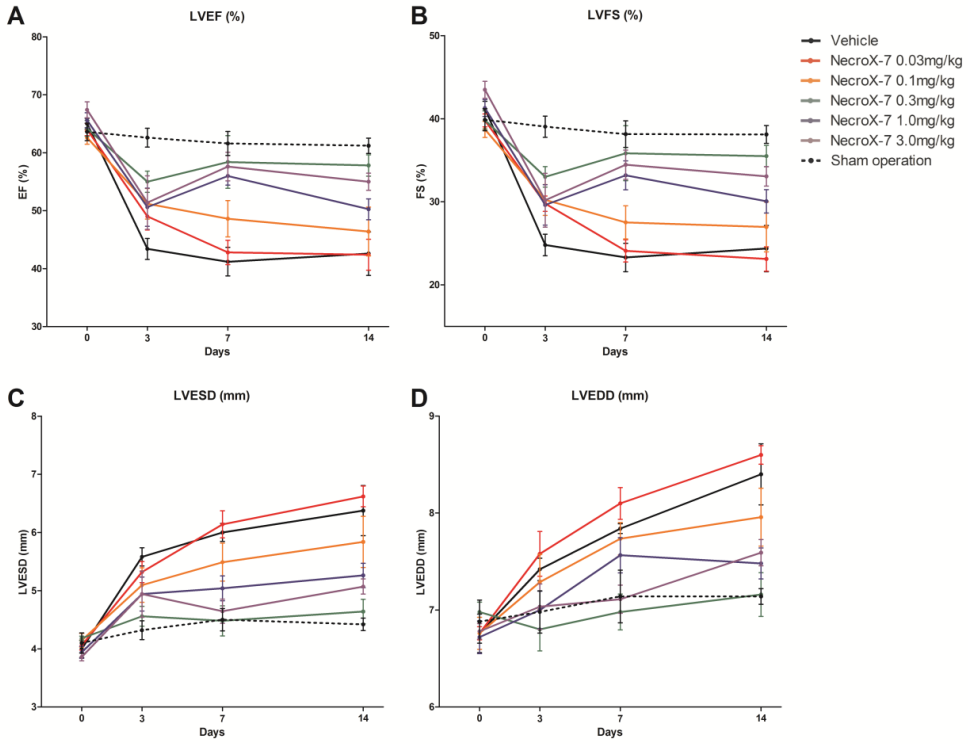

Figure 5. Echocardiographic analysis. Echocardiograms were performed before ischemia-reperfusion (I/R) injury, 3rd, 7th, and 14th day after surgery. **(A)** EF and **(B)** FS were significantly impaired in rats pretreated with vehicle (5% dextrose) or 0.03 mg/kg of NecroX-7. NecroX-7 0.1 mg/kg was also ineffective. Higher dosages of NecroX-7 showed cardioprotective effect against I/R injury. **(C)** ESD and **(D)** EDD showed that NecroX-7 could prevent LV remodeling. LV cavity sizes were significantly smaller in rats pretreated with NecroX-7 0.3 mg/kg or higher concentrations.

Black-line; vehicle, red-line; NecroX-7 0.03 mg/kg, orange-line; NecroX-7 0.1 mg/kg, green-line; NecroX-7 0.3 mg/kg, blue-line; NecroX-7 1.0 mg/kg, purple-line; NecroX-7 3.0 mg/kg, black dotted-line; sham operation.

Discussion

NecroX-7, a novel necrosis inhibitor, showed myocardial protective effect in rat I/R injury model. Necrosis area measured at 12 hours after IR injury was significantly smaller in NecroX-7 treated group. LV systolic function was preserved in NecroX-7 pretreated groups, while LV remodeling was attenuated. Fibrosis area, measured at 14^{th} day after I/R injury, also revealed beneficial effect of NecroX-7. The MED of NecroX-7 was 0.3 mg/kg whereas the lower concentrations were ineffective but revealed a dose-response relationship.

Mechanism of myocardial ischemia-reperfusion injury

Reperfusion injury, paradoxically caused by restored blood flow to the ischemic myocardium, leads to the additional death of cardiomyocytes (4). Also myocardial I/R injury is cited as a reason behind the still high rates of death and heart failure those cannot be further decreased, and thus, is an important issue to be overcome (9). During reperfusion, oxidative stress generates substantial necrotic cell death of myocardium (25). ROS induces dysfunction of the sarcoplasmic reticulum and sarcolemmal membrane damage, which are attributable to the increase in intracellular and mitochondrial Ca^{2+}, resulting in the hypercontracture and death of cardiomyocytes (26, 27). Rapid restoration of pH during reperfusion is

another contributor of I/R injury (5, 28). These series of events lead to the opening of mPTP as a consequence. Previous experiments established that oxidative stress, Ca^{2+} overload, and rapid restoration of acidosis result in the opening of mPTP and mitochondrial dysfunction (29-31). Induction of inflammatory cytokines such as IL-1β, IL-6, and TNF-α is a resultant consequence of I/R injury, while the cytokines activates further inflammatory response (32-34).

Myocardial protective effect of NecroX-7

In the present study, we used anti-MHC antibody to detect the exposed myosin heavy chain in damaged sarcolemmal membrane. Among the few methodologies capable of detecting of necrosis, the use of anti-MHC antibody provides a safe, reliable, and specific quantification of necrosis and *in vivo* binding of anti-MHC antibody to necrotic myocytes was established by a series of previous reports (20-22, 35-37). Therefore, the significant difference in the anti-MHC antibody positive area directly indicates the cardioprotective effect of NecroX-7 against I/R injury induced myocardial necrosis.

Cardioprotective effect of NecroX-7 was also confirmed by the release of inflammatory cytokines. Harmful injuries to myocardium, including I/R injury as in the present study, trigger a cytokine cascade which is initiated by the release of TNF-α (34, 38, 39). *Deten et al* showed that mRNA expressions of IL-1β and IL-6 were elevated few hours after myocardial infarction (40). In the present study, pretreatment with NecroX-7 reduced the releases of IL-1β,

IL-6 and TNF-α 12 hours after I/R injury. This result implies that NecroX-7 could prevent cell death at early stage of necrotic pathway and inhibit further inflammatory reaction at the reperfused myocardium.

Myocardial fibrosis was substantially suppressed regardless of the measurement criteria, showing smaller absolute area, shorter length, and lower percentage of fibrosis. Moreover, NecroX-7 preserved LV systolic function and attenuated LV remodeling on echocardiograms. Inhibition of necrotic cell death immediately after I/R injury prevented myocardial fibrosis and remodeling. Considering that prognosis of myocardial injury is significantly compromised by LV remodeling (41, 42), the effect of NecroX-7 is not limited to the short-term cardioprotection but includes long-term prognostic benefit.

The results of our study support the previously established mechanisms of NecroX-7 (13-17). This novel chemical agent showed scavenging activity against ROS and reactive nitrogen species (13). NecroX-5, one of the derivatives of NecroX series, provided a strong protection of mitochondria against I/R injury by reducing oxidative stress, preserving mitochondrial membrane potential, improving mitochondrial oxygen consumption, and attenuating mitochondrial Ca^{2+} accumulation, as a mitochondrial Ca^{2+} uniporter inhibitor (17). Inhibition of NADP oxidase activity was another important mechanism of cytoprotection by NecroX-7 (16). Additionally, *Lee et al* reported that NecroX-7 could improve the efficacy of transplantation of fibroblasts into rat hearts, suggesting its potent cytoprotective effect and

promising role in cell therapy (10, 15).

Clinical perspective of NecroX-7

It should be noted that only a single bolus intravenous injection, during myocardial ischemia before reperfusion, showed significant cardioprotective effects. This feature of NecroX-7 has a tremendous clinical potential for treatment of patients with acute myocardial infarction (AMI), for whom revascularization should be established as soon as possible (43). In such a situation, the easier and faster methods are more favorable. A bolus of NecroX-7 might be administered during a preparatory period before definitive revascularization therapy in AMI patients. This convenience would facilitate the clinical application of NecroX-7.

In addition, NecroX-7 has a potential to be applied in various disease conditions where I/R injury is involved, including brain, liver, kidney, lung, and other organs (7). Postoperative or post-transplantation organ preservations are also possible candidates. Tissue and organ damages of these conditions are attributable to oxidative stress, which elicits the opening of mPTP and necrotic cell death (7, 44). Broad spectrum of experimental studies and proof-of-concept trials are required for relevant applications of NecroX-7.

The minimal effective dose of NecroX-7

NecroX-7 protected myocardium against I/R injury in dose-dependent manner. Cardioprotective effect of NecroX-7 reached plateau in

concentrations of 0.3 mg/kg or higher. Although the higher concentrations of NecroX-7 resulted in slightly larger myocardial necrosis and fibrosis, there were no significant differences compared to 0.3 mg/kg of NecroX-7. Also we could observe a dose-response relationship among the concentrations of 0.3 mg/kg or lower. As this threshold was concordant over the various experimental protocols in this study, we concluded that the MED of NecroX-7 in rat I/R model is 0.3 mg/kg. These pharmacological profiles provide valuable preclinical data as well as facilitate the first-in-human clinical trials (45, 46).

Limitations

This study is subject to several limitations. First, serum concentrations of NecroX-7 were not provided. However, serum and tissue concentrations of lower dosages of NecroX-7 were not reliable because their values were below the reference range, and moreover, pharmacokinetic and pharmacodynamic information were not the main scope of this study. Second, we could not suggest molecular mechanisms of NecroX-7 on myocardial protection. Although we showed the differences in inflammatory cytokines which are indirect evidence of the inhibitory effect of NecroX-7 against necrotic cell death, further experimental studies are required. Despite these limitations, this study contains valuable insights regarding cardioprotection against I/R injury and facilitates the first-in-human trial through suggesting the MED.

Conclusion

Pretreatment with NecroX-7 reduces myocardial necrosis and preserves cardiac function and geometry in rat I/R injury model. The MED of NecroX-7 was 0.3 mg/kg. NecroX-7 is a potent candidate as a cardioprotective agent against I/R injury.

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초 록

서론: 새로 개발된 괴사억제제 NecroX-7은 미토콘드리아 투과성 변이공 (mitochondrial permeability transition pore)의 개방을 차단함으로써 허혈-재관류 손상의 주요 기전인 세포 괴사(necrosis)를 억제한다. 본 연구에서는 생체 내 (in vivo) 허혈-재관류 손상 모델에서 NecroX-7의 심장보호효과를 확인하고 최소유효용량을 알아보았다.

방법: 랫드의 좌전하행 관상동맥을 45분간 결찰한 뒤, 결찰 부위를 해제함으로써 허혈-재관류 손상 모델을 만들었다. 대조약제 (5% dextrose) 또는 다양한 용량의 NecroX-7 (0.03, 0.1, 0.3, 1.0, and 3.0 mg/kg)을 재관류 5분 전에 정맥투여 하였으며, 각 군당 5마리의 랫드를 사용하였다. 항미오신항체(anti-myosin antibody)를 사용하여, 허혈-재관류 손상 후 12시간 뒤에 심근괴사의 면적을 측정하였다. 심초음파검사를 허혈-재관류 손상 전, 3일째, 7일째, 14일째에 시행하였으며, 14일째에 심근 섬유화 면적을 측정하였다.

결과: 심근괴사 면적은 0.3 mg/kg의 NecroX-7을 투여받은 군이 대조약제를 투여받은 군에 비해 유의하게 작았다 (17.0±1.2 [0.3 mg/kg of NecroX-7] versus 39.3±3.3% [vehicle], P=0.004). 투여된 NecroX-7이 0.3 mg/kg 미만인 경우에는 심근괴사를 억제하는 효과가 관찰되지 않았

다. 14일째에 측정한 좌심실 구혈율은 NecroX-7을 투여받은 군에서 57.8±1.9% 였던 반면, 대조약제 군에서는 42.6±3.8%로 현저한 차이를 보였으며 (P=0.016), 좌심실 수축기말 직경 및 이완기말 직경은 NecroX-7을 투여받은 군에서 유의하게 작았다. 심근 섬유화 면적은 0.3 mg/kg 이상의 NecroX-7을 투여받은 군에서 유의하게 작게 나타났다. 혈중 염증성 사이토카인 (inflammatory cytokine) 농도 역시 NecroX-7의 심장보호효과를 입증하였으며, 최소유효용량 (0.3 mg/kg)도 동일한 결과를 나타냈다.

결론: 허혈-재관류 손상 모델에서 NecroX-7 전처치는 심근괴사 및 섬유화를 억제하고, 심장 기능을 보존하며, 심장 재형성을 억제하였다. NecroX-7의 최소유효용량은 0.3 mg/kg로 나타났다. NecroX-7은 허혈-재관류 손상에 대한 심근보호제로서 강력한 후보물질이다.

주요어: 허혈-재관류 손상, 괴사 억제제, NecroX-7, 심근보호

학번: 2011-21872

Table 1. Quantification of necrosis

	Vehicle	NecroX-7					Overall P
		0.03 mg/kg	0.1 mg/kg	0.3 mg/kg	1.0 mg/kg	3.0 mg/kg	
Necrosis area (% of total LV area)	39.3 ± 3.3	30.2 ± 2.5 *	26.5 ± 5.2	17.0 ± 1.2 **‡	19.1 ± 2.3 *†	22.1 ± 3.6 *	0.007

Values are means ± standard error of means (SEM). Area of necrosis was provided as the ratio of necrosis area to total left ventricular (LV) area (%).

* P value <0.05 versus Vehicle, ** P value <0.01 versus Vehicle, † P value <0.05 versus 0.03 mg/kg, and ‡ P value <0.01 versus 0.03 mg/kg.

Abbreviations; LV = left ventricular.

Table 2. Inflammatory cytokines

	Vehicle	NecroX-7					Sham op
		0.03 mg/kg	0.1 mg/kg	0.3 mg/kg	1.0 mg/kg	3.0 mg/kg	
IL-1β (pg/mL)	238.4 ± 35.0	213.2 ± 5.2	202.4 ± 6.8	165.2 ± 12.7	159.9 ± 19.4	160.6 ± 25.9	178.8 ± 5.2
IL-6 (pg/mL)	308.6 ± 40.7	349.2 ± 20.4	296.2 ± 37.2	232.7 ± 54.9	194.5 ± 32.3	271.1 ± 38.4	175.4 ± 19.7
TNF-α (pg/mL)	12.1 ± 2.0	12.6 ± 2.4	11.3 ± 1.3	9.5 ± 0.7	9.5 ± 1.5	9.2 ± 2.5	8.4 ± 1.0

Values are means ± standard error of means (SEM). Inflammatory cytokines were measured 12 hours after ischemia-reperfusion injury.

Abbreviations; IL = interleukin, TNF = tumor necrosis factor.

Table 3. Quantification of fibrosis area

	Vehicle	NecroX-7					Overall P
		0.03 mg/kg	0.1 mg/kg	0.3 mg/kg	1.0 mg/kg	3.0 mg/kg	
Fibrosis area	16738.4	16087.6	12003.0	7393.4	7350.6	11158.0	0.094
(arbitrary unit)	± 4122.3	± 2879.3	± 1751.5	± 2003.3	± 1854.8 †	± 801.5	
Fibrosis length	542.0	495.5	410.6	294.5	240.4	398.0	0.049
(arbitrary unit)	± 103.2	± 29.9	± 52.4	± 64.6 †	± 58.2 *‡	± 91.9	
Fibrosis percentage	22.4	20.3	15.1	6.8	7.5	11.2	0.004
(% of total LV area)	± 5.3	± 2.4	± 2.4	± 1.9 *‡	± 2.0 *‡	± 1.0 ‡	

Values are means ± standard error of means (SEM). Area of fibrosis was provided as the ratio of necrosis area to total left ventricular (LV) area (%).

* P value <0.05 versus Vehicle, ** P value <0.01 versus Vehicle, † P value <0.05 versus 0.03 mg/kg, and ‡ P value <0.01 versus 0.03 mg/kg.

Table 4. Echocardiographic parameters

	Vehicle	NecroX-7					Sham op	Overall P
		0.03 mg/kg	0.1 mg/kg	0.3 mg/kg	1.0 mg/kg	3.0 mg/kg		
EF (%)								
Baseline	65.0 ± 1.0	64.0 ± 1.1	62.6 ± 1.1	64.2 ± 1.4	65.6 ± 1.3	67.4 ± 1.4	63.6 ± 1.5	0.168
Day 3	43.4 ± 1.8	49.0 ± 2.4	51.2 ± 2.6 *	55.0 ± 1.8 **	50.6 ± 3.3	51.4 ± 4.6	62.6 ± 1.6 **‡	0.164
Day 7	41.2 ± 2.5	42.8 ± 2.1	48.6 ± 3.1	58.4 ± 4.6 *†	56.0 ± 1.6 **‡	57.6 ± 2.5 *†	61.6 ± 2.1 **‡	0.005
Day 14	42.6 ± 3.8	42.4 ± 2.7	46.4 ± 4.2	57.8 ± 1.9 *‡	50.3 ± 1.8	55.0 ± 1.5 *‡	62.0 ± 1.6 *‡	0.007
FS (%)								
Baseline	41.2 ± 0.9	39.8 ± 0.8	38.7 ± 1.0	39.8 ± 1.1	41.3 ± 1.0	43.5 ± 1.0	39.9 ± 1.3	0.038
Day 3	24.8 ± 1.3	29.8 ± 0.9 **	30.2 ± 1.9	33.0 ± 1.2 **	29.6 ± 2.4	30.2 ± 3.2	39.0 ± 1.3 **‡	0.141
Day 7	23.3 ± 1.7	24.1 ± 1.3	27.5 ± 2.0	35.8 ± 3.3 *†	33.2 ± 1.7 *†	34.5 ± 1.8 *†	38.1 ± 1.6 **‡	0.005
Day 14	24.4 ± 2.8	23.1 ± 1.5	27.0 ± 3.0	35.5 ± 1.3 *‡	30.1 ± 1.4 †	33.1 ± 1.2 *‡	38.1 ± 1.1 *‡	0.006

ESD (mm)								
Baseline	4.0 ± 0.2	4.1 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	4.1 ± 0.2	0.149
Day 3	5.6 ± 0.2	5.3 ± 0.2	5.1 ± 0.3	4.6 ± 0.2 **†	4.9 ± 0.3	5.0 ± 0.4	4.3 ± 0.2 **‡	0.193
Day 7	6.0 ± 0.2	6.1 ± 0.2	5.5 ± 0.3	4.5 ± 0.3 *†	5.0 ± 0.2 *†	4.7 ± 0.2 **‡	4.5 ± 0.2 **‡	0.001
Day 14	6.38 ± 0.4	6.6 ± 0.2	5.8 ± 0.4	4.6 ± 0.2 *‡	5.3 ± 0.2 †	5.1 ± 0.1 *‡	4.4 ± 0.1 *‡	0.006
EDD (mm)								
Baseline	6.8 ± 0.2	6.8 ± 0.1	6.8 ± 0.2	7.0 ± 0.1	6.7 ± 0.2	6.8 ± 0.1	6.9 ± 0.2	0.647
Day 3	7.4 ± 0.1	7.6 ± 0.2	7.3 ± 0.3	7.0 ± 0.2	7.0 ± 0.2	7.0 ± 0.2	7.0 ± 0.2	0.327
Day 7	7.8 ± 0.1	8.1 ± 0.2	7.7 ± 0.2	7.1 ± 0.2 **‡	7.6 ± 0.2	7.1 ± 0.1 **‡	7.1 ± 0.3 *†	0.005
Day 14	8.4 ± 0.3	8.6 ± 0.1	8.0 ± 0.3	7.2 ± 0.2 **‡	7.5 ± 0.2 *†	7.6 ± 0.1 ‡	7.1 ± 0.1 *‡	0.006

Values are means ± standard error of means (SEM). Echocardiograms were performed at baseline, 3rd, 7th, and 14th day after ischemia-reperfusion injury. Overall P values indicate the differences between vehicle and various dosages of NecroX-7 groups at each time point.

* P value <0.05 versus Vehicle, ** P value <0.01 versus Vehicle, † P value <0.05 versus 0.03 mg/kg, and ‡ P value <0.01 versus 0.03 mg/kg.

Abbreviations; EF = ejection fraction, FS = fractional shortening, ESD = end-systolic dimension, EDD = end-diastolic dimension.