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Dexmedetomidine preconditioning for myocardial protection in ischaemia-reperfusion injury in rats by downregulation of the high mobility group box 1-toll-like receptor 4-nuclear factor κB signalling pathway

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Summary
Pharmacological preconditioning reduces myocardial infarct size in ischaemia-reperfusion (I-R) injury. Dexmedetomidine, a selective α₂-adrenoceptor agonist, has a proven cardioprotective effect when administered prior to I-R, although the underlying mechanisms for this effect are not fully understood. We evaluated whether dexmedetomidine preconditioning could induce a myocardio-protective effect against I-R injury by inhibiting associated inflammatory processes through downregulation of the high mobility group box 1 (HMGB1)-toll-like receptor 4 (TLR4)-nuclear factor κB (NF-κB) signalling pathway. Seventy rats were randomly assigned to seven groups: a control and six test groups, involving I-R for 30 and 120 minutes, respectively, in isolated rat hearts and different pretreatment protocols with dexmedetomidine (10 nmol/L) as well as yohimbine (1 μmol/L) and recombinant HMGB1 peptide (rHMGB1; 20 μg/L), alone or in combination with dexmedetomidine. Cardiac function was recorded; myocardial HMGB1, TLR4, and NF-κB activities and levels of tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were measured as were lactate dehydrogenase (LDH) and creatine kinase (CK) in coronary outflow. Dexmedetomidine preconditioning significantly improved cardiac function (P<.05), downregulated the expression of HMGB1-TLR4-NF-κB, reduced levels of TNF-α and IL-6 in isolated ventricles during I-R injury, and significantly reduced CK and LDH levels in coronary outflow (P<.05). All of these effects were partially reversed by yohimbine (P<.05) or rHMGB1 (P<.05). Dexmedetomidine preconditioning alleviated myocardial I-R injury in rats through inhibition of inflammatory processes associated with downregulation of the HMGB1-TLR4-NF-κB signalling pathway via activation at α₂-adrenergic receptors.

KEYWORDS
dexmedetomidine, high mobility group box 1-toll-like receptor 4-nuclear factor κB, myocardial protection, preconditioning
1 | INTRODUCTION

Immune and inflammatory pathways initiated by the innate immune system have been implicated in myocardial ischaemia-reperfusion (I-R) injury. High mobility group box 1 (HMGB1)—a highly conserved nuclear protein released from necrotic cells and secreted by activated macrophages, natural killer cells, and mature dendritic cells—has been implicated in modulation of myocardial I-R injury. In addition, previous studies have shown that levels of HMGB1 in serum were increased in patients with acute myocardial infarction and were associated with poor outcomes.7

Toll-like receptor 4 (TLR4) signalling has been implicated in cardiac dysfunction induced by haemorrhagic shock and has, moreover, been linked to the production of proinflammatory mediators following myocardial I-R.8-10 Ischaemia is a potent trigger for HMGB1 release, and TLR4 is a known pathway for HMGB1 in a potential feed-forward mechanism.5,11 HMGB1 binds to TLR4, leading to the activation of downstream signalling molecules such as nuclear factor κB (NF-κB), thereby promoting the release of proinflammatory cytokines, including tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6).5,12,13 Therefore, these data indicate an important role of HMGB1-TLR4-NF-κB signalling in the evolution of myocardial I-R injury.

Dexmedetomidine, a selective α2-adrenoreceptor agonist, offers good perioperative hemodynamic stability, reduced intraoperative anaesthetic requirements, and has proven anti-inflammatory effects.14 Perioperative dexmedetomidine use is associated with decreased postoperative mortality for up to 1 year, with decreased incidence of postoperative complications and delirium in patients undergoing cardiac surgery.15 Moreover, dexmedetomidine showed cardioprotective properties when administered prior to I-R events (dexmedetomidine preconditioning).16,17 A recent study demonstrated that dexmedetomidine mitigated the expression of several inflammatory molecules, including nitric oxide, TNF-α, interleukin-1β (IL-1β), and IL-6, through inhibition of the NF-κB signalling pathway and activation of α2-adrenergic receptors.18 Additionally, the study showed that dexmedetomidine inhibited the expression of proinflammatory cytokines (TNF-α and IL-6) and HMGB1 in serum as well as that of HMGB1 mRNA in lung tissues of mice with sepsis induced by cecal ligation and puncture (CLP).19 Gu et al.20 reported that dexmedetomidine inhibited activation of TLR4 signalling and generation of HMGB1, thereby inducing renoprotective effects against I-R injury.

At present, mechanisms underlying cardioprotective properties of dexmedetomidine preconditioning in I-R injury remain unexplored. This study was designed to test our hypotheses that: (i) dexmedetomidine preconditioning can exert a myocardial protective effect against global I-R injury in a Langendorff perfusion system; and (ii) this protective effect is exerted through the downregulation of the HMGB1-TLR4-NF-κB signalling pathway via activation of α2-adrenergic receptors.

2 | RESULTS

2.1 | Myocardial function

The study design is shown in Figure 1. Seventy isolated rat hearts were perfused via the ascending aorta in a Langendorff perfusion system (ALC-HP, Shanghai, China) for 20 minutes prior to 30 minutes of normothermic global ischaemia that was followed by 120 minutes of reperfusion. After a 20-minute equilibration, specimens were randomly assigned to one of the following groups: C, control group (C group), no ischaemia; 2, ischaemia-reperfusion group (I-R group), 30 minutes of global ischaemia followed by 120 minutes of reperfusion; 3, dexmedetomidine group (D group), dexmedetomidine 10 nmol/L, 25 minutes before ischaemia; 4, dexmedetomidine-yohimbine group (DY group), yohimbine 1 μmol/L and dexmedetomidine 10 nmol/L, 25 minutes before ischaemia, respectively; 5, yohimbine group (Y group), yohimbine 1 μmol/L, 30 minutes before ischaemia; 6, dexmedetomidine-rHMGB1 group (HD group): recombinant HMGB1 peptide (rHMGB1) 20 μg/L and dexmedetomidine 10 nmol/L, 30 and 25 minutes before ischaemia, respectively; and 7, rHMGB1 group (H group) rHMGB1 20 μg/L, 30 minutes before ischaemia.

Dexmedetomidine preconditioning significantly improved post-ischaemic cardiac functional recovery in an ex vivo isolated rat heart model, as evidenced by higher left ventricular pressure peak rates of positive and negative changes (±dP/dt) and higher left ventricular developed pressure (LVDP), and these effects were reversed by the...
2.2 Expression of HMGB1-TLR4-NF-κB by western blot analysis

We detected HMGB1-TLR4-NF-κB expression in isolated left ventricles after 120 minutes of reperfusion using western blot analysis. Compared to the control group, I-R injury induced an increase in HMGB1-TLR4-NF-κB expression following 30 minutes of ischemia and 120 minutes of reperfusion (P<.05). Dexmedetomidine significantly suppressed HMGB1-TLR4-NF-κB expression in isolated left ventricles (P<.05), but this was partially reversed by yohimbine, an α2-adrenergic receptor antagonist. Moreover, co-treatment with rHMGB1, enhanced HMGB1-TLR4-NF-κB activity, and this was partially reduced by dexmedetomidine (P<.05; Figure 3A-D).

2.3 Analysis of cytokine levels

Compared with the control group, I-R injury resulted in increased serum levels of TNF-α and IL-6 after 120 minutes of reperfusion (P<.05). Dexmedetomidine significantly decreased levels of cardiac TNF-α (P<.05) and IL-6 (P<.05), and this effect was partially reversed by rHMGB1 (P<.05; Figure 4A,B).

Both LDH and CK levels were nearly undetectable in the control group. After I-R, LDH and CK levels were markedly elevated in the other six groups, as compared to the C group (P<.05). Dexmedetomidine preconditioning prevented this marked increase in LDH and CK levels (P<.05), and this was partly reversed by yohimbine or rHMGB1 co-treatment (P<.05; Figure 4C,D).

2.4 Immunohistochemical analysis of HMGB1 and NF-κB

Positive protein expression of HMGB1 and NF-κB was identified by blue cytoplasmic staining. Both HMGB1 and NF-κB were strongly expressed in group I-R, as indicated by the increased numbers of HMGB1- and NF-κB-positive cells (P<.05). However, HMGB1 and NF-κB expression decreased following dexmedetomidine treatment,
TABLE 1 Haemodynamic values across study groups

<table>
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<tr>
<th></th>
<th>C</th>
<th>IR</th>
<th>D</th>
<th>DY</th>
<th>Y</th>
<th>HD</th>
<th>H</th>
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<td>+dp/dt (mm Hg/s)</td>
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<tr>
<td>Baseline</td>
<td>2192.11±65.98</td>
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<td>2219.00±99.83</td>
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<td>1577.42±131.44*</td>
<td>1325.84±122.50*</td>
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<td>120 minutes</td>
<td>2064.66±69.11</td>
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<td>1110.98±84.45*</td>
<td>1027.58±65.80*</td>
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<td>Baseline</td>
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<td>1463.36±61.89</td>
<td>1440.23±99.87</td>
<td>1485.75±106.65</td>
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<td>60 minutes</td>
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<td>1191.22±107.39</td>
<td>863.23±97.74*</td>
<td>863.24±59.35*</td>
<td>1008.80±31.08*</td>
<td>945.76±63.32*</td>
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<td>120 minutes</td>
<td>1187.19±48.67</td>
<td>834.73±61.05*</td>
<td>1031.37±17.67</td>
<td>886.64±72.51*</td>
<td>785.81±58.56*</td>
<td>796.34±60.01*</td>
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<td>81.62±2.84</td>
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<td>50.11±7.42*</td>
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<td>64.01±1.45</td>
<td>54.07±7.63*</td>
<td>52.34±4.52*</td>
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<td>20.60±1.88*</td>
<td>23.42±1.88*</td>
<td>26.45±3.46*</td>
<td>22.02±3.03*</td>
<td>24.45±3.23*</td>
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<td>120 minutes</td>
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<td>23.18±1.08*</td>
<td>23.01±1.08*</td>
<td>24.63±2.21*</td>
<td>23.86±3.50*</td>
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<td>CF (mL/min)</td>
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<td>Baseline</td>
<td>13.94±0.88</td>
<td>14.12±0.93</td>
<td>15.66±1.12</td>
<td>14.98±1.33</td>
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<td>60 minutes</td>
<td>12.06±0.81</td>
<td>8.84±0.59</td>
<td>11.06±0.60</td>
<td>8.78±1.59*</td>
<td>7.96±1.15*</td>
<td>8.36±0.42*</td>
<td>7.9±0.24*</td>
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<td>120 minutes</td>
<td>11.14±0.76</td>
<td>7.32±0.22*</td>
<td>9.5±0.52</td>
<td>7.48±1.62*</td>
<td>6.76±0.75*</td>
<td>7.1±0.55*</td>
<td>6.04±0.56*</td>
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<td>HR (b.p.m.)</td>
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<tr>
<td>Baseline</td>
<td>244.01±8.43</td>
<td>248.75±8.27</td>
<td>240.07±6.14</td>
<td>239.32±11.172</td>
<td>237.91±5.66</td>
<td>247.25±9.15</td>
<td>248.97±7.51</td>
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<tr>
<td>60 minutes</td>
<td>239.03±7.57</td>
<td>8.27±11.59</td>
<td>240.98±12.14</td>
<td>210.49±11.13</td>
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<td>229.94±10.61</td>
<td>231.96±13.15</td>
<td>228.59±8.56</td>
</tr>
</tbody>
</table>

*P<.05 vs C group.

#P<.05 vs D group.
but this effect was partially reversed by yohimbine (P<.05). Moreover, co-treatment with rHMGB1 enhanced HMGB1 and NF-κB expression, but this effect was partially reduced by dexmedetomidine (P<.05; Figure 5).

3 | DISCUSSION

In this study, we investigated the mechanisms underlying the cardioprotective properties of dexmedetomidine preconditioning, associated with the HMGB1-TLR4-NF-κB signalling pathway, in I-R. Dexmedetomidine preconditioning alleviated myocardial I-R injury in rats by inhibiting the associated inflammatory process through down-regulation of the HMGB1-TLR4-NF-κB signalling pathway by activation of α2-adrenergic receptors.

Perioperative cardiac complications such as myocardial ischaemia and infarction are predominant causes of morbidity and mortality in patients undergoing noncardiac surgery.21 Following an acute myocardial infarction, re-establishing coronary blood flow through rapid reperfusion strategies, such as thrombolysis or primary angioplasty, is essential to salvage viable myocardial tissues.22 However, reperfusion of ischaemic myocardium carries with it an inherent risk that, paradoxically, I-R could cause localized myocardial inflammation, accompanied by apoptosis—a form of myocardial cell damage.23 A growing body of evidence shows that inflammatory processes, including leukocyte recruitment, play a major role in the extension of myocardial damages after I-R.1,2,4

Studies have shown that HMGB1 functions as a proinflammatory cytokine and promotes progression of myocardial I-R injury.3,5,6 Following release from necrotic cells, apoptotic cells, or macrophages, HMGB1 functions as a proinflammatory stimulus that upregulates TNF-α and IL-6 expression,2,7 indicating that this mechanism reinforces the inflammatory process. The first 40 peptide segments of the B-box could reduce myocardial ischaemia and reperfusion injury and inhibit the release of TNF-α and IL-6,25 suggesting that inhibition of HMGB1 expression could suppress the inflammatory process. A previous study has shown that the HMGB1-TLR4 pathway contributes to the secretion of IL-17A and, thereby, promotes myocardial I-R injury.26 NF-κB is a key transcription factor in TLR4-mediated signalling and plays a critical role in stimulating immune-mediated and inflammatory responses of gene expression.8,11,27 NF-κB activation has been observed in cardiac tissue from patients with congestive heart failure and in I-R-exposed hearts.28-31 Numerous studies have shown that inhibition of NF-κB activation results in attenuation of I-R injury, with a concomitant improvement in functional recovery, downregulation of inflammatory cytokines, chemokines, and adhesion molecule gene expression.29-32 Taken together, these data suggest that inhibiting the HMGB1-TLR4-NF-κB signalling pathway may be a potential therapeutic target for protecting the myocardium from I-R injury.

The present study showed that dexmedetomidine has cardioprotective properties when administered prior to I-R events.15,18,20 However, the underlying mechanisms mediating this effect are yet to be elucidated. Our results showed that the expression of cytokines and TLRs and hemodynamic performance significantly worsened in isolated left ventricles after I-R injury following 120 minutes of reperfusion. Parameters of cardiac function including LVDP, ±dp/dt, and LVEDP recovered to a greater extent after global I-R in cases with dexmedetomidine preconditioning. Moreover, we found that dexmedetomidine preconditioning downregulated the expression of the HMGB1-TLR4-NF-κB factors, decreased levels of TNF-α and IL-6 in isolated left ventricles after I-R injury, and reduced CK and LDH levels in CF. However, these dexmedetomidine-induced beneficial effects on ventricular function were partially inhibited by the α2-adrenergic receptor antagonist yohimbine. This evidence suggests, therefore, that the cardioprotective effects of
Dexmedetomidine are mainly mediated by a molecular response within cardiomyocytes following α₂-adrenergic stimulation. Another study has shown that preconditioning with xylazine, an α₂-adrenergic receptor agonist, significantly reduced infarct size and improved hemodynamic function in an ex vivo model, and this effect was similarly blunted by yohimbine.³³ On the other hand, administration of yohimbine alone did not influence ventricular function.

Furthermore, the protective effect of dexmedetomidine can be mitigated by co-treatment with rHMGB1. Therefore, dexmedetomidine could exert a protective effect on I-R-induced injury, possibly through blockade of the HMGB1-TLR4-NF-κB signalling pathway. Previous studies have shown that I-R-induced HMGB1 release could promote the release of TNF-α and IL-6, and, therefore, plays an important role in myocardial I-R injury.³⁵,⁶ Treatment with rHMGB1 worsened I-R injury, whereas inhibition of HMGB1 expression attenuated myocardial I-R injury and inhibited the release of TNF-α and IL-6.⁵

Our study has some limitations. First, the ex vivo isolated heart model was established to investigate the effect of dexmedetomidine on global ischaemia; however, the creation of this model may have evoked more severe damage to the heart than in an in vivo model. The dexmedetomidine concentrations used in this study were based on concentrations used in clinical settings.³⁴ However, it would be difficult to extrapolate these data to humans because of interspecies differences specific to pharmacokinetics and pharmacodynamics.

In conclusion, dexmedetomidine preconditioning inhibited myocardial inflammation and provided myocardial protection against I-R injury in rats, partly by downregulation of the HMGB1-TLR4-NF-κB signalling pathway by activation of α₂-adrenergic receptors. This study may facilitate an understanding of the specific effects of dexmedetomidine on the heart, while but excluding the central sympathetic effects.

4 | METHODS

4.1 | Animals

Male adult Sprague-Dawley rats (8-10 weeks old; 300-350 g) were housed under standard conditions (room temperature, 22°C; 12 hour light/dark cycle) with free access to food and water. All experimental procedures were approved by the Animal Ethics Committee of Soochow University and were performed in accordance with the National Institutes of Health "Guidelines for the Care and Use of Laboratory Animals".

4.2 | Experimental protocols

Animals were anaesthetized with intraperitoneal (ip) chloral hydrate (400 mg/kg) and heparinized with sodium heparin (1000 U/kg). A thoracotomy was performed and the heart was rapidly excised, immersed in ice-cold modified Krebs-Henseleit bicarbonate (KHB) buffer solution (all mmol/L: NaCl 119; KCl 6.0; CaCl₂ 1.24; NaHCO₃ 20.1; KH₂PO₄ 1.24; MgSO₄ 1.24; glucose 11.2), and then immediately placed in a temperature-regulated heart chamber, to be perfused via the ascending aorta in a Langendorff perfusion system (ALC-HP). The KHB solution was perfused at a constant perfusion pressure of 75-80 mm Hg and equilibrated with a mixture of 95% O₂ and 5% CO₂ to a pH of 7.35-7.45 at 37°C. Seventy hearts were perfused for 20 minutes prior to 30 minutes of normothermic global ischaemia, followed by 120 minutes of reperfusion. After a 20-minute equilibration, the specimens were randomly assigned to seven groups.

For measurement of cardiac parameters, a latex balloon containing normal saline was inserted into the left ventricle via the mitral valve and attached to a pressure transducer (YP200; Medease Science and Technology Co. Ltd., Nanjing, China) to monitor the left ventricular end diastolic pressure (LVEDP). The LVEDP was adjusted to 4-10 mm Hg.

![FIGURE 4](image-url) The levels of TNF-α and IL-6 in reperfused left ventricles and LDH and CK in CF were markedly elevated compared to that in the C group. Dexmedetomidine preconditioning significantly prevented an increase of LDH and CK levels, which were partially reversed by yohimbine or rHMGB1 co-treatment. Data are expressed as means ± standard error of the mean, n = 5 per group. TNF-α, tumour necrosis factor α; IL-6, interleukin 6; CK, creatine kinase; LDH, lactate dehydrogenase; C, control group; I-R, ischaemia-reperfusion group; D, dexmedetomidine group; DY, dexmedetomidine-yohimbine group; Y, yohimbine group; HD, dexmedetomidine-rHMGB1 group; H, rHMGB1 group. *P<.05 vs C group; †P<.05 vs D group.

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FIGURE 5 The localization of HMGB1-NF-κB in the rat heart was determined by immunohistochemical analysis. The numbers of HMGB1- and NF-κB-positive cells were increased as compared with the C group. However, the expression of HMGB1 and NF-κB was obviously decreased by dexmedetomidine, and these effects were partially reversed by yohimbine or rHMGB1. Data are expressed as mean ± standard error of the mean, n = 5 per group. NF-κB, nuclear factor κB; HMGB1, high mobility group box 1 (HMGB1); C, control group; I-R, ischaemia-reperfusion group; D, dexmedetomidine group; DY, dexmedetomidine-yohimbine group; Y, yohimbine group; HD, dexmedetomidine-rHMGB1 group; H, rHMGB1 group. *P<.05 vs C group; #P<.05 vs D group.

and then maintained at a constant volume throughout the experiment. A pressure amplifier (MedLab-U/4C501H, Nanjing, China) was used to measure hemodynamic parameters: HR, LVSP, LVEDP, and ±dp/dt. The LVEDP (in mm Hg) was calculated as: LVEDP = LVSP − LVEDP. While inducing ischaemia, hearts were placed in an organ bath chamber filled with normal saline at 37°C without pacing. A computerized pressure amplifier continuously recorded ±dp/dt, LVDP, and LVEDP. Haemodynamic and coronary outflow (CF) data were acquired after stabilization of a 20 minute baseline and following reperfusion cycles of 60 and 120 minutes. The prepped hearts were excluded from the study if baseline LVDP or heart rate were less than 50 mm Hg or 200 b.p.m., respectively.

4.3 Western blot analysis

The HMGB1-TLR4-NF-κB protein levels were determined by western blotting. After reperfusion, the left ventricle was dissected free, snap-frozen in liquid nitrogen, and stored at −80°C until further processing. Whole protein extraction from frozen tissue samples was done with RIPA buffer (Beyotime, Shanghai, China) containing 2% polymethyl sulfoxide (PMSF) on ice for 30 minutes. Extracts were normalized for equal amounts of total protein, measured by the bicinchoninic acid (BCA) method. Whole protein (80 μg) was separated on 10% SDS-polyacrylamide electrophoresis gels and transferred to polyvinylidene fluoride (PVDF) membranes where they were blocked with 5% non-fat milk in Tris-buffered saline (TBS) for 2 hours and incubated overnight at 4°C with the specified primary antibodies for HMGB1 (1:1000; ab182566; Abcam, Cambridge, MA, USA), TLR4 (1:200; ab22048; Abcam), and NF-κB (1:500; ab7970; Abcam), respectively; thereafter, the specimens were incubated for 2 hours with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000; Beyotime). GAPDH (Multi Science, Zhejiang, China) was used as a loading control for comparison between samples. Immunoreactivity was evaluated by a peroxidase-based chemiluminescence detection kit (Beyotime) and the signal intensities of bands in the immunoblots were quantified by densitometry using IMAGE-PRO PLUS 6.0 software.

4.4 Cytokine analysis

A commercial enzyme-linked immunosorbent assay kit (ELISA; Multi Science, Zhejiang, China) was used to detect levels of TNF-α and IL-6 in the myocardium. Absorbance of standards and samples was determined by using spectrophotometry at 450 nm with a microplate reader (MD190; Molecular Devices, Sunnyvale, CA, USA) and results were plotted against the linear portion of the standard curve.

Furthermore, levels (IU/L) of lactate dehydrogenase (LDH) and creatine kinase (CK) in CF after 120 minutes of reperfusion were analyzed using commercialized assay kits (A032 and A020-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

4.5 Immunohistochemical analysis

The localization of HMGB1 and NF-κB was determined by immunohistochemical analysis. After reperfusion, hearts were fixed in 4% formaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 24 hours, dehydrated in alcohol, clarified in xylene, embedded in paraffin wax, and cut into 4 μm sections. Standard immunoperoxidase techniques were used to detect HMGB1 and NF-κB by applying the corresponding antibodies for HMGB1 (1:100) and NF-κB (1:200) to each individual section at 4°C overnight. Immunostaining was done using an HRP-labeled streptavidin biotin kit (SP9001/9002; ZSGB-BIO, Beijing, China). Sections were counterstained with hematoxylin (Beyotime). Controls were obtained by replacing the primary antibody with phosphate-buffered saline. All immunostained sections were examined by light microscopy (Olympus, BX60, Kyoto, Japan). The degree of HMGB1 and NF-κB activation was
expressed as the percentage of HMGB1- and NF-κB-positive cells to total cells.

4.6 | Statistical analysis

Data are expressed as mean ± standard error of the mean. Statistical analysis was conducted using GraphPad Prism 5.0. Analyses of hemodynamic and CF data were conducted by repeated measure analysis of variance (ANOVA) followed by the Tukey post hoc test. Levels of proteins and cytokines were analyzed with one-way ANOVA followed by Tukey post hoc test. P-values less than .05 were considered statistically significant.

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AUTHOR CONTRIBUTIONS

Yu-fan Yang managed study design, research experiments, data collection and analysis, and manuscript preparation. Ke Peng developed study hypotheses and undertook data collection, and manuscript editing. Hong Liu was involved in manuscript preparation, presentation of data analytical findings, and manuscript editing. Xiao-wen Meng and Jing-jing Zhang assisted with experimental studies and data collection. Fu-hai Ji oversaw quality parameters pertaining to the integrity of the entire study, study design, and manuscript revision.

DISCLOSURE

None.

REFERENCES


**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.