L-NAME improves doxycycline and ML-7 cardioprotection from oxidative stress

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1. ABSTRACT

Matrix metalloproteinase-2 (MMP-2) mediated degradation of myosin light chain 1 (MLC1) and troponin I (TnI) contributes to myocardial ischemia/reperfusion (I/R) injury. Modifications of MLC1 triggered by oxidative stress are mediated by myosin light chain kinase (MLCK), nitric oxide synthase (NOS), and MMP-2. Previous studies have shown that inhibiting both MLCK and MMP-2 protects against I/R injury. Here, we hypothesized that the addition of NOS inhibitor (L-NAME) at subprotective concentration to the mixture of subprotective concentrations of ML-7 and doxycycline (Doxy), will increase a synergistic cardioprotection of Doxy and ML-7 during I/R. Isolated rat hearts were subjected to global ischemia without or with administration of the mixture of inhibitors. Markers of I/R injury were measured in hearts and coronary effluents. Addition of L-NAME to the mixture of Doxy and ML-7 led to full recovery of heart contractility in comparison to combination of Doxy and ML-7. Improved heart contractility was associated with reduced degradation of TnI and MLC1. The combined administration of NOS, MMP-2 and MLCK inhibitors provides a novel strategy to protect heart from I/R injury.

2. INTRODUCTION

Ischemia and reperfusion (I/R) lead to a significant increase of oxidative stress, which in turn triggers a cascade of pathophysiological events and consequently to heart damage. One of the main factors contributing to the pathogenesis of I/R injury is the enhanced activity of matrix metalloproteinase 2 (MMP-2) (1,2) accompanied by an increased affinity towards myosin light chain 1 (MLC1) and troponin I (TnI) and their consequent degradation (3–7). Although several studies on MMPs have described their extracellular role in long-term remodeling of the extracellular matrix, recent studies on their crucial intracellular roles have emerged (8–12), namely demonstrating that MMP-2 degrades myocardial contractile proteins such as troponin I, MLC1 and...
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Consequently, the development of pharmacological agents that selectively inhibit MMP-2 activity became increasingly attractive since it could provide a novel effective strategy in the treatment of heart injury induced by oxidative stress. Using a rat model of I/R, we have previously shown that MMP-2 is rapidly activated during I/R, and pharmacological inhibition of MMP-2 with the broad spectra MMP inhibitor - doxycycline (Doxy), improves recovery of cardiac mechanical function (1).

Increased MMP-2-mediated degradation of MLC1 results from an increase in the affinity to MMP-2. We have previously established that posttranslational modification of MLC1 is responsible for its increased degradation by MMP-2. Specifically, we showed that phosphorylation of MLC1 triggered by I/R (4) and nitration/nitrosylation as a consequence of increased synthesis of peroxynitrite (ONOO-) affects the degree of MLC1 degradation by MMP-2 (5,6). In addition, our most recent work shows that MMP-2 also regulates MLC1 levels under physiological conditions (5,16), with the complete inhibition of intracellular MMP-2 resulting in higher MLC1 levels and increased contractility in aerobically perfused cardiomyocytes (16). Aside from affecting MMP-2-mediated degradation, other groups have identified MLC modifications in other important physiological roles (6,8,9,11). Nitration of MLC has been reported in the process of vascular aging (17,18), and the phosphorylation of MLC2 controls the sensitivity of myofilaments to calcium in the regulation of heart contractility (19–21).

Given that both MMP-2 activity and MLC modifications have various physiological roles, full pharmacological blockade would result in a multitude of side-effects that may be as detrimental as I/R injury itself. Cardiac cytotoxicity is observed with the currently available MMP-2 and kinase inhibiting drugs when higher doses are used (1,22–26). We have previously shown that co-administration of low concentrations of drugs that prevent both phosphorylation and degradation of MLC1 improves cardiac mechanical function after I/R (27). Additionally, in a model of hypoxia-reoxygenation with use of isolated cardiomyocytes we showed that the combination of a subthreshold concentration of a NOS inhibitor with subthreshold concentrations of MLCK and MMP-2 inhibitors resulted in full recovery of cardiomyocyte contractility (28). Thus, we propose that an alternative to monotherapy at full therapeutic dose (often limited by cytotoxicity) is the use of combination therapy at lower therapeutic concentrations without loss of protective effects with the added advantage of limiting adverse physiological side effects. These agents, with different modes of action, can synergistically protect the heart from MI or I/R injury.

In this study we hypothesize that the simultaneous pharmacological reduction of post-translational phosphorylation of contractile proteins together with the pharmacological inhibition of NOS and MMP-2 activity may protect the heart from I/R injury. We showed that the addition of NOS inhibitor at subthreshold or lower concentration into previously used mixture of Doxy and ML-7 improved the synergistic effect of these drugs and led to complete protection of mechanical function of heart after I/R.

3. MATERIALS AND METHODS

This study conforms to the Guide to the Care and Use of Experimental Animals published by the Polish Ministry of Science and Higher Education and was approved by the local Ethics Committee for Experiments on Animals at the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland.

3.1. Isolated heart perfusion according to Langendorff

Male Wistar rats weighting 300–350 g, in the age of 9–10 weeks, were used as a surrogate model for the analysis of cardioprotection. Rats were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.) and hearts rapidly excised. Immediately after removal, spontaneously beating hearts were rinsed in ice-cold Krebs-Henseleit buffer containing 118 mmol/l NaCl, 4.7. mmol/l KCl, 1.2. mmol/l KH$_2$PO$_4$, 1.2. mmol/l MgSO$_4$, 3.0. mmol/l CaCl$_2$, 25 mmol/l NaHCO$_3$, 11 mmol/l glucose and 0.5. mmol/l EDTA, pH 7.4., and cannulated by the aorta on a Langendorf system and maintained at 37°C. Hearts were perfused at a constant pressure of 60 mmHg with Krebs-Henseleit buffer at pH 7.4., at 37°C and gassed continuously with 5% CO$_2$/95% O$_2$. After stabilization, hearts were subjected to global, no-flow ischemia and followed by aerobic reperfusion. Coronary flow (CF), heart rate (HR), left ventricular developed pressure (LVDP) were determined. Cardiac mechanical function was expressed as the recovery of RPP (rate pressure product at 75 min versus 25 min of perfusion). RPP was calculated as the product of heart rate and left ventricular developed pressure (systolic minus diastolic ventricular pressures). Coronary effluents for biochemical studies were collected at the beginning of reperfusion (45 min) to reach the constant (15 ml) volume (Figure 1).

3.2. Protocol of global ischemia and reperfusion

After 25 min of aerobic perfusion, hearts were subjected to 20 min global, no-flow ischemia, followed by 30 min of aerobic reperfusion (Figure 1). Aerobic controls were perfused aerobically for 75 min. Hearts subjected to 25 min of stabilization, 20 min of ischemia...
and 30 min of reperfusion without drugs were used as I/R control for hearts subjected to stabilization/ischemia and reperfusion with addition of drugs. In the following treatment groups, doxycycline (1–10 μM), ML-7 (0.5–5 μM), and non-selective inhibitor of NOS (L-NAME, 2–50 μM) were administered separately or in the mixture into perfused hearts. ML-7, Doxy and L-NAME (all from Sigma Aldrich, St. Louis, USA) were dissolved in ethanol/ddH₂O up to 50 mg/ml and then diluted in Krebs-Henseleit buffer to the final concentration. The concentration of ethanol infused during the heart was equal to 0.025% (v/v). I/R group was also treated with ethanol vehicle to check if the concentration of 0.025% (v/v) of ethanol affects the mechanical function of the hearts.

3.3. Concentration of proteins

Each sample of fifteen milliliters of coronary effluents, collected at 45 min of perfusion, were concentrated in Amicon Ultra-15 Centrifugal Filter Units with Ultracel-10 membrane (EMD Millipore, Billerica, MA, USA) for 30 min, 3500 x g. The concentrates were rinsed 3 times with 1 ml of ddH₂O and then diluted in Krebs-Henseleit buffer to the final concentration. The concentration of ethanol infused during the heart was equal to 0.025% (v/v). I/R group was also treated with ethanol vehicle to check if the concentration of 0.025% (v/v) of ethanol affects the mechanical function of the hearts.

3.4. Determination of LDH activity

Lactate Dehydrogenase Activity Assay Kit (Sigma Aldrich) was used to determine the activity of LDH in concentrated coronary effluents collected during perfusion. Briefly, lactate dehydrogenase catalyzes the interconversion of pyruvate and lactate with the reduction of NAD to NADH, specifically detected calorimetrically (450 nm). Lactate dehydrogenase served as a marker of tissue damage.

3.5. Immunoblot analysis of MLC1

MLC1 content in concentrated coronary effluents was determined by western blot. Twenty microliters of effluent were analyzed on 12% SDS-PAGE. Proteins transferred on PVDF membrane (Bio-Rad) were detected with mouse monoclonal anti-ventricular specific MYL3 (ventricular isoform of MLC1) IgG antibody 1:1000 (Thermo Scientific Pierce Antibodies, Rockford, USA, cat. no. MA5–15513). Secondary antibody goat anti-mouse IgG 1:1000 (BioRad) was used for detection of the immune complex. VersaDoc 5000 and Quantity One software (all from BioRad) were used for fluorescence and band density measurement.

3.6. Troponin I content in rat hearts and coronary effluents

Troponin I level in doubly concentrated coronary effluents was measured using ELISA Kit for Rat Troponin I cardiac muscle derived from Wuhan ElAaB Science Co. (Wuhan, China). Before biochemical analysis, coronary effluents concentrated with Amicon Ultra-15 Centrifugal Filter Units with
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Ultracel-10 membrane (see section 3.3.) were concentrated again in Amicon Ultra concentrating vessels (EMD Millipore) to achieve the sensitivity of the ELISA test. Briefly, 300 μl of concentrates were filtered using Microcon Ultracel YM-10 (EMD Millipore). Final volume of filtrate was adjusted to the same volume for each sample (200 μl).

Rat troponin I was tied with antibody specific to rat cardiac muscle troponin I and was detected by biotin-conjugated polyclonal antibody and avidin conjugated to horseradish peroxidase (HRP). TMB substrate solution was used to develop the reaction.

3.7. Statistical analysis

GraphPad Prism v.5 was used to perform the statistical analysis. ANOVA, ANOVA with Kruskal–Wallis post hoc analysis or Student’s t-tests was used as appropriate. Results were expressed as means±SEM. P<0.05 was the criterion for statistical significance.

4. RESULTS

4.1. Determination of the protective threshold for cardiac mechanical function

We first wanted to determine the threshold for protection of cardiac function against I/R injury for the inhibitors of MMP-2, MLCK and iNOS (Figure 2A,B,C). Recovery of cardiac mechanical function was calculated at the end of the perfusion protocol and is presented as percentage of RPP measured at the end of the 25 min stabilization period. Cardiac mechanical function was decreased by ~60% in hearts subjected to I/R in comparison to aerobically perfused hearts (Figure 2A-C). Protection of cardiac mechanical function by all tested inhibitors revealed to be concentration dependent. For Doxy (inhibitor of MMP-2), 10 μM resulted in the full protection of contractility, whereas 1 μM did not and represents a subthreshold concentration for mechanical function of I/R hearts (Figure 2A). For ML-7 (inhibitor of myosin light chain kinase) at the protective concentration was 1 μM and the subthreshold 0.5. μM (Figure 2B). For the iNOS inhibitor (L-NAME), the protective and subthreshold concentrations were 50 and 2 μM, respectively (Figure 2C).

4.2. Protective effect of co-administration of subthreshold concentrations of Doxy, ML-7 and L-NAME against cardiac I/R injury

After determining protective and subthreshold concentrations for all three inhibitors we proceeded to administer either a combination of MMP-2 and MLCK inhibitors or MMP-2, MLCK and iNOS together. Co-administration of Doxy and ML-7 only partially protected cardiac contractility recovery (Figure 3), whereas addition of L-NAME to the mixture resulted in the full protection of mechanical function of I/R hearts (Figure 3, Table 1). The increased recovery in contractile function, following I/R, in the hearts treated with the combination of 3 inhibitors was accompanied by a ~50% decrease in released LDH, a nonspecific marker of tissue damage (Figure 4). Additionally, release of troponin I (TnI) and MLC1 (MMP-2 substrates and specific markers of heart injury) was reverted to baseline aerobic levels (Figure 5A and B).

5. DISCUSSION

The vast majority of therapeutic strategies for protection of heart from ischemia/reperfusion (I/R) injury are based on single drug therapies with high (effective) concentrations of pharmacological agents (1,29–33). It is well accepted, that one of the main factors contributing to the pathogenesis of I/R injury is an increased activity of matrix metalloproteinases (MMPs) (1,2) and degradation of contractile proteins (3–6). Recently we showed that the co-administration of a combination of low, subthreshold concentrations of MMP-2 and MLCK inhibitors is as effective and protective against I/R as the same inhibitors given alone at full protective doses (27,28). In addition, protective doses of single drug often exhibit undesired effects. For example Doxy, ML-7 and other inhibitors of metalloproteinases or MLCK can be cytotoxic and are potentially deleterious for other physiological processes involving these proteins (1,8,20,35–37). These data suggest that the use of the synergistic
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Figure 3. Recovery of cardiac mechanical function after administration of two or three inhibitors at subthreshold concentrations: Doxy (1 µM), ML-7 (0.5. µM) and L-NAME (2 µM). Recovery of cardiac mechanical function was expressed as the difference of RPP measured at the end of perfusion (75 min) as a percentage of the RPP measured after 25 min of aerobic perfusion (stabilization period/baseline). N = 8–10 per group; *p<0.05 vs Aero; #p<0.05 vs I/R. Aero, aerobic control; I/R, ischemia/reperfusion; Doxy, doxycycline; ML-7, MLCK inhibitor; L-NAME, non-selective inhibitor of NOS.

Table 1. Hemodynamic parameters of hearts subjected to ischemia/reperfusion and perfused aerobically

<table>
<thead>
<tr>
<th>Component</th>
<th>Aerobic control</th>
<th>I/R</th>
<th>Doxy (1 µM) + ML-7 (0.5 µM) + L-NAME (2 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>HR</td>
<td>369.4 ± 134.7</td>
<td>120.1 ± 79.1</td>
<td>158.7 ± 93.0</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>83.7 ±13.4</td>
<td>56.5 ± 49.5</td>
<td>60.9 ± 29.6</td>
</tr>
<tr>
<td>CF (ml · min⁻¹)</td>
<td>11.5 ± 3.0</td>
<td>4.1 ± 2.4</td>
<td>6.8 ± 4.5</td>
</tr>
<tr>
<td>RPP recovery (%)</td>
<td>104.6 ± 8.5</td>
<td>45.4 ± 2.6</td>
<td>106.5 ± 9.1</td>
</tr>
</tbody>
</table>

HR, heart rate; LVDP, left ventricular developed pressure; CF, coronary flow; RPP- rate pressure product; I/R- ischemia/reperfusion; Doxy- doxycycline; ML-7, myosin light chain kinase inhibitor; L-NAME- nitric oxide synthase inhibitor

effect of two or more drugs could represent a safer alternative to monotherapy and a novel approach in the prevention or treatment of I/R injury.

We have recently demonstrated that the use of subthreshold concentrations of inhibitors of nitric oxide/peroxynitrite (NO/ONOO⁻) production, in addition to a combination of inhibitors of MMP activity and MLC phosphorylation, results in a higher protection of cardiomyocyte contractility, in comparison to when only 2 inhibitors are used (28). Since these observations have been made in isolated right ventricular cardiomyocytes we wanted to validate the use of this approach to a more relevant pathological model. Here we studied the synergistic effect of subthreshold concentrations of Doxy (inhibitor MMP activity) ML-7 (inhibitor of MLC1 phosphorylation) and L-NAME (inhibitor of NO synthase) on contractility of hearts subjected to I/R. The use of isolated rat heart model let us to assess the cardioprotective effect of tested inhibitors on recovery of heart function, taking into account the possible interactions and reciprocal regulation of several types of cells included in the structure of myocardium. We are aware that the final result of above mixture will be at least affected by response of cardiomyocytes, cardiac endothelium, fibroblasts, epicardium cells, macrophages and smooth muscle.
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Figure 4. Cardioprotective effect of subthreshold doses of Doxy (1μM), ML-7 (0.5 μM) and L-NAME (2 μM) on degradation of cardiac tissue by assessment of LDH in coronary effluents collected at the beginning of reperfusion. N = 8–10 per group; *p<0.0.5 vs Aero; #p<0.0.5 vs I/R. Aero, aerobic control; I/R, ischemia/reperfusion; Doxy, doxycycline; ML-7, MLCK inhibitor; L-NAME, non-selective inhibitor of NOS; CF- coronary flow; mU/ml, milli international enzyme units per milliliter.

Figure 5. Quantitative analysis of TnI (A) by ELISA and MLC1 level (B) by immunoblot analysis in coronary effluents (collected at the beginning of reperfusion) after co-administration of subthreshold doses of Doxy (1μM), ML-7 (0.5 μM) and L-NAME (2 μM), separately and in the cocktail into perfused hearts subjected to I/R. Data presented as a ratio of TnI or MLC1 and CF. *p<0.0.5 vs Aero; #p<0.0.5 vs I/R; n=3 per group in immunoblot and ELISA analysis; Aero, aerobic control; I/R, ischemia/reperfusion; Doxy, doxycycline; ML-7, MLCK inhibitor; L-NAME, non-selective inhibitor of NOS; CF- coronary flow.
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cells. For this reason the aim of the current study was to confirm the cardioprotective effect previously tested inhibitors on the function of the whole organ ex vivo.

Improvement of cardiac mechanical function has been previously demonstrated using Doxy, in a MMP-2 dependent manner (1). Additionally the co-administration of subthreshold concentrations of MLCK inhibitors and Doxy also improves the functional outcomes of the heart (27). The triple targeting of 1) MMP-2 activity with Doxy, 2) MLCK activity with ML-7 and 3) NOS activity with L-NAME has been reported to protect contractility of isolated right ventricular cardiomyocytes (28). Because two drug cocktail showed protective effect limited to reinstatement of 60% of heart contractile function, in the current study we proposed the preventive strategy consisting of three drugs, Doxy, ML-7 and L-NAME, at their subthreshold concentrations. Here we show that the administration of a combination of subthreshold concentrations of Doxy (1 µM), ML-7 (0.5 µM) and L-NAME (2 µM) in rat hearts subjected to global ischemia followed by aerobic reperfusion resulted in full recovery of cardiac mechanical function (Figure 3A), although these concentrations did not produce any contractile protective effect when used separately (Figure 2A, B, C). An improved functional outcome of hearts after ischemia/reperfusion was a result of significantly increased coronary flow and better buffer supply after I/R following the cocktail administration (Figure 3B) in comparison to I/R control.

It is well established that the restoration of blood flow to myocardium subjected to ischemia leads to the development of ischemia/reperfusion (I/R) injury, in part mediated by oxidative stress due to an increased formation of oxygen radicals (ROS) in the area of I/R (38–40). Oxidative stress also triggers the increased expression of either inducible or endothelial NO synthases (iNOS and eNOS) and subsequent production of peroxynitrite (ONOO−) (38,41,42), which in turn leads to the activation of matrix metalloproteinases (MMPs) resulting in heart damage (10–12, 15,43,44). Additionally oxidative stress has been shown to induce both phosphorylation and nitration/nitrosylation of myocardial contractile proteins, such as myosin light chains 1 and 2 (MLC1 and MLC2) and TnI, which led to increased MMP-2 mediated degradation of contractile proteins and consequently to contractile dysfunction (3,4,13,14). For this reason Doxy (inhibitor of MMP-2), ML-7 (inhibitor of MLCK) and L-NAME (non-selective inhibitor of NOS) could be important components in the development of novel cardioprotective strategies.

Previous studies have showed that protective doses of Doxy and ML-7 resulted in reduction of protein degradation in isolated hearts subjected to I/R (1,27,45). Protein degradation (in part) can likely be attributed to bursts of ROS and pro-inflammatory cytokines triggered by ischemia and reperfusion (39,40). High levels of ROS can directly damage the cell membrane, ultimately leading to cell death (46). Additionally, high ROS can lead to the spontaneous formation of peroxynitrite, a highly reactive oxidant resulting from the reaction of nitric oxide with superoxide, that has the potential to directly activate MMPs (43,47). In the current study we show that targeting the mechanisms of cardiac contractile proteins degradation by co-administering a combination of low, subthreshold concentrations of Doxy, ML-7 and L-NAME significantly reduces heart injury during I/R (Figure 4). We believe that decreased heart injury during global ischemia and reperfusion was the result of the multidirectional activity of combined mixture. Moreover, given that all MMP-2 and NOS activity as well as MLC modifications play various physiological roles such as: embryogenesis, organogenesis, wound healing, tissue remodeling and regulation of arteriolar vasconstriction and capillary/venule permeability, neurotransmission, vascular tone and gene transcription (44,48–50), full pharmacological blockade would result in a multitude of side-effects resulting from the above. Moreover, some of these pharmacological agents have a direct cytotoxic effects. Doxycycline showed the cytotoxic effect (an inhibition of cell growth and viability) in human lung fibroblasts at concentration 0.45 µM or lower, and the effect was proportional to drug concentration (51). L-NAME potentiates arachidonic acid toxicity in rat and human hepatocytes which express CYP2E1 (52,53). It well known that the organization and stiffness of the cell cytoskeleton are determined inter alia by the forces generated by actin and myosin II. In the previous study it was shown that dephosphorylation of myosin light chain 2 (by inhibition of MLCK with 20 µM ML-7) leads to destabilization of the cytoskeleton and consequently to cell death (54). For this reason lower dosing of drugs may allow control of pathological overactivation/activity without completely disrupting physiological roles.

Co-localization of MMP-2 with structural proteins of thin myofilaments in hearts subjected to I/R has been established (7). Hence, MMPs may cause direct contractile dysfunction by mediating the proteolytic degradation of troponin I, myosin light chains and myosin heavy chain (3,4,6, 55). In the current study we show that ischemia and reperfusion generating massive oxidative stress led to failure of cardiomyocyte contractile apparatus (Figure 5A, B). This was counteracted by the co-administration of subthreshold concentrations of Doxy and ML-7, in combination with non-selective inhibitor of NOS, which decreased the proteolytic degradation of MLC1 and TnI in myocardium.

In conclusion, this study provides new perspectives for a novel approach to prevent
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heart from I/R injury by combining subthreshold concentrations of MMP-2, MLCK and NOS inhibitors. The use of low concentrations of above inhibitors allow for normal, physiological function of MMP-2, MLCK and iNOS. Moreover, as these drugs are used at very low concentrations (not protective doses when used separately), the potential dose-dependent toxicity and harmful drug–drug interactions should be minimal. As shown above, administration of inhibitors cocktail leads to full recovery of cardiac contractile function with concomitant preservation of MLC1 and TnI. Hence, this promising approach can prove to be a valid strategy for future protection of the heart against I/R injury in clinical practice. Taking into account that similar mechanism are observed during hypoxia followed by reoxygenation during perinatal asphyxia (56) and organ transplantations (57), the results of these study may have paramount implications for pharmacological prevention/treatment of injury resulting from reoxygenation of newborns with asphyxia or for better preparation of donor’s heart and other organs for transplantation.

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Abbreviations: CF: coronary flow; Doxy: doxycycline; HR: heart rate; H/R: hypoxia/reoxygenation; eNOS: endothelial nitric oxide synthase; iNOS: inducible NOS; I/R: ischemia/reperfusion; L-NAME: non-selective inhibitor of iNOS/eNOS; LVDP: left ventricular developed pressure.
pressure; MLC- myosin light chain; MLC1: myosin light chain type 1; MLC2: myosin light chain type 2; MLCK: myosin light chain kinase; ML-7: inhibitor of MLCK; MMPs: matrix metalloproteinases; MMP-2: matrix metalloproteinase 2; MYL3: ventricular isoform of MLC1; NO: nitric oxide; NOS: nitric oxide synthase; ONOO-: peroxynitrate; PVDF: polyvinylidene fluoride; ROS: reactive oxygen species; RPP: the difference of systolic and diastolic ventricular pressures; TnI: troponin I;

**Key Words:** Ischemia, Reperfusion, Isolated rat heart, Doxycycline, ML-7, L-NAME

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