

Mitochondrial-Targeted Antioxidants Given at Reperfusion Protect Cardiac and Hindlimb Muscles against Ischemia/Reperfusion Injury

Keywords: Myocardial ischemia/reperfusion injury; Hindlimb ischemia/reperfusion; Mitochondrial antioxidants; Nitric oxide; Hydrogen peroxide

Abstract

Background and purpose: The principal cause of cardiomyocyte dysfunction and necrosis resulting from Ischemia/Reperfusion (I/R) injury is the production and release of Reactive Oxygen Species (ROS) from damaged mitochondria. Mitochondrial ROS-mediated oxidation of cellular proteins and lipids disrupts both cellular metabolism and organelle integrity, leading to depletion of ATP stores and an increase in intracellular calcium (Ca^{2+}) levels. Thus, attenuation of I/R-induced ROS production has been a therapeutic strategy to salvage damaged cardiomyocytes and thereby limit cardiac functional impairment and infarct size [1]. Previous studies have tested the effectiveness of mitochondrial-targeted antioxidants. Mitoquinone (MitoQ) was effective in reducing I/R injury when given only prior to prolonged ischemia, while Szeto-Schiller (SS)-31 was effective when given either prior to ischemia or at the beginning of reperfusion. This study was undertaken to determine whether these agents are effective in limiting myocardial and hindlimb I/R injury when given during the first 5min of reperfusion only, and thereby support the premise that mitochondrial damage underlies reperfusion-induced cell death during the initial minutes of reperfusion.

Experimental approach: Male Sprague-Dawley rats (275-325 g) were randomized into myocardial or hindlimb I/R groups. Isolated, retrogradely perfused hearts were subjected to global I(30 min)/R(45 min). Hearts were treated with MitoQ (1-20 μ M), SS-31 (10-100 μ M), or plasma (control) added to the perfusate at the onset of reperfusion and was assessed for cardiac function and infarct size. In the hindlimb experiments, either hydrogen peroxide (H_2O_2) or Nitric Oxide (NO) sensors were placed randomly in both the right and left femoral veins of the same animal. One limb was subjected to I(30 min)/R(45 min) by reversibly clamping the femoral artery/vein, while the other limb served as a sham. The animal received an intravenous (i.v.) bolus of either MitoQ (1.8 mg kg^{-1}), SS-31 (2.5 mg kg^{-1}), or saline (control) at the beginning of reperfusion. The difference in blood H_2O_2 or NO between the femoral vein of ischemic and sham limbs in each animal was continuously measured to assess the effects of the drugs.

Key results: In the myocardial I/R model, MitoQ and SS-31 given upon reperfusion significantly improved cardiac function and reduced infarct size compared to untreated control hearts. In the hindlimb I/R model, elevations in blood H_2O_2 levels and reductions in blood NO, both indices of elevated ROS, were significantly attenuated by both MitoQ and SS-31 given upon reperfusion compared to saline treated control animals.

Conclusion and implications: The results indicate that mitochondrial-derived ROS is a major contributor to reperfusion injury and that MitoQ and SS-31 work expeditiously to attenuate ROS in that both agents improve cardiac function and limit infarct size when administered only at the onset of reperfusion. The data suggest that MitoQ or SS-31 would be an effective adjuvant to reduce ischemia reperfusion injury in acute myocardial infarction patients receiving percutaneous coronary intervention, thrombolytic therapy, or undergoing coronary by-pass surgery.



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Introduction

The incidence of Myocardial Infarction (MI) in the United States is 800,000 annually [2]. Prompt treatment to reestablish the blood flow via percutaneous coronary intervention, thrombolytic therapy, or coronary artery by-pass surgery is critical to limit tissue damage, preserve cardiac function and improve clinical outcomes. Paradoxically, abrupt reperfusion of the ischemic myocardium will cause tissue injury, accounting for up to 50% of the final size of the infarct [3]. Consequently, over the years, numerous studies have attempted to identify agents that limit this Ischemia/Reperfusion (I/R) injury. However to date, there are no FDA approved treatments for cardiac I/R injury. Therefore, it is critical to understand the underlying mechanisms to develop effective therapeutic strategies to mitigate I/R induced cardiac damage [4,5].

Reestablishing perfusion of coronary blood restores the delivery of essential substrates (i.e., oxygen and fatty acids) required to restore ATP levels and normalize pH, however these substrates are also responsible for I/R injury with the majority of cell death occurring during the initial few minutes of reperfusion [6-8]. During severe ischemia, mitochondrial respiration stops, thereby dissipating the membrane potential across the inner membrane and lowering ATP levels. Additionally, calcium (Ca^{2+}) accumulation through reversal of the plasma membrane sodium (Na^+)/ Ca^{2+} exchanger and Ca^{2+} leakage through the sarcoplasmic reticulum ryanodine receptor channel (RyR2) favors mitochondrial Ca^{2+} overload, resulting in arrhythmias and hypercontracture [7,9]. Reperfusion is associated with a shift in mitochondria from the production of ATP to Superoxide (SO), specifically from complexes I and III [10,11]. The increase in mitochondrial Ca^{2+} and Reactive Oxygen Species (ROS) leads to the activation of the Mitochondrial Permeability Transition Pore (MPTP), which dissipates mitochondrial membrane potential and uncouples oxidative phosphorylation, further impairing ATP production [11]. Collectively, these events lead to mitochondrial membrane peroxidation and the release of cytochrome c through the ruptured outer mitochondrial membrane, stimulating apoptotic and necrotic pathways [12].

Under I/R conditions, the mitochondria are a major source

of ROS as they constitute 1/3 of the heart volume; therefore mitochondrial-targeted antioxidants, such as mitoquinone (MitoQ) and SS-31, should be effective pharmacological agents to limit I/R injury [13-15]. The targeted attenuation of mitochondrial ROS would be beneficial by two mechanisms: 1) inhibition of lipid peroxidation would reduce cellular damage and 2) a reduction in peroxynitrite anion (-ONOO) formation by increasing blood Nitric Oxide (NO) bioavailability when mitochondrial-derived SO combines with NO [16]. Moreover, attenuating mitochondrial-derived SO would also reduce oxidation of the endothelial NO synthase (eNOS) essential co-factor, tetrahydrobiopterin (BH₄) to dihydrobiopterin (BH₂). It is well known that when BH₂ is increased during reperfusion, eNOS becomes uncoupled and produces SO instead of NO [17-19]. Thus, reducing mitochondrial-derived ROS would also reduce additional ROS generated by uncoupled eNOS during reperfusion [18,20]. Although both MitoQ and SS-31 concentrate their antioxidant properties within mitochondria, their mechanisms targeting the mitochondria differ. MitoQ is covalently attached to a lipophilic Triphenylphosphonium Cation (TPP), which allows it to easily permeate the phospholipid bilayer and concentrate several hundred-fold in the mitochondria [21]. The mitochondrial respiratory chain reduces MitoQ to its active form, ubiquinol, which has been shown to effectively prevent mitochondrial damage and lipid peroxidation [13,22]. In contrast, Szeto-Schiller (SS) peptides contain an alternating cationic and aromatic amino acid with a basic amino acid, e.g., Arg, Lys, providing two positive charges. This alternating cationic-aromatic structural motif enables SS peptides to permeate the plasma cell membrane despite carrying a 3+ charge at physiological pH [14,15]. Thus, SS peptides uptake by mitochondria is energy independent, non-saturable and unaffected by the mitochondrial membrane potential.

Additionally, SS-31 contains a dimethyl tyrosine residue that imparts its antioxidant effect. Either agent should reduce I/R-induced mitochondrial ROS production by buffering ROS production at the inner mitochondrial membrane, thereby inhibiting cytochrome c release, mitochondrial swelling, and mitochondrial membrane peroxidation [21,23].

Previous studies with MitoQ or SS-31 have reported effective cardioprotection when administered as a pretreatment or throughout the entire myocardial I/R protocol [13,23,24]. More recently, SS-31 was found to limit the I/R injury when administered during ischemia in a hindlimb I/R model [25]. Since pretreatment is often not an option in the clinical scenario of an acute MI, a mitochondrial-directed antioxidant that is effective when administered exclusively during the reperfusion phase would offer more clinical utility. Thus, the primary goal of this study was to test the efficacy of the mitochondrial-directed antioxidants MitoQ and SS-31 when given only during the onset of reperfusion. The effectiveness of these antioxidants in reducing I/R damage was evaluated in myocardial and hindlimb I/R models by measuring post-reperfused cardiac function and infarct size, and blood NO/H₂O₂ levels, respectively.

Methods

Experimental animals and ethical statement

All animal procedures complied with the legal and ethical guidelines established by the Institutional Animal Care and Use

Committee at PCOM for care and use of animals. Male Sprague-Dawley (SD) rats 275-325 g (8-10 weeks) (Charles River, Springfield, MA) were housed in polypropylene cages (2 rats in each) lined with wood shavings and provided free access to food and water until the day of the experiment.

Isolated rat heart preparation

Male SD rats (275-325 g) were anesthetized by Intraperitoneal (i.p.) injection of sodium pentobarbital (60 mg kg⁻¹) containing sodium heparin (1000 U) for anticoagulation. After opening the peritoneum, blood (8 ml) was drawn from the abdominal aorta into a 10 ml syringe that contained 1 ml of citrate phosphate buffer in order to isolate plasma. The heart was isolated from the rat and retroperfused with a modified Krebs' buffer through the aorta using the Langendorff protocol [26]. As described in previous studies, the aorta was cannulated with a modified 18 gauge syringe needle and secured using 0 grade silk thread. The preparation was lowered into a temperature-controlled reservoir that was filled with Krebs' buffer maintained at pH of 7.35-7.45 by aerating continuously with 95% O₂-5% CO₂ at 37 °C. A constant pressure of 80 mmHg was maintained throughout the experiment. The Krebs' buffer contained: 25 mM NaHCO₃, 17 mM dextrose, 5.9 mM KCl, 120 mM NaCl, 0.5 mM EDTA, 2.5 mM CaCl₂, and 1.2 mM MgCl₂. Cardiac function parameters: Left Ventricular End-Systolic Pressure (LVESP), Left Ventricular End-Diastolic Pressure (LVEDP), maximal rate of left ventricular developed pressure generation (+dP/dt_{max}) and decline (-dP/dt_{min}), heart rate, and coronary flow were recorded throughout the entire experiment using a pressure transducer (SPR-524, Millar Instruments, Inc., Houston, TX) positioned in the left ventricle and an in-line flow meter (T106, Transonic Systems, Inc., Ithaca, NY). Left Ventricular Developed Pressure (LVDP) was calculated continuously by subtracting LVEDP from LVESP. Data were continuously recorded and stored using a Powerlab station acquisition system (PowerLab/8Sp, AD Instruments, Grand Junction, CO) [18,19,27].

Myocardial I/R procedure

After the baseline period (15 min), global ischemia was induced for 30 min followed by a 45 min reperfusion period. Hearts were randomized to receive either 5 ml of plasma (control), plasma containing SS-31 (10 μM, 25 μM, 50 μM, 100 μM), or Mito Q (1 μM, 10 μM, 20 μM). Stock concentrations of SS-31 (50 mM) and MitoQ (20 mM) were prepared in deionized H₂O and further diluted in plasma. Drug + plasma or plasma alone was administered to the heart during the first 5 min of reperfusion at a rate of 1 ml/min using an infusion pump. At the end of the experiment, the heart was cross-sectioned into 2 mm sections from apex to base. The heart cross-sections were subjected to 1% Triphenyltetrazolium Chloride (TTC) staining for 15 min at 37 °C to determine infarct size as previously described [20,27,28].

Hindlimb I/R procedure

Male SD rats were anesthetized with an i.p. injection of sodium pentobarbital (60 mg kg⁻¹ induction dose, 30 mg kg⁻¹ maintenance doses as needed). The hindlimb was dissected to expose the femoral veins and arteries bilaterally. Both femoral veins were cannulated with a 24-gauge catheter housing a calibrated H₂O₂ or NO microsensor (100 μm diameter, World Precision Instruments (WPI) Inc., Sarasota,

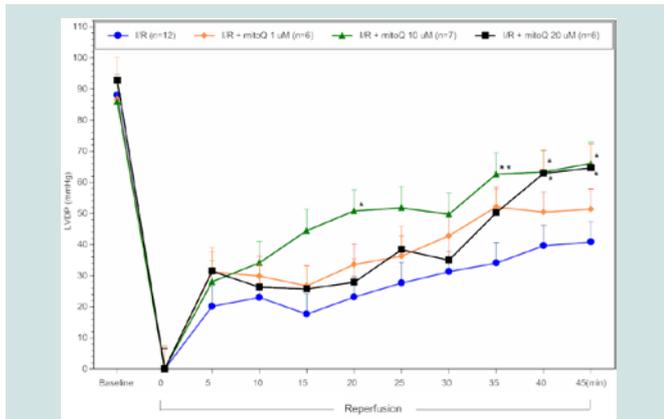


Figure 1: LVDP timecourse for MitoQ I/R hearts. All data were analyzed using ANOVA with the Student-Newman-Keuls test. Final LVDP (45 min reperfusion) was significantly improved in MitoQ I/R treated hearts (10 & 20 μM) compared to control I/R hearts (*p<0.05; **p<0.01 compared to control I/R).

FL) that were inserted into the catheters bilaterally. The microsensors were connected to a free radical analyzer (Apollo 4000, WPI, Inc., Sarasota, FL). This arrangement allowed for real-time measurements of H₂O₂ or NO release as previously described [18,20,29]. One limb was subjected to I/R via clamping of the femoral artery and vein for 30 min followed by reperfusion for 45 min. Meanwhile, the other limb was used as a non-ischemic sham control in the same rat. The rats were randomized to receive either MitoQ (1.8 mg kg⁻¹), SS-31 (2.5 mg kg⁻¹), or saline (non-drug control) *via* i.v. bolus into the left jugular vein at the beginning of reperfusion. These doses were estimated to result in blood levels of 10 and 50 μM, respectively. These concentrations were chosen because they resulted in significant cardioprotective effects in the isolated rat heart experiments. Blood H₂O₂ or NO release was continuously monitored and values were recorded at 5 min intervals. Values measured from the I/R limb were subtracted from the sham limb to determine the net relative difference in H₂O₂ or NO release over the course of the experiment. At the conclusion of the experiment, while still under anesthesia, the rats were sacrificed by opening the thoracic cavity and removing the heart.

Materials

MitoQ (MW=579 g mol⁻¹) used in this study was completed with cyclodextrin (MW=1135 g mol⁻¹) to improve water solubility (total MW=1714 g mol⁻¹, Vosun Shop, Suzhou China). SS-31 peptide: D-Arg-Dmt-Lys-Phe-Amide (MW=640 g mol⁻¹) was synthesized by Genemed Synthesis, Inc., San Antonio, TX. TTC was purchased from Sigma-Aldrich, St. Louis, MO. All other reagents were purchased from Fisher Scientific, Fair Lawn, NJ.

Statistical analysis

All data in the text and figures are presented as means±standard error of the mean (S.E.M.). In the myocardial I/R studies, the data were analyzed post hoc by analysis of variance (ANOVA) with the Student-Newman-Keuls test, while a Student’s t-test was used to analyze the data from the hindlimb I/R experiments. In all cases, a p value <0.05 was considered to be statistically significant.

Results

Effects of MitoQ and SS-31 on cardiac function and infarct size

We examined the effects of MitoQ and SS-31 by measuring the following cardiac function parameters throughout the experiment (Table 1): LVEDP, LVESP, heart rate, coronary flow, +dP/dt_{max} and -dP/dt_{min}. Sham hearts were not subjected to ischemia and maintained near normal cardiac function parameters throughout the 90 min experimental period. There were no significant differences between the initial and final values in all cardiac function indices and minimal cell death (infarct size was less than 0.05%; data not shown). Also, there were no significant differences in baseline (pre-ischemia) cardiac function values among all experimental groups. I/R hearts treated by infusion of MitoQ (10 or 20 μM) and SS-31 (50 μM) during the first 5 min of reperfusion exhibited significantly improved postreperused cardiac contractile function and reduced infarct size compared to controls. Post-reperused LVDP at the end of reperfusion recovered to 59±12% (1 μM; p>0.05, n=6), 77±6% (10 μM; p<0.05, n=7), 70±10% (20 μM; p<0.05, n=6) in I/R MitoQ treated hearts when compared to control I/R hearts that recovered to 46±6% (n=12) of initial baseline values. In SS-31 treated hearts, post-reperused LVDP at the end of reperfusion recovered to 38±3% (10 μM; p>0.05, n=6), 48±7% (25 μM; p>0.05, n=6), 80±6% (50 μM; p<0.01, n=6), and 62±8% (100 μM; p>0.05, n=6) compared to control I/R hearts that recovered to 46±6% (n=12) of baseline values. As shown in (Figure 1), MitoQ infusion (10 μM and 20 μM) resulted in a significant improvement in post-reperused LVDP from 35 to 45 min compared to controls. In contrast, similar concentrations of SS-31 (10 and 25 μM, Figure 2), showed no significant improvement in cardiac function. Improvement in post-reperused cardiac function was observed when the SS-31 concentration was further increased to 50 μM. As illustrated in (Figure 2), at this concentration of SS-31(50 μM), a significant improvement in LVDP was observed at 15 min post-reperfusion, and sustained throughout the reperfusion period (45 min) compared to control I/R hearts (p<0.01). However, similar to MitoQ, the improvement in post-reperused LVDP was associated with a significant increase in final post-reperused LVESP (131±11 mmHg [MitoQ 20 μM]; 127±5 mmHg [SS-31 50 μM] vs. 102±6 mmHg [Control]; p<0.05) and not a decrease in LVEDP.

Unlike cardiac function, all doses of MitoQ (except 1 μM) and SS-31 used in these studies significantly improved (reduced) infarct size

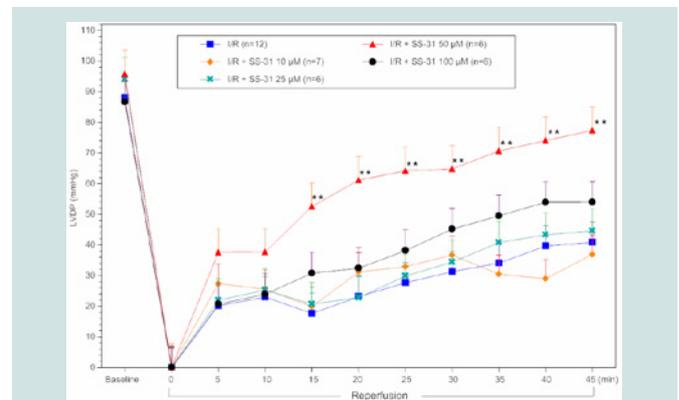


Figure 2: LVDP timecourse for SS-31 I/R hearts. All data were analyzed using ANOVA with the Student-Newman-Keuls test. Final LVDP (45min reperfusion) was significantly improved in SS-31 treated (50 μM) hearts compared to control I/R hearts (**p<0.01 compared to control I/R).

Table 1: Cardiac function initial (baseline) and final values for control I/R, I/R + MitoQ (1-20 μM), I/R + SS31 (10-50 μM) treated hearts and infarct size. LVESP, left ventricular end systolic pressure; LVEDP, left ventricular end diastolic pressure; LVDP, left ventricular developed pressure; maximal rate of LV pressure generation (+dP/dt_{max}) and decline (-dP/dt_{min}). Data expressed as mean±SEM. *p<0.05; **p<0.01 vs control I/R. #p<0.05; ##p<0.01 vs I/R + SS-31(10 μM). † p<0.05; †† p<0.01 vs I/R + SS-31 (25 μM) ω p<0.05; ωω p<0.01 vs. SS-31 (100 μM).ψ p<0.05 vs I/R + MitoQ (1μM). ψψ p<0.01 vs I/R + MitoQ (1 μM).

	I/R Control (plasma) (n=12)	I/R + MitoQ 1 μM (n=6)	I/R + MitoQ 10 μM (n=7)	I/R + MitoQ 20 μM (n=6)	I/R + SS-31 10 μM (n=7)	I/R + SS-31 25 μM (n=6)	I/R + SS-31 50 μM (n=6)	I/R + SS-31 100 μM (n=6)
Initial LVESP (mmHg)	99±4	99±4	96±2	104±3	94±2	104±3	107±3	96±2
Initial LVEDP (mmHg)	11±1	12±1	10±1	11±1	7±1	10±1	11±1	9±0.3
Initial LVDP (mmHg)	88±4	87±4	86±2	93±2	87±2	94±3	96±3	87±2
Final LVESP (mmHg)	102±6	115±6	115±8	131±11*	85±6	103±5	127±5 *##††ω	103±10
Final LVEDP (mmHg)	61±5	63±5	49±6 ψ	66±7	52±6	59±3	50±7	49±6
Final LVDP (mmHg)	41±5	51±10	66±5*	65±9*	33±5	45±7	77±5**##†† ωω	54±7#
Initial+dP/dt _{max} (mmHg/s)	2349±78	2218±72	2226±60	2342±40	2211±25	2270±66	2216±48	2182±41
Final+dP/dt _{max} (mmHg/s)	882±113	912±193	1381±110*ψ	1294±148*ψ	674±120	970±98	1565±162**##††ωω	1069±76#
Initial-dP/dt _{min} (mmHg/s)	-1570±77	-1436±50	-1420±36	-1486±50	-1448±37	-1629±40	-1540±70	-1429±59
Final-dP/dt _{min} (mmHg/s)	-769±56	-858±157	-1025±115	-948±187	-526±80	-704±101	-1256±120**##††	-886±149#
Initial Coronary Flow (mL/min)	17±1	15±1	17±2	17±2	17±1	21±1	18±3	16±1
Final Coronary Flow (mL/min)	8±0.4	9±1	12±3	8±1	8±1	10±1	11±1	9±1
Initial Heart Rate (BPM)	278±16	251±14	273±12	277±14	266±5	282±20	261±7	300±9
Final Heart Rate (BPM)	250±14	240±20	238±23	243±21	232±8	257±15	265±16	262±12
Infarct Size (%)	45±2	54±6	27±3 ** ψ ψ	23±2 ** ψ ψ	36±2 *	25±4 **##	19±2**##	21±2**##

when compared to controls as shown in (Table 1). The concentrations of MitoQ and SS-31 that showed the greatest effect, reduced infarct size to 23±2% (20 μM, p<0.01, n=6) and 19±2% (50 μM, p<0.01, n=6), respectively, compared to the control I/R group, which showed an infarct size of 45±2%. The lowest infarct sizes also correlated with robust and significant recovery in post-reperfused LVDP at 45 min observed in MitoQ treated [(20 μM); p<0.05, n=6]; SS-31 treated [(50 μM); p<0.01, n=6]) compared to control I/R hearts (n=12) (Table 1).

Effects of MitoQ and SS-31 on ROS and NO bioavailability in blood

We compared the effects of MitoQ and SS-31 in reducing blood ROS as measured as a decrease in H₂O₂ and an increase in bioavailability of NO in a rat hindlimb I/R model. Our data depicts the relative difference between the I/R limb and the non-ischemic or sham limb in animals treated with drug or saline (control). As shown in (Figure 3), rats treated with a bolus of MitoQ (1.8mg kg⁻¹ ~10 μM in blood) at the onset of reperfusion had a significant reduction in blood levels of H₂O₂ beginning at 10 min after infusion and maintained this effect throughout the reperfusion period compared to controls receiving saline. Similarly, rats treated with SS-31 (2.5 mg kg⁻¹ ~ 50 μM in blood) at reperfusion had significantly decreased H₂O₂ levels compared to the control group. This effect was observed at 15 min and maintained throughout the reperfusion period (Figure 4). In contrast to blood H₂O₂ levels, NO bioavailability was significantly higher beginning at 20 min post reperfusion in rats treated with MitoQ (1.8 mg kg⁻¹, ~10 μM in blood) or SS-31 (2.5 mg kg⁻¹, ~ 50 μM in blood) compared to saline controls (Figure 5), and this effect was maintained throughout the reperfusion period (Figure 6).

Discussion and Conclusion

The major findings of this study are: 1) Both MitoQ and SS-

31 treatment initiated at the onset of reperfusion restored post-reperfused cardiac function and reduced infarct size compared to controls in isolated rat hearts subjected to 30 min of ischemia and 45 min of reperfusion; 2) MitoQ and SS-31 significantly decreased blood H₂O₂ and increased blood NO bioavailability following hindlimb I/R injury compared to saline controls. The increased NO bioavailability effect was most likely due to a reduction in NO scavenging by SO. Together, these results indicate that mitochondrial-targeted antioxidant agents are effective in reducing I/R injury when given only at reperfusion by reducing mitochondrial ROS production and improving NO bioavailability.

Myocardial I/R Model

The *ex vivo* myocardial I/R model has proven to be reliable to screen potential drug candidates to reduce reperfusion-induced cellular injury [20,26,27,30,31]. Drug candidates that have shown reduction in infarct size and improved post-reperfused function in *ex vivo* studies are then tested using *in vivo* myocardial I/R models [32-34]. Previous studies have shown that both MitoQ and SS-31 were able to reduce infarct size when given as a pretreatment or preconditioning, i.e., prior to the initiation of a prolonged ischemia period [13,24,35]. The novelty of our study is that we tested both of these mitochondrial-directed antioxidants when given only briefly, during the first 5 min of reperfusion following 30 min of ischemia. This is an important test to evaluate the therapeutic benefit of such compounds since pretreatment is often not an option prior to percutaneous coronary intervention. In addition, many potential drug candidates that have worked when given as a pretreatment in preclinical myocardial I/R studies, e.g., cyclosporine and exenatide, have not translated to the clinical setting of myocardial infarction

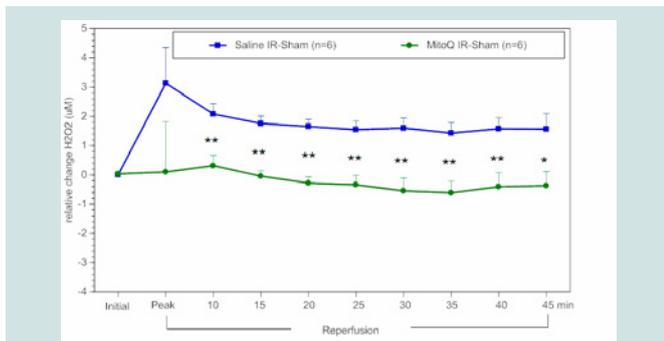


Figure 3: Timecourse of the relative difference in H₂O₂ release of I/R limbs compared to the other sham limbs (I/R–sham) during reperfusion in MitoQ (1.8 mg kg⁻¹) or saline treated animals. The blue squares represent the relative difference in H₂O₂ release between the sham limb and I/R limb in the saline treated (control) group. The green circles represent the relative difference in H₂O₂ release between the sham limb and I/R limb in the MitoQ treated group. All data were analyzed using Student's t-test (*p<0.05; **p<0.01 compared to saline control).

[36,37]. In part, this may be due to the inefficient targeting to key sources of ROS or other necrotic pathways active at the beginning of reperfusion or limited accessibility of the therapeutic agent to its target pathway. It is more likely that agents that are effective in preconditioning the myocardium fail as postconditioning agents because cardioprotective pathways prior to the ischemia are different from cardioprotective pathways after the ischemic insult [27,30]. In general, the effectiveness of these mitochondrial-directed therapeutic agents, specifically when given upon the onset of reperfusion and for only a brief time period (5 min), strongly support the hypothesis that the mitochondria play a critical role in mediating reperfusion-induced cell death in the early minutes following the reestablishment of flow.

A. Cardiac function

Interestingly in this study, MitoQ (10-20 µM) and SS-31 (50 µM) were found to be the most effective doses in restoring post-reperfused cardiac function. Specifically, the component of LVDP that seems to be responsible for this improved function was the significant elevation in LVESP (i.e., MitoQ 20 µM and SS-31 50 µM) compared to controls. This may be related to improving mitochondrial function, which in turn would lead to greater ATP production and an improvement in cardiac contractility during reperfusion [3].

The unique finding in our study is that both MitoQ and SS-31 given only during the first 5min of reperfusion was sufficient to restore cardiac function, thus, need not be given throughout reperfusion or as pretreatment (prior to ischemia) as reported in previous studies [13,24,35]. Mechanistically, this may be related to inhibiting mitochondrial ROS during reperfusion and reducing cardiolipin peroxidation, thus maintaining the electron transport chain function, preserving mitochondrial membrane potential and ATP synthesis, and maintaining normal Ca²⁺ handling by the cardiomyocyte leading to enhancement of post-reperfused cardiac function [7]. Previous reports on the cardioprotective effect of MitoQ on I/R injury in isolated rat heart have described using this agent prior to prolonged ischemia (preconditioning) to limit mitochondrial damage during the ischemic period, which in turn would also presumably preserve

mitochondrial function at reperfusion [13,23,24]. By contrast, the results from our study implicate that inhibiting mitochondrial-derived ROS production briefly during the onset of reperfusion is sufficient to restore post-reperfused cardiac function and attenuate infarct size. In general, the present findings further support the premise that MitoQ inhibits lethal ROS induced I/R injury [4,24]. A previous study using SS-31 on isolated guinea pig hearts subjected to I/R (30 min ischemia and 90 min reperfusion) reported that SS-31 infused during baseline (prior to ischemia) and throughout the reperfusion period or throughout the reperfusion period only (no baseline infusion) significantly improved post-reperfused cardiac contractile function [35]. By contrast, we found in the present study that SS-31 or MitoQ infused during only the first 5 min of a 45 min reperfusion period was sufficient to significantly reduce I/R injury, most likely via a reduction in mitochondrial produced ROS.

As shown in (Figure 2), while treatment with a 10 µM concentration of SS-31 significantly reduced infarct size, it did not restore cardiac function, suggesting that the viable myocardium was stunned and unable to fully recover post-reperfusion cardiac function within 45 min of reperfusion. Stunning is a phenomena where the heart does not recover function immediately following reperfusion that is not accounted for by cardiomyocyte death or reduced blood flow and may take several days to weeks before returning to normal. Consistent with this observation, another study of SS-31, also known as Bendavia, reported that the compound reduced infarct size in two of three myocardial I/R models: (*in vivo* sheep and *ex vivo* guinea pig hearts showed a significant reduction of infarct size, and *in vivo* rabbit hearts showed a trend of reduced infarct size). However, cardiac function (i.e., LVDP of guinea pig hearts *ex vivo* and ejection fraction in sheep and rabbit hearts *in vivo*) did not recover in any of the three models [38]. Collectively, the data from the current study and others suggest that post ischemic administration of SS-31 is effective in reducing infarct size over a relatively broad dose range, but myocardial stunning may persist since cardiac function did not recover over the reperfusion period tested (45 min). Interestingly, studies on isolated mouse liver mitochondria [23], showed that over the same concentration range used in the present study, SS-31 concentration dependently inhibited ROS in isolated mitochondria

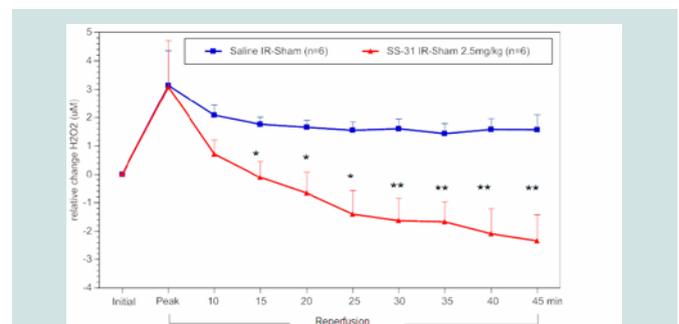


Figure 4: Time course of the relative difference in H₂O₂ release of I/R limbs compared to their sham limbs (I/R–sham) during reperfusion in SS-31(2.5 mg kg⁻¹) or saline treated animals. The blue squares represent the relative difference in H₂O₂ release between the sham limb and I/R limb in the saline treated (control) group. The red triangles represent the relative difference in H₂O₂ release between the sham limb and I/R limb in the SS-31 treated group. All data were analyzed using Student's t-test (*p<0.05; **p<0.01 compared to saline control).

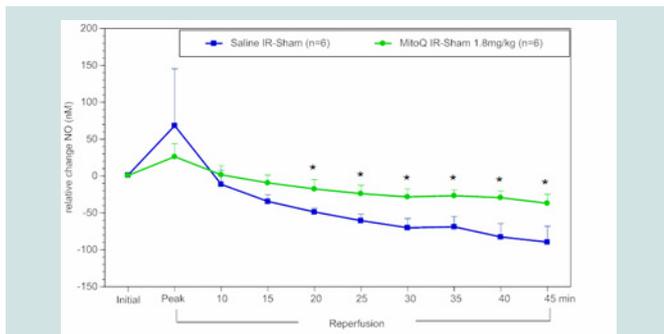


Figure 5: Time course of the relative difference in NO release of I/R limbs compared to their sham limbs (I/R-sham) during reperfusion in MitoQ (1.8 mg kg⁻¹) or saline treated animals. The blue squares represent the relative difference in NO release between the sham limb and I/R limb in the saline treated (control) group. The green circles represent the relative difference in NO release between the sham limb and I/R limb in the MitoQ treated group. All data were analyzed using Student's t-test (*p<0.05 compared to saline control).

induced by 3-nitropropionic acid, a potent complex II toxin. These results, along with the hindlimb I/R studies described earlier suggest that inhibiting mitochondrial-derived ROS underlies the improved post-reperfused cardiac function and reduced infarct size found with SS-31 and MitoQ treatment. Collectively, the data indicate that MitoQ significantly improved final post-reperfused LVDP compared to controls, and reached a maximum effect between 10 to 20 µM with this mitochondrial anti-oxidant agent (Table 1). While SS-31 also exhibited a concentration dependent effect to improve cardiac function as indicated by the increase in final LVDP (i.e., 10 µM[33±5mmHg]; 25 µM[45±7mmHg] vs. 50 µM[77±5mmHg]), the 100 µM[54±7mmHg] exhibited a decrease in post-reperfused cardiac function compared to hearts treated with 50 µM. These latter results suggest that SS-31 at 100 µM may induce post-reperfused cardiac stunning. A similar effect was also reported in *ex vivo* guinea pig hearts and *in vivo* rabbit and sheep hearts in the Bendavia study [38].

B. Infarct size

In a previous study, Adlam et al. provided MitoQ (500 µM) in drinking water as pretreatment for two weeks and reported a significant reduction in lactate dehydrogenase activity in coronary effluent of isolated hearts [13], in cytochrome c release from mitochondria into the cytosol and in caspase 3 upregulation, all indicating a reduction in cardiac tissue damage. By contrast, in this study, both MitoQ (10 and 20 µM) and SS-31 (10-100 µM) reduced infarct size when compared to untreated controls when given during the initial 5 min of reperfusion (Table 1). This finding is clinically relevant since it indicates that these mitochondrial-directed compounds could be effective after ischemic injury has occurred and thus, could be given in a clinical setting to limit I/R injury, during percutaneous coronary intervention, thrombolytic treatment, or by-pass surgery. Likewise, Szeto reported that SS-31 is effective in reducing infarct size in isolated hearts subjected to global ischemia when given throughout the 90 min reperfusion period [14]. In contrast, in the present study we found SS-31 was effective in both improving cardiac function and reducing infarct size when given during only the first 5 min of reperfusion (Table 1). Collectively, the data show that MitoQ exhibited a concentration dependent effect in

the reduction of infarct size (1 µM [54±6] vs. 10 µM[27±3] and 20 µM[23±2]) that paralleled the improvement in post-reperfused LVDP. By contrast, SS-31 treated hearts showed a concentration-dependent effect in reducing infarct size, and this effect was maximally obtained in the 50 µM[19±2%] to 100 µM[21±2%] concentration tested. The effects of SS-31 in this study are consistent with the findings in the Bendavia study (38) and suggest SS-31 can still exert myocardial tissue salvaging effects (i.e., reduced cell death) without an accompanying increase in post-reperfused cardiac function (e.g., LVDP) since only the 50 µM exhibited significant improvement in post-reperfused cardiac function compared to controls. Moreover, the infarct sparing effects of SS-31 in concentrations that did not result in significantly improved post-reperfused cardiac function compared to controls during the 45 min reperfusion period suggest that the heart may be experiencing stunning and could potentially recover cardiac function as stunning subsided.

Collectively, the effects of MitoQ (10 & 20 µM) and SS-31 (all concentrations) on infarct size are similar despite differences in their mode of entry into the mitochondria. The positive charge on the MitoQ molecule is essential for entry into the negatively-charged mitochondrial membrane potential [13,21]. By contrast, SS-31 can enter the mitochondria independently of the mitochondrial membrane potential [14]. During global ischemia, mitochondrial membrane potential likely dissipated. Nevertheless, the results of this study would suggest that both of these mitochondrial-directed antioxidants are able to rapidly enter the mitochondria within the first 5 minutes of reperfusion to preserve mitochondrial membrane potential and decreased mitochondrial ROS production compared to untreated control hearts.

Hindlimb I/R model

We used the hindlimb I/R animal model to measure changes in blood H₂O₂ and NO in real-time in rats treated with MitoQ and SS-31 at reperfusion. We chose this model due to the ability to measure blood H₂O₂ as an index of blood ROS levels that may occur in the coronary circulation during reperfusion injury, thought to result from both endothelial and cardiomyocyte damage. Moreover, our previous studies have shown consistently that agents which reduce blood H₂O₂

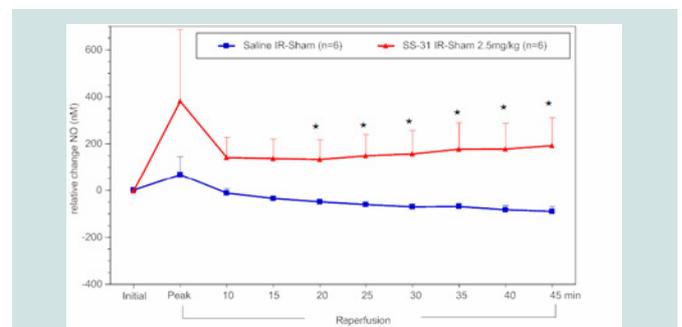


Figure 6: Time course of the relative difference in NO release of I/R limbs compared to their sham limbs (I/R-sham) during reperfusion in SS-31 (2.5 mg kg⁻¹) or saline treated animals. The blue square line represents the relative difference in NO release between the sham limb and I/R limb in the saline treated (control) group. The red triangles represent the relative difference in NO release between the sham limb and I/R limb in the SS-31 treated group. All data were analyzed using Student's t-test (*p<0.05 compared to saline control).

and increase blood NO in hindlimb I/R also improve post-reperfused cardiac function and reduce infarct size [18,19,20,31,39].

Inhibition of mitochondrial-derived ROS release would attenuate the quenching of endothelial-derived NO (i.e., less -OONO formation) and thus lead to restoration of post-reperfused cardiac function, reduction of infarct size, and inhibition of inflammatory cytokines released from ROS-damaged tissue [4,24,40]. As expected, the bioavailability of blood NO following I/R injury was significantly greater in the groups treated with MitoQ or SS-31 compared to the controls, which only received saline at reperfusion. This is likely related to the decrease in blood H₂O₂ in treated animals. Blood H₂O₂ serves as an index of SO, given the short half-life of SO. When SO scavenges NO, it produces the harmful -ONOO, which further exacerbates reperfusion-induced ROS tissue damage and leads to a further decrease in the bioavailability of NO [16]. Moreover, attenuating mitochondrial-derived ROS would also indirectly attenuate additional ROS release from uncoupled eNOS (oxidation of BH₄), Nox2 (ROS mediated cytokine release) and xanthine oxidase (ROS mediated microvascular dysfunction), thereby inhibiting all four known sources of ROS during reperfusion [18,20,31,41]. This is consistent with our findings in (Figures 3-6), which shows an inverse relationship between lowering blood H₂O₂ and increasing blood NO with both MitoQ and SS-31 treatment. Recently, Cai et al. 2018 reported that SS-31 stabilized Superoxide Dismutase (SOD) and catalase levels determined by western blot in the hindlimb I/R model compared to control, which showed a relative decrease in SOD and catalase [25]. It is well known that SOD converts SO to H₂O₂ and catalase converts H₂O₂ to H₂O to attenuate ROS production; therefore, the stabilizing effects of SS-31 on SOD and catalase activities in I/R injury would cause a further reduction of ROS [17,25]. Our results extend these findings and show that MitoQ and SS-31 treatment at the onset of reperfusion result in a decrease in blood H₂O₂ and an increase in blood NO compared to controls. Collectively, these new findings are consistent with our previous studies showing reduction of blood H₂O₂ and increased NO bioavailability in hindlimb I/R correlates with improved post-reperfused cardiac function and decreased infarct size in myocardial I/R [18,19,20,31,39].

The role of mitochondria in I/R injury

Preserving the integrity of these energy-producing organelles is of vital importance to maintain cardiac function and reduce I/R injury. It is well known that mitochondria are key sources of ROS, especially during the reperfusion phase of I/R [33]. Therefore, it is plausible that MitoQ and SS-31 act to scavenge mitochondrial-derived ROS when given just during reperfusion. MitoQ is selectively concentrated within the mitochondria due to the large membrane potential gradient. It is reduced by the respiratory chain to ubiquinol, which is then oxidized to ubiquinone and accumulates in the mitochondria. Its antioxidant effects are recovered when ubiquinone is converted back to ubiquinol by the respiratory chain, which is active during reperfusion [13]. In contrast, the two positively charged amino acids of SS-31 enable this molecule to be concentrated in the mitochondria in an energy independent, non-saturable manner. This difference may be significant in that the mitochondrial membrane potential may be disrupted during reperfusion and therefore limit the effectiveness of MitoQ. Our results show that MitoQ (10 and 20

µM) was cardioprotective when given upon reperfusion after a 30 min ischemic period, which indicates that either the mitochondrial membrane potential was sufficiently maintained despite I/R or MitoQ can accumulate in the mitochondria despite dissipation of the mitochondrial membrane potential. Collectively, the data from both mitochondrial-directed agents would suggest that mitochondria are a major component of reperfusion-induced cell death, and consequently that attenuating mitochondrial-derived ROS at the beginning of reperfusion can mitigate the deleterious effects of ROS on infarct size and post-reperfused cardiac function.

Future studies

Considering that I/R induced ROS is a major component of reperfusion injury, inhibiting more than one major source of I/R derived ROS may lead to even further reduction in infarct size. It is well known that mitochondrial dysfunction, NADPH oxidase, uncoupled eNOS, and xanthine oxidase are the principal sources of I/R derived ROS. Therefore, inhibiting mitochondrial-derived ROS combined with a Nox2 inhibitor should be more effective in reducing the deleterious effects of reperfusion injury than either agent alone. Thus, it would be interesting to test a combination of a Nox2 inhibitor (e.g., apocynin or Nox2ds peptide) and a mitochondrial antioxidant (e.g., MitoQ or SS-31) on post-perfused cardiac function, infarct size, and blood H₂O₂/NO in myocardial and hindlimb I/R, respectively. Such studies would help determine the extent of the contribution of ROS to I/R injury.

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