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A drug cocktail for protecting against ischemia-reperfusion injury

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1. ABSTRACT

Ischemia followed by reperfusion (I/R) of cardiomyocytes causes release of a large amount of inducible nitric oxide (NO) synthase (iNOS) followed by an increase of asymmetric dimethylarginine (ADMA). ADMA disrupts NO signaling by switching of the NOS activity from NO to the production of reactive oxygen species (ROS). Previously, we have shown that pretreatment of the hearts by co-administration of sub-threshold concentrations of doxycycline, a matrix metalloproteinase (MMPs) inhibitor, ML-7 an inhibitor of myosin light-chain kinase (MLCK) and L-NAME a non-selective NOS inhibitor protects the heart against I/R injury. In this study, we replaced the L-NAME with 1400W (selective inhibitor of iNOS) in the drug cocktail that was Langendorff-perfused into the hearts of Wistar rats before (prevention) or after (treatment) the induction of I/R. This pre-treatment resulted in full protection of contractility, decreased production of iNOS and ADMA and normalized the bioavailability of NO in the I/R hearts. Thus, the formulated drug cocktail protects the heart from I/R injury.

2. INTRODUCTION

Ischemia/reperfusion (I/R) injury which occurs after coronary artery bypass surgery or other ischemic episodes leads to numerous metabolic, morphological and contractile changes in the myocardium.

Nitric oxide (NO) is an endothelium-derived relaxing factor with the potential to protect the heart against the I/R injury (1). NO production requires nitric oxide synthase (NOS), which catalyzes the oxidation of L-arginine to L-citrulline. In presence of oxygen, three different isoforms of NOS catalyze the synthesis of endogenous NO. These include neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (1). nNOS controls neuronal functions, while eNOS acts as a homeostatic regulator of essential cardiovascular functions including vascular tone and homeostasis, prevention of leukocyte adhesion and platelet aggregation, and vascular inflammation (2). iNOS, which protects against cytotoxic effects, when it is induced by cytokine stimulation, may promote cellular damage. During I/R, release of a large amount of iNOS is followed by an increase in the synthesis of asymmetric dimethylarginine (ADMA) which acts as an endogenous inhibitor of NOS.

Constitutively expressed NO serves as a radical scavenger, by reacting with superoxide and hydroxyl radicals and formation of the innocuous anions, nitrate and nitrite, respectively (3-4). After ischemia, NO to act as a radical scavenger during myocardial reperfusion (3). In presence of superoxide ($O_2^{\bullet-}$), however, NO forms peroxynitrite ($ONOO^-$) which can cause damage to the myocardium (5). Overproduction of $ONOO^-$ in heart tissue also activates matrix metalloproteinase 2 (MMP-2), the protease responsible for intra-cellular degradation of contractile proteins such as myosin light chains 1 (MLC1) (6). Also, during I/R, an increased phosphorylation of MLC1 increases its degradation by MMP-2 (7). In ischemic hearts, in addition to MLC1, MMP-2 degrades other contractile proteins such as MLC2 (6), troponin I (TnI) (8), and titin (9). Such degradation of contractile proteins can lead to disturbances in myocardial contractility and heart failure.

Given that I/R injury engages multiple factors such as MMPs, NO or ROS, it follows that pervention of the I/R injury requires a complex cocktail to prevent damages induced by these factors. However, the use of multiple drugs, at their normal therapeutic levels, may disturb normal physiological processes and cause numerous side-effects (10). Thus, it follows that low doses of these drugs might still be effective if they exhibit synergistic or additive effects to protect the myocardial tissue against injury while maintaining normal tissue homeostasis. We showed the effectiveness of use of such sub-optimal doses in prevention of I/R injury by co-administration of doxycycline, as an inhibitor of MMP-2 and ML-7 as an inhibitor of phosphorylation of MLC1 and L-NAME as a NOS inhibitor. This treatment resulted in almost full recovery of contractile function of the heart in experimental models of myocardial I/R injury (10). To improve this cocktail and to fully protect the myocardium from I/R injury, here, we replaced L-NAME with 1400W which acts as a selective inhibitor of iNOS.

3. MATERIALS AND METHODS

3.1. Experimental animals

Pathogen-free, adult male Wistar rats (300-325 grams) were obtained from Mossakowski Medical Research Center, Polish Academy of Sciences, Warsaw, Poland. Rats were housed in cages (two rats/cage) and kept at controlled temperature (22 ± 2 °C), humidity (55 ± 5%) and light/dark (12/12 hours) cycle. Rats were supplied with food and water *at libitum*. All experimental procedures in the animals were approved by the Ethics Committee at the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland and were performed in accordance with the published Guide of the Polish Ministry of Science and Higher Education for the Care and Use of Experimental Animals.

3.2. Pharmacological agents

N-((3-(aminomethyl)phenyl)methyl) ethanimidamide dihydrochloride (1400W), doxycycline (Doxy), 1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-7) were obtained from Sigma Aldrich (St. Louis, USA). All other chemicals were of analytical grade. Drugs were dissolved in ethanol/distilled H_2O, and then, immediately before administration, they were diluted to its final concentration with Krebs-Henseleit buffer. The ethanol concentration infused into the

heart was 0.025% (v/v) which failed to affect the mechanical function of the heart. Doxy and ML-7 were used as reported previously (10-11) and the optimal concentration of 1400W was determined experimentally.

3.3. Experimental design and Langendorff-perfusion of hearts

Rats were anesthetized by intra-peritoneal injection of phenobarbital (160 mg/kg), and the hearts were rapidly excised. 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_2PO_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 the aorta on a Langendorff apparatus. 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 a mixture of 95% O_2 and 5% CO_2. The above procedure was completed within 30 seconds. A latex balloon was filled with water and then it was connected to a pressure transducer (EMKA Technologies, Paris, France). The baloon was, then, introduced into the left ventricle via a mistrial valve. The volume of the balloon was adjusted to achieve a stable left ventricular end-diastolic pressure of 8-10 mmHg during stabilization and reperfusion and coronary flow (CF), heart rate (HR), and left ventricular developed pressure (LVDP) were monitored. Hearts that showed a CF >28 ml/min or < 10 ml/min were excluded from the study. The hemodynamic indicators of the heart function were monitored by determining the rate- pressure product (RPP) calculated as the product of the heart rate and pressure developed in the left ventricle (intra-ventricular pressure of left ventricular x heart rate/1000). After an initial stabilization period of 25 min (aerobic perfusion), hearts were subjected to 20 min of global ischemia (no-flow), followed by 30 min aerobic reperfusion (10, 12). Animals were equally and randomly categorized into groups of 6-10 rats per group including aerobic control without drug, aerobic control with drug, acute myocardial I/R injury without and with drug. Controls were comprised of hearts that were perfused aerobically for 75 min with or without drugs as well as I/R control hearts that were subjected to stabilization/ischemia/reperfusion without administration of drugs. In the experimental groups, selective inhibitor of iNOS (1400W, 0.5-1 μM), and doxycycline (1 μM) and ML-7 (0.5 μM) were first separately perfused into the hearts during the last 10 min of stabilization immediately prior to global ischemia (prevention), or in the first 10 min of reperfusion- after global ischemia (treatment) (10, 12) (Figure 1).

Coronary effluents were collected at the beginning of reperfusion when the constant (15 ml) volume was reached and these were concentrated to a final volume of 1 ml in Amicon Ultra-15 Centrifugal Filter Units with Ultracel-10 membrane (EMD Millipore, USA) (10). Coronary effluents were aliquoted and frozen -80°C and at the end of experiments, heart tissues were frozen in liquid nitrogen and stored at -80°C. Percent recovery of myocardial function was calculated as a differences between RPP at 75 and 25 min of aerobic perfusion protocol.

3.4. Preparation of homogenates of heart tissues

Frozen heart tissue was crushed in liquid nitrogen and then mechanically homogenized in an ice cold buffer comprised of 50 nM Tris-HCl (pH 7.4), 3.1 mM sucrose, 1 nM DTT, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin and 0.1% Triton X-100. Homogenates were centrifuged at 30000 xg for 10 min at 4°C and the supernatants were collected and stored at -80°C. Total protein concentration in tissue homogenates was quantified using Bradford based assay (BioRad, Hercules, USA) and standardized by using bovine serum albumin.

3.5. Measurement of nitric oxide (NO)

Concentration of nitric oxide was assessed in heart homogenates by Nitric Oxide Assay Kit (Abcam, UK) and expressed as nmol/mg of total protein. The assay provides two-step measurement of total nitrate and nitrite. The first step converts nitrate to nitrite utilizing nitrate reductase. The second step uses Griess Reagent to convert nitrite into a deep purple azo compound. Spectrophotometric determination of the

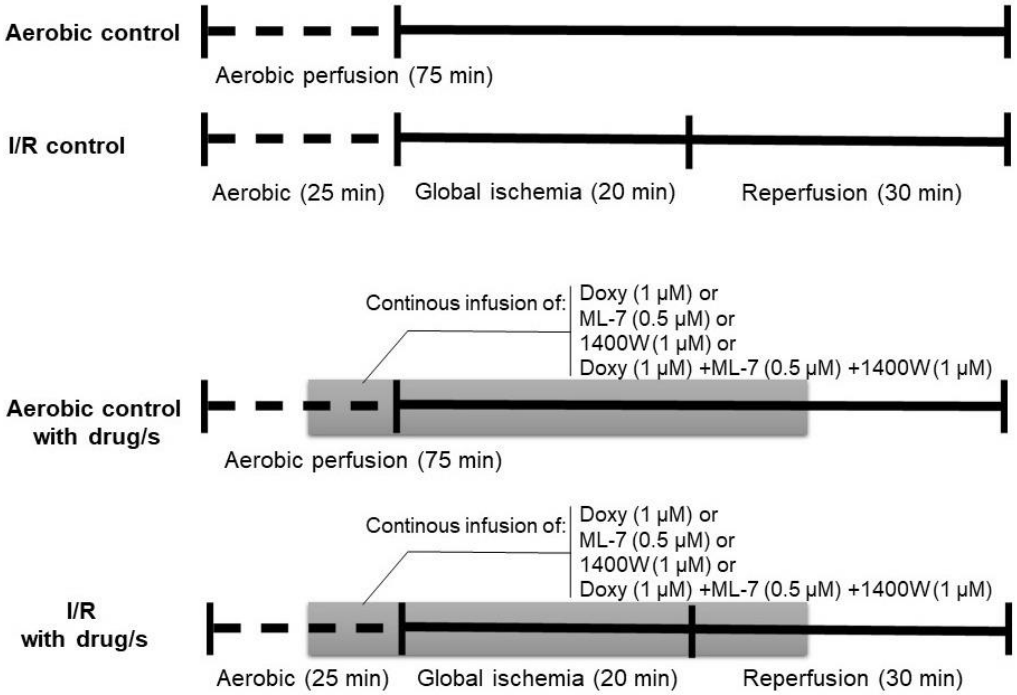

Figure 1. Diagram representing the perfusion protocol for experimental groups. The drugs were administered during the last 10 min of stabilization, prior to the global ischemia (prevention), as well as within the first 10 min of reperfusion after global ischemia (treatment).

azochromophore, that reflects nitric oxide amount in the sample, was measured with Multi Scan Go spectrophotometer (Thermo Fisher Scientific, USA).

3.6. Measurement of endogenous asymmetrical dimethylarginine (ADMA)

The concentration of ADMA in the heart homogenates, was assessed by a rat ADMA competitive inhibition enzyme immunoassay (ELISA) Kit (Cusabio, USA) in plates pre-coated with goat-anti rabbit antibody and was expressed as ng/mg of total protein. Samples were added to the microtiter plate wells with an antibody specific for ADMA and HRP-conjugated ADMA. The competitive inhibition reaction was carried out by using HRP-labeled ADMA and unlabeled ADMA and a specific antibody to ADMA.

3.7. Immunoblotting of iNOS

The iNOS in heart homogenates was assessed by Western blotting. Briefly, 60 µg of total protein extracted from heart homogenates resolved in 12% SDS-PAGE were transferred onto PVDF membrane (Bio-Rad, Germany). iNOS was detected using a mouse anti-rat iNOS polyclonal antibody 1:5000 (Abcam, ab 21775) followed by washing and then applying a goat anti-mouse IgG horseradish peroxidase conjugate 1:1000 (Bio-Rad). The blots were developed using ClarityTM Western ECL substrate (Bio-RAD). The bands were detected and their densities were measured by ChemiDocTM MP System and Quantity One Software (Bio-Rad). iNOS was expressed as densitometric units of iNOS (AU) normalized to total protein amount.

3.8. Measurement of ventricular isoform of myosin light chain 1 (MLC1)

MLC1 in heart homogenates was quantified and expressed as µg/mg of total protein, by using a rat MYL3 ELISA Kit (LSBio, USA), according to the manufacturer's instruction that uses a biotinylated anti-rat MLC1 antibodies followed by detection using a streptavidin-horseradish peroxidase complex.

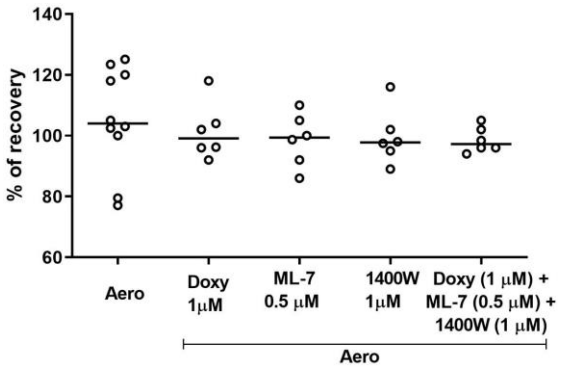

Figure 2. The effect of low dose inhibitors and the inhibitor mixture on recovery of contractile function.

3.9. Measurement of activity of matrix metalloproteinase 2 (MMP2)

MMP2 was detected in cardiac homogenates by gelatin zymography (13-14). Prior to electrophoresis, protein content was measured using Bradford Protein Assay (Bio-Rad Laboratories, Germany), and bovine serum albumin (98% heat shock fraction, Sigma-Aldrich) was used as a protein standard. Briefly, homogenates adjusted to the same protein concentration were mixed with 4x Laemmli sample buffer (Bio-Rad, Germany). Samples containing 20 µg of protein were then subjected to denaturing but non-reducing conditions by using 8% polyacrylamide gel co-polymerized with 2 mg/ml gelatin and 0.1% SDS. After electrophoresis, gels were rinsed three times for 20 min each in 2.5% Triton X-100. Gels were then washed with incubation buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 150 mM NaCl and 0.05% NaN_3) for 20 min at room temperature and then for 18 hourd at 37 °C. Protein bands were stained using 0.05% Coomassie Brilliant Blue G-250 in a mixture of 30% methanol and 10% acetic acid, and then destained with aqueous solution of 4% methanol, 8% acetic acid until the bands were clearly visible. Developed gels were scanned with GS-800 calibrated densitometer (model PowerLook 2100 XL-USB) and MMP-2 activity was measured using Quantity One v. 4.6.9 software (Bio-Rad). MMP activity expressed in arbitrary units (AU) as activity per microgram of total protein.

3.10. Statistical analysis

The statistical analysis was performed with GraphPad Prism v.8. Kruskal–Wallis test with Dunn's test or ANOVA with Tukey post hoc analysis. To assess the correlation between variables, Pearson's correlation was used. Data are presented as the mean ± SEM. A probability value of <0.05 was considered as being statistical significant.

4. RESULTS

Harmful effect of drugs (1400W, Doxy, ML- 7) on contractility of myocardium under aerobic condition was prevented by careful adjustment of concentration of the drug in perfusates (Figure 2).

4.1. The effect of 1400W on contractility of hearts undergoing I/R injury

Main factors that contribute to the pathogenesis of I/R injury are increased generation of peroxynitrite, increased activity of MMPs and degradation of contractile proteins. The sub-optimal concentration of inhibitors of MMP-2 and MLCK to protect of cardiac function against I/R injury have already been determined to be around 1 µM (10). For this reason, here we identified the optimal and sub-optimal doses of 1400W, on contractility of hearts that were subjected to I/R (Figure 3). Recovery of mechanical function of the heart was calculated, at the end of the perfusion protocol (75 min), by the heart rate and the left ventricular pressure, and presented as percentage of RPP measured at the end of the 25 min stabilization period. The mechanical function of the heart was decreased approximately by 40% in hearts that were subjected to I/R in comparison to hearts that were subjected to aerobic perfusion (100% of recovery). 1400W fully protected the heart contractility (average recovery 93.2%) from IR injury at 5 µM while a lower dose of 1 µM (sub-threshold) afforded no protection (Figure 3).

4.2. Protective effect of an inhibitor mixture of MMP-2, MLCK and iNOS against I/R injury

We developed a cocktail of drugs that was comprised of inhibitors of MMP-2, MLCK and iNOS

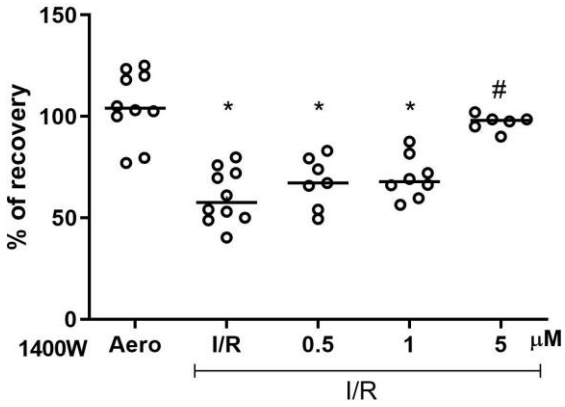

Figure 3. Concentration dependent recovery of mechanical function of the hear by 1400W. *p<0.05 vs Aero; #p<0.05 vs I/R20. Aero - aerobic control; I/R – ischemia/reperfusion.

and such a cocktail was introduced into the heart either 10 minutes before or after induction of I/R. Co-administration of low doses of 1400W (1 μM), Doxy (1 μM) and ML-7 (0.5 μM) resulted complete recovery of contractile function of I/R hearts (Figure 4A-B).

I/R injury led to increased iNOS (Figure 5A) and ADMA (Figure 5B) while it decreased production of NO as measured as total nitrite/nitrate content (Figure 5C). The recovery from I/R injury by the drug cocktail was accompanied by a significant decrease in iNOS production (Figure 4) (Figure 5A) and ADMA (Figure 5B) while it decreased nitrite/nitrate to levels comparable to those in hearts perfused under aerobic conditions (control hearts, Figure 5C). There was a strong positive correlation between production of iNOS and ADMA (Figure 5D) and strong negative correlation between ADMA and NO in the hearts subjected to I/R (Figure 5E). In comparison to the levels of iNOS, ADMA and nitrite/nitrate in I/R hearts perfused without inhibitors, 1400W when used alone at sub-threshold concentration, had no effect on iNOS, ADMA and nitrite/nitrate levels (Figure 5A-C).

MLC1 which is a specific marker of heart injury, was decreased in the heart tissues by approximately 30% in hearts that were subjected to I/R while pre-treatment of the hearts with the inhibitor mixture prevented such a decrease in MLC1 (Figure 6A). Also, pre-treatment of the heart subjected to I/R with the same mixture, normalized the increased activity of MMP-2 (Figure 6B) showing existence of a strong negative correlation between MLC1 and MMP-2 in the hearts subjected to I/R (Figure 6C).

5. DISCUSSION

Under physiological conditions, most of the NO in the vascular system originates from endothelial cells by the constitutive activity of nitric oxide synthase that maintains homeostasis of cardio-vascular functions (15-17). Yet, NO is also involved in tissue damage not directly, rather from generation of peroxynitrite, free radicals of nitric oxide as well as superoxide radicals ($O_2^{\bullet-}$). The main factors that contribute to the pathogenesis of I/R injury in the heart includes increased expression of iNOS, subsequent increased production of $ONOO^-$ and enhanced activation of MMP-2 (18).

ADMA is a natural endogenous competitive inhibitor of NOS, which under physiological conditions, is produced by the degradation of methylated proteins and is metabolized via hydrolytic conversion to citrulline and dimethylamine by the enzyme, dimethylarginine dimethylaminohydrolase-1 (19-20). ADMA inhibits NOS and decreases the bio-available NO, causing attenuation of endothelium-dependent vaso-dilation, hyper-aggregability of platelets, and enhanced monocyte adhesion (21). However, in many cardiovascular diseases, ADMA is also known as a risk factor (19). ADMA disrupts NO signaling (NOS uncoupling) in endothelial cells by switching the production of NO to ROS (21–23). ADMA-enhanced “iNOS uncoupling” is followed by generation of large amounts of superoxide radicals ($O_2^{\bullet-}$) and toxic levels of peroxynitrite ($ONOO^-$) which inhibit ADMA metabolism by DDAH, activate MMP-2 and lead to nitration/nitrosylation of myosin light chains (MLC1s). We showed here that increased production of iNOS during I/R led to increased production of ADMA and identified a strong positive correlation between iNOS and ADMA production. The increased ADMA was associated with the inhibition of iNOS, and decreased NO production (nitrite level). Yet, the increased ADMA and decreased NO production in hearts subjected to I/R did not protect the mechanical function of the hearts that were subjected to I/R.

The increase in $ONOO^-$ leads to cellular

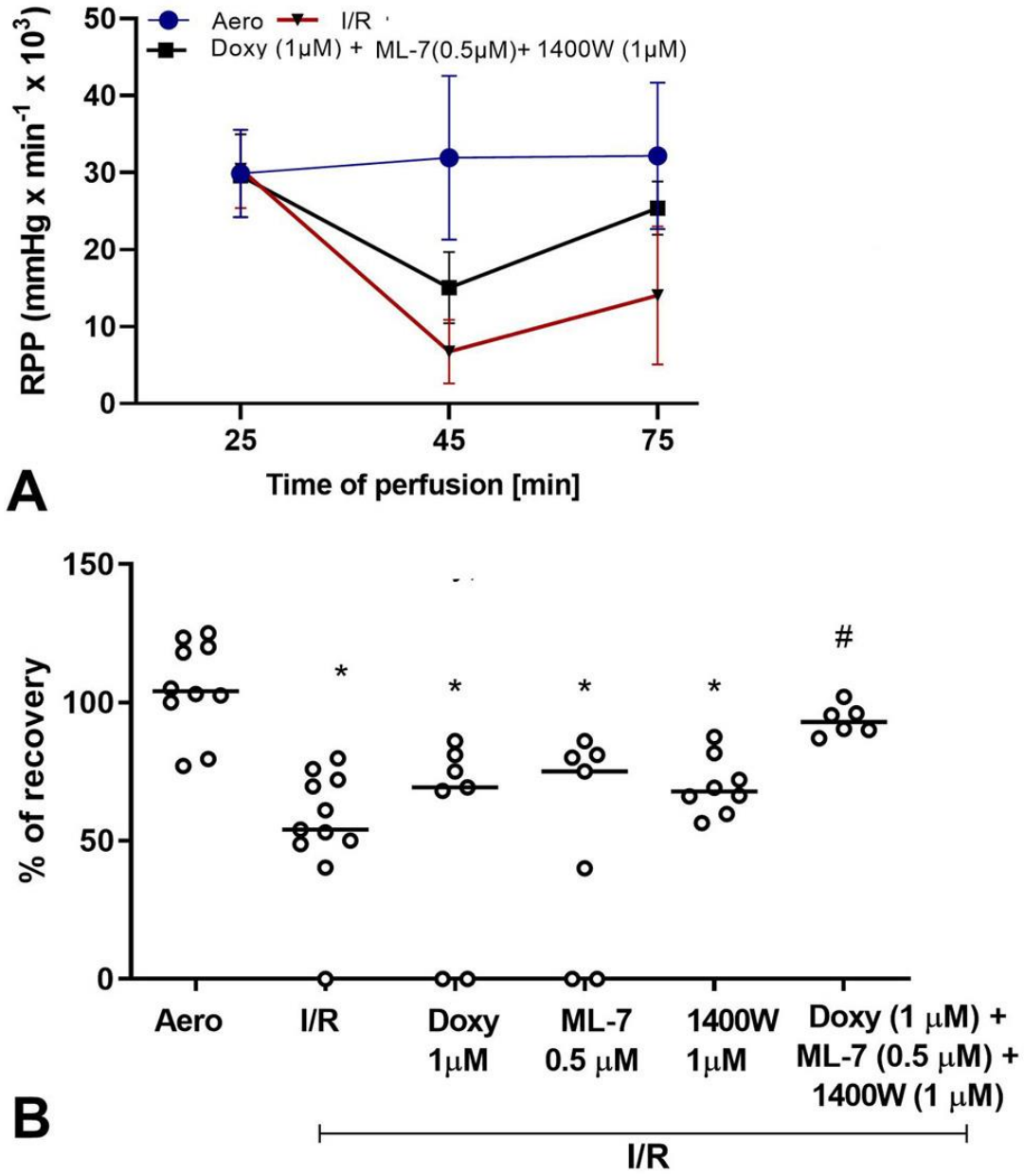

Figure 4. Protective effect of drug cocktail comprised of low dose inhibitors on recovery of contractile function of the heart. (A) RPP calculated as the product of the heart rate and pressure developed in the left ventricle (intraventricular pressure of left ventricular x heart rate/1000). (B) Percent recovery was calculated as a differences between RPP at 75 and 25 min of perfusion protocol. *p<0.05 vs Aero; #p<0.05 vs I/R20. Aero - aerobic control; I/R – ischemia/reperfusion.

damage by irreversibly nitrating tyrosine residues in proteins. Increased levels of plasma ADMA is one of the most reliable predictors of mortality in patients with myocardial infarction (19). During I/R, $ONOO^-$ targets bio-molecules as well as contractile proteins of the myocardium by indirectly activating MMP-2 (24, 25). In clinical settings, during ischemia and post-ischemic reperfusion, I/R leads to a significant loss of contractility and mechanical function of myocardium as a result of injury due to generation of

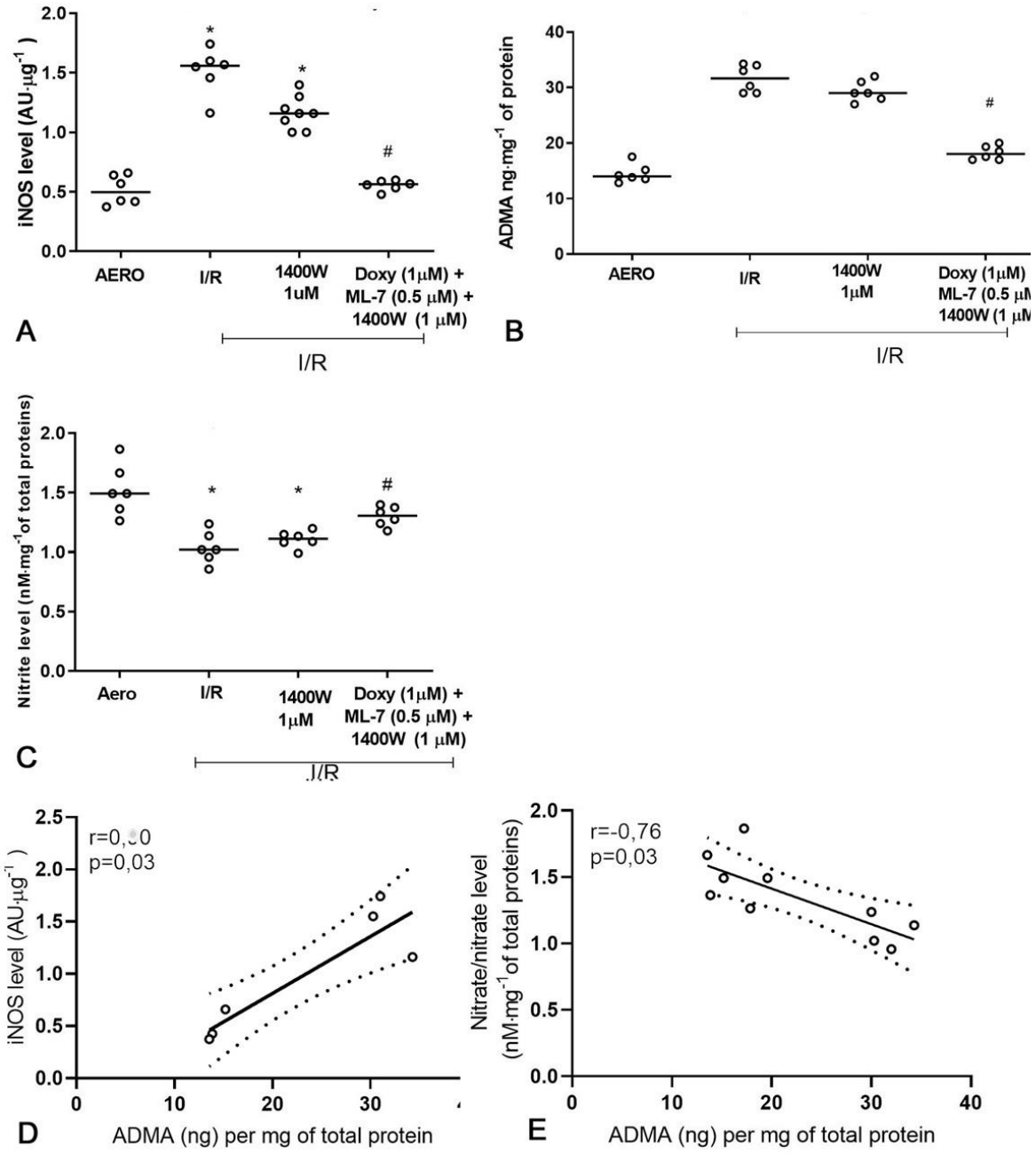

Figure 5. The effect of a drug cocktail comprised of low dose of doxycycline (DOXY; 1 μM), ML-7 (0.5 μM), 1400W (1 μM) on A: iNOS: B: ADMA and C: NO content D: Correlation between iNOS and ADMA, E: Nitrate and ADMA in homogenates from hearts. *p<0.05 vs Aero; #p<0.05 vs I/R20. Aero - aerobic control; I/R – ischemia/reperfusion.

oxidative stress leading to increased ROS and changes in iNOS (26). Under physiological conditions, MMP-2 is necessary for regulation of MLC levels (6, 27). Oxidative stress induces phosphorylation, nitration and nitrosylation of myocardial contractile proteins such as MLC1 and MLC2 (6, 13, 28). Phosphorylation and nitration/nitrosylation of MLCs increase their

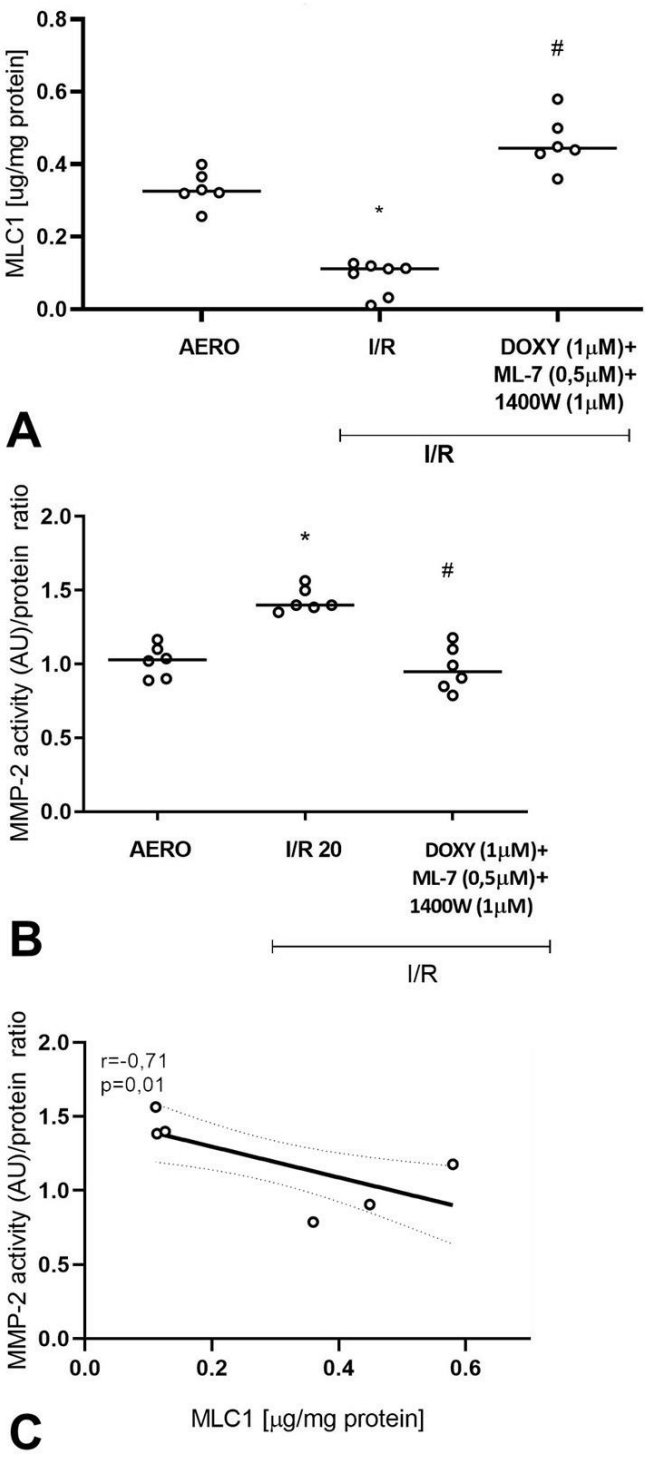

Figure 6. The effect of a drug cocktail on A: MLC1 amount B: MMP-2 activity C: Correlation between MMP-2 and MLC1 in homogenates from hearts.). *p<0.05 vs Aero; #p<0.05 vs I/R20. Aero - aerobic control; I/R – ischemia/reperfusion.

degradation by MMP-2, which leads to dysfunction of heart contractility (6-7, 9, 13, 29).

Based on present knowledge, it follows that recovery from I/R injury and myocardial damage demands the use of a drug cocktail that can jointly inhibit MMP-2 activity, can reduce phosphorylation of MLC and can selectively inhibit iNOS (18, 25, 30-31). For example, we previously showed that among synthetic MMP inhibitors, while rhosphoramidon failed to inhibit MMPs, phenanthroline was the most effective inhibitor of MMPs, followed by doxycycline as well as tetracyclines (8, 32-33). We showed that, doxycycline, when used at 30 µM, resulted in 70% recovery of contractile function of myocardium after I/R (29). Based on such a background, reduction of post-translational phosphorylation of MLC-1 contractile protein (by ML-7), together with the pharmacological inhibition of MMP-2 activity (by doxycycline) should protect the heart from I/R injury through synergistic drug effects. Consistent with the effect of sub-therapeutic use of a drug cocktail, combined sub-threshold concentrations of doxycycline (1 µM) with ML-7 (1 µM) led to 50-60% protection of cardiac function after I/R (27). However, to reduce side effects, we also propose to use a drug combination at sub-therapeutic levels of each drug, injected either prior to (prevention) or after (treatment) the induction of I/R. Consistent with such a concept, we showed that co-administration of doxycycline, ML-7 and L-NAME at low concentrations resulted in almost full protection of cardiac contractility in experimental models of myocardial I/R injury (10). As shown here, contractility of the hearts subjected to I/R decreased by ~40% in comparison to aerobically perfused hearts. In the model of I/R using isolated cardiomyocytes, the protective effect of 1400W on cell contractility was documented (12). To show the effectiveness of a drug mixture that can prevent or allow recovery from I/R at sub-optimal doses, we replaced L-NAME, a non-selective inhibitor of NOS, with 1400W which acts as a selective inhibitor of iNOS) (10). We showed here that 1400W, in a dose of 5 µM, led to almost full recovery of mechanical function of the heart in comparison to I/R control. It has already been reported that iNOS-ADMA-NO pathway is engaged during oxidative stress (34). Here, we showed that administration of sub-threshold concentrations of 1400W improved the synergistic effect of doxycycline and ML-7 and led to 90% protection of mechanical function of the heart after I/R. Cardio-protective effect of the drug mixture that we employed led to decreased iNOS production and subsequent increase of NO. The release of ADMA was reverted to baseline aerobic levels leading to increased bioavailability of NO and NO-dependent cardio-protection. Additionally, pretreatment of the hearts using our inhibitor cocktail prevented I/R induced increase in MMP-2 activity and protected MLC1 from degradation.

In conclusion, we show here that co-administration of a mixture of drugs that simultaneously inhibit iNOS, MMP-2 and MLCK, at sub-therapeutic levels, can afford both protection (prevention) as well as recovery (treatment) from I/R induced damage in the myocardium. Thus, this mixture might be useful in a clinical setting that involves I/R such as those which occur in myocardial infarction, coronary artery bypass or organ transplantation.

6. ACKNOWLEDGMENTS

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Abbreviations: 1400W: N-((3-(aminomethyl)phenyl)methyl) ethanimidamide dihydrochloride; ADMA: asymmetrical dimethylarginine; eNOS: endothelial NOS; I/R: ischemia/reperfusion; iNOS: inducible NOS; MLC1: ventricular isoform of myosin light chain; MLCK: myosin light-chain kinase; MMP-2: matrix metalloproteinase 2; nNOS: neuronal NOS; NO: nitric oxide; NOS: nitric oxide synthase $O_2^{\bullet}$: superoxide; $ONOO^{-}$: peroxynitrite; ROS: reactive oxygen species.

Key Words: Ischemia-Reperfusion Injury, MMP-2, NO, ADMA, iNOS, Isolated Rat Hearts

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