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Nitrite therapy after cardiac arrest reduces ROS generation, improves cardiac and neurological function and enhances survival via reversible inhibition of mitochondrial complex I

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Clinical Summary

Cardiac arrest results in significant morbidity and mortality driven mainly by the cardiac and neurological injury resulting from global ischemia and reperfusion injury. Although resuscitation rates can exceed 65% for some rhythms, between 50-75% of these patients will die before hospital discharge and up to a third of survivors will suffer significant brain injury. Only hypothermia has shown clinical benefit as a post-resuscitation therapy in a subset of patients. Clearly additional therapies are needed. The recent finding that nitrite acts as an ischemic reservoir for enzyme independent nitric oxide generation has resulted in numerous animal studies where it has proven beneficial in reducing focal ischemic organ injury. Based on promising results in focal heart and brain ischemia, Dezfulian et al. have adapted a mouse model of cardiac arrest to model the high clinical mortality and myocardial and neurological dysfunction associated with cardiac arrest. Within this model, nitrite therapy given at the start of resuscitation resulted in significant improvements in survival and myocardial and neurological function in survivors. The authors further investigate the potential mechanism for cardioprotection which involves nitrite's role as a mitochondrial antioxidant early in resuscitation. The significant benefits attributed to nitrite, along with its ease of delivery and known primate and human safety data make this a promising therapy for a condition with few current therapeutic options.

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Subject Codes: [151] Ischemic biology - basic studies, [91] Oxidant stress, [130] Animal models of human disease, [25] CPR and emergency cardiac care

Disclosures

Dr. Gladwin is co-inventor on NIH/Government patents for the use of nitrite salts for cardiovascular indications and the use of nitrite to detoxify hemoglobin based oxygen carriers.

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Abstract

Background—Three-fourths of cardiac arrest survivors die prior to hospital discharge or suffer significant neurological injury. Excepting therapeutic hypothermia and revascularization, no novel therapies have been developed that improve survival or cardiac and neurological function after resuscitation. Nitrite (NO_2^-) increases cellular resilience to focal ischemia-reperfusion injury in multiple organs. We hypothesized that nitrite therapy may improve outcomes after the unique global ischemia-reperfusion insult of cardiopulmonary arrest.

Methods and Results—We developed a mouse model of cardiac arrest characterized by 12-minutes of normothermic asystole and a high cardiopulmonary resuscitation (CPR) rate. In this model, global ischemia and CPR was associated with blood and organ nitrite depletion, reversible myocardial dysfunction, impaired alveolar gas exchange, neurological injury and an approximate 50% mortality. A single low dose of intravenous nitrite (50 nmol=1.85 μmol/kg=0.13 mg/kg) compared to blinded saline placebo given at CPR initiation with epinephrine improved cardiac function, survival and neurological outcomes. From a mechanistic standpoint, nitrite treatment restored intracardiac nitrite and increased S-nitrosothiol levels, decreased pathological cardiac mitochondrial oxygen consumption due to reactive oxygen species formation and prevented oxidative enzymatic injury via reversible specific inhibition of respiratory chain complex I.

Conclusion—Nitrite therapy after resuscitation from 12-minutes of asystole rapidly and reversibly modulated mitochondrial reactive oxygen species generation during early reperfusion, limiting acute cardiac dysfunction and death, as well as neurological impairment in survivors.

Keywords

cardiopulmonary resuscitation; heart arrest; ischemia; nitric oxide; reperfusion

Introduction

Nitrite (NO_2^-), historically considered inert, functions as a reservoir for nitric oxide (NO) [1]. During physiological hypoxia and pathological ischemia, nitrite is reduced to NO regulating hypoxic vasodilation, cellular respiration, mitochondrial reactive oxygen species (ROS) generation, angiogenesis[2], and cellular death programs[3]. Nitrite in human plasma exists at concentrations of 100–300 nM[3–5] and may be reduced to NO by iron-containing enzymes[3, 6] including hemoglobin, myoglobin, neuroglobin, xanthine oxidoreductase, endothelial NO synthase, mitochondrial electron transport chain proteins and the hepatic cytochrome P450 system. The rate and extent of nitrite reduction is coupled to deoxygenation and proton generation. Thus NO generation is coupled to oxygen and pH gradients[3, 7] and maximized in ischemic tissues.

Nitrite therapy limits cellular injury and apoptosis after ischemia and reperfusion[6]. Nitrite therapy is cytoprotective in numerous animal models of focal ischemia-reperfusion injury[6] including rodent heart[8], brain[9], liver[8] and kidney, canine heart[10] and primate brain[11]. Systemic nitrite reduction by ceruloplasmin knockout[12] or dietary nitrate/nitrite elimination[13] increased infarction volume in the liver and heart after experimental ischemia. These studies indicate that physiological systemic nitrite levels modulate host resilience to ischemia. The established safety of human and animal nitrite dosing[14] and its potent effects in limiting major organ injury suggest that nitrite represents an ideal therapy for cardiac arrest.

Cardiac arrest results in global multi-organ ischemic injury associated with significant morbidity and mortality[15, 16]. Over 70% of those resuscitated die prior to hospital discharge[15, 16]. Excepting the selective application of hypothermia and revascularization, no novel post-resuscitation therapies have been developed that improve survival or cardiac and neurological function[15]. We explored nitrite therapy in a mouse model of cardiac arrest. We show evidence that nitrite therapy improves cardiac function, survival and neurological function in survivors. Mechanistically, we show that nitrite specifically and reversibly inhibits cardiac complex I, limiting oxidative reperfusion injury. Nitrite's ease of delivery, established human safety and efficacy in murine cardiac arrest suggest its promise as a novel therapy after cardiac arrest.

Methods

Mouse Cardiac Arrest Model

In initial model building we adapted mouse cardiac arrest models from prior reports[17, 18] with the goal of nearly 100% resuscitation and 50% 24 hour mortality. To modulate mortality we extended asystolic time from 8 to 12 minutes and added epinephrine to increase return of spontaneous circulation (ROSC). Seven to twelve week old (24–30g) C57BL/6 male mice were used as approved by the NIH IACUC. Anesthesia intraperitoneal ketamine/xylazine (100/15 mg/kg respectively) preceded surgical preparation consisting of orotracheal intubation and placement of right carotid and jugular catheters. Ventilation (ASV, Harvard Instruments, Cambridge, MA) for 10 minutes (minimum) at a rate of 110 bpm and a volume of 15 ml/kg preceded cardiac arrest and produced normal blood gases (Table 1). EKG, arterial blood pressure, exhaled carbon dioxide and rectal temperature were recorded at baseline and during 60 minutes post-resuscitation (Powerlab, ADInstruments, Colorado Springs, CO). Asystole was induced by 100 μl potassium chloride (0.09 g/kg; minimum 2.5% [w/v]) IV bolus and temperature maintained at 36.5°C for 12 minutes. Cardiopulmonary resuscitation (CPR) was performed with rapid (375–425) finger compressions, resumption of mechanical ventilation (25% tidal volume increase) and 10 μg IV epinephrine (500 μl) prior to CPR and 2 μg (100 μl) 1 minute post-CPR. Animals received either 50 nmol (1.85μmol/kg=0.13 mg/kg,) sodium nitrite (in 0.9% saline) or saline placebo IV in randomized, blinded fashion at CPR initiation. This dose and timing of delivery were based on prior work[8].

To minimize variability we paired inbred animals to nitrite or placebo based on similar weight, sex, age, delivery date and where possible holding cage. Pairing appeared effective (Table 2). Post-resuscitation care was uniform between groups. A model summary is provided as Figure 1. Sham control animals received anesthesia, surgery and ventilation but no cardiac arrest and the same post-resuscitation care until the similarly timed endpoint. Shams were drawn from the same batch as experimental animals.

Blood Gases and Blood/Tissue Nitrite Levels

Carotid arterial blood was obtained in a heparinized syringe either 55 minutes after anesthesia (sham) or 5 minutes after CPR. Blood was utilized for blood gas analysis (Nova Biomedical, Waltham, MA) or mixed 4:1 with nitrite preservation solution[4] or centrifuged to isolate plasma for nitrite measurements. Animals were perfused[19] with tissue nitrite preservation solution (1 mM KCN, 0.2% NP-40, 0.8 mM ferricyanide, 0.5 mM NEM, 100 μM DTPA) and brains homogenized for nitrite measurements. Snap frozen hearts obtained 15 minutes after CPR were sectioned at −20°C, placed into ice cold nitrite preservation solution and homogenized for nitrite measurements. All nitrite measurements were determined by tri-iodide based gas-phase reductive chemiluminescence using an NO analyzer (GE Analytic, Boulder, CO) as described previously[20, 21] and tissue levels normalized to protein content (BCA Protein Assay, Pierce, Rockford, IL).

Left ventricular echocardiography

Animals had lines removed, wound closure and chest hair removal before transportation for echocardiogram 60 minutes after CPR or sham surgery. Animals were secured to the Vevo 770 (VisualSonics, Toronto, ON, Canada) platform and temperature/EKG were monitored (temperature maintained at >35°C, heart rate >300 beats per minute, supplemental oxygen delivered via nose cone). Parasternal long-axis 2D images of the left ventricle (LV) were obtained using the RMV707B scanhead 75–90 minutes post-CPR. M-mode images were used to measure end-systolic and –diastolic ventricular size and fractional shortening and ejection fraction (EF) calculated with the manufacturer's software (v.2.3.0).

Cardiac MRI

Anesthetized animals (1–1.5% isoflurane) were MRI imaged 24 hours post-CPR or sham surgery. MRI experiments were carried out in a 7.0T, 16-cm horizontal Bruker MR imaging system (Bruker, Billerica, MA) with Bruker ParaVision 3.0.2 software. Magnevist (Bayer HealthCare, Montville, NJ) diluted 1:10 with sterile 0.9% saline, was administered subcutaneously at a dose of 0.3 mmol/kg. Six short axis slices were used to determine EF and ventricular volumes using CAAS-MRV-FARM software (Pie Medical Imaging, Netherlands).

Histology

Mice were transcardiac perfused with saline followed by 10% buffered formalin[19] and brains removed, further fixed, paraffin-embedded, sectioned and stained with hematoxylin and eosin. Seven serial high powered (400×) fields of bilateral CA1 at Bregma -1.5 mm were examined and live and dead cells counted and normalized to hippocampal length as described elsewhere[17].

Mitochondria Isolation

Heart mitochondrial isolation was performed by differential centrifugation as described elsewhere[22] and protein concentration determined. Fresh mitochondria were used for respirometry, ROS and ATP generation assays and aliquots stored at −80°C for subsequent Complex I activity assays as described previously[23].

Aconitase Activity

Hearts snap frozen 15 minutes after CPR or sham surgery were homogenized in the manufacturer's commercial buffer and lysates prepared by three cycles of freeze/thaw. Aconitase activity was determined spectrophotometrically (340 nm) monitoring NADPH formation using the Bioxytech Aconitase-340 kit (Oxis Research).

Statistical Analysis

Data appears as means±standard error (SEM) with analysis performed using GraphPad Prism 5 (La Jolla, CA). Continuous data were compared between three groups using one-way ANOVA with post-hoc Bonferroni adjustment, between two groups using paired Student's t-test for variables that are normally distributed and Wilcoxon for variables that are not normally distributed and for variables measured at multiple time points from same subjects using repeated measures ANOVA. Mitochondrial experiments were performed as multiple paired experiments at discrete times and therefore analyzed at each time utilizing a paired t-test. Mortality was assessed by Kaplan-Meier survival analysis (log rank test). A two-tailed $p<0.05$ was considered statistically significant

Statement of Responsibility

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Cardiac arrest physiological effects

Cardiac arrest resulted in metabolic (lactic) and respiratory acidosis and oxygen depletion (Table 1). Ischemic times and resuscitation rates were similar between groups (Table 2). Cardiac arrest led to transient hyperemia soon after resuscitation (Figure 1B) as described by others[17]. One hour after resuscitation, placebo treated mice exhibited signs of myocardial depression based on decreased blood pressure, increased tachycardia (Figure 1C) and decreased ejection fractions (Figure 1D) compared to pre-arrest baseline. Twenty four hours later LVEF had normalized but right ventricular (RV) failure remained based on RV dilation and diminished RVEF (Figure 1E). Since right sided pressures were not assessed, it's unclear whether this was due to pulmonary hypertension or loss of RV contractility.

Cardiac arrest depletes systemic nitrite which is reversed by IV therapy

Nitrite is reduced to NO and NO-modified proteins during focal ischemia in rodents[13, 24]. We tested the hypothesis that basal systemic levels of nitrite would be reduced by ischemic consumption during cardiac arrest and IV nitrite could restore levels (Figure 2A). In placebo treated mice, ischemia depleted whole blood nitrite levels (0.64±0.05μM) and nitrite therapy repleted these levels (1.01±0.06 μM) to near baseline (1.15±0.10μM). These findings were mirrored in plasma (Figure 2B), heart (Figure 2E/F) and a similar trend noted in brain (Figure 2C). S-nitrosation of the cardiomyocyte L-type calcium channel[25] and mitochondrial complex I[23, 25] are protective post-translational modifications resulting from nitrite therapy or ischemic preconditioning. We found that total S-nitrosothiol modified protein concentration (Figure 2F) did not change with ischemia but nitrite therapy significantly increased these levels ($p<0.05$).

Nitrite repletion improves cardiac function

Increased systemic nitrite in treated mice was associated with improved cardiac function. Just before CPR, animals exhibited similar vascular loading based on identical asystolic pressures (Table 2). Nitrite didn't decrease diastolic blood pressure, a coronary perfusion pressure surrogate, during CPR and ROSC occurred sooner ($p=0.034$) than placebo treatment. Consistent with improved cardiac function, nitrite-treated mice exhibited trends towards less tachycardia than placebo-treated controls (68.7±12.1 vs. 94.7±17.8 beat per minute increase) and less hypotension (9.1 ± 3.2 vs. 12.6±4.2 mm Hg decrease).

Nitrite significantly improved post-arrest LVEF (54.4±2.4%; Figure 3A/C) compared to placebo-treated mice (43.5±2.9%; $p=0.007$). Heart rate and blood pressure were similar between treatment groups throughout post-ROSC monitoring (data not shown, $p>0.2$). Given similar baseline volume loading pre-CPR and similar mean blood pressures prior to imaging, the LVEF improvements likely indicate improved LV contractility. RVEF measured by MRI (Figure 3B/D) 24 hours post-CPR was significantly better in nitrite treated mice (54.7±1.3%) than placebo (42.8±1.7%; $p< 0.001$). Consistent with improved pulmonary perfusion, gas exchange 5 minutes post-CPR was improved with nitrite therapy compared to placebo (Table 1).

Nitrite repletion improves survival and survivor neurological function

Death after ROSC occurred 1–6 hours post-CPR after which all animals survived until the subsequent day (Figure 4). We observed bradycardia and hypotension progressing to asystole

in mice dying early (while monitored) consistent with death from post-ischemic myocardial stunning and heart failure. Nitrite therapy significantly improved survival (19/25 [76%]) compared to placebo (12/25 [48%]; hazard ratio 2.72 [95% confidence interval 1.1–6.7]; p=0.033).

Since a third of cardiac arrest survivors have neurological disability, we blindly assigned neurological scores[18] to all 1 day surviving mouse pairs (n=11) (Figure 5A). The median score for nitrite-treated mice (11) was significantly better than placebo (9; p=0.020). In all pairs, placebo-treated mice exhibited equal or more severe impairment than nitrite-treated mice. We measured rectal temperature 22 hours post-resuscitation (Figure 5B) since rodent hypothermia correlates with severity of injury[26]. Nitrite-treated mice had better thermoregulation (34.9±0.4° C) than placebo-treated mice (32.4±0.9°C; p = 0.013). Sham surgery didn't impair neurological scores (all 12) or thermoregulation (36.5±0.5°C). Hippocampal CA1, know to be selectively vulnerable to global ischemia, showed no histological injury at 24 hours consistent with prior observations[27]. Seventy-two hour survival experiments were therefore performed (n=3) demonstrating increased CA1 neuronal injury in placebo- compared to nitrite-treated mice (Figure 5C, D).

Nitrite specifically and reversibly inhibits complex I

Based on prior observations[23, 28] we hypothesized that nitrite therapy *in vivo* would transiently S-nitrosate and inhibit mitochondrial complex I resulting in a decrease in reperfusion ROS. Heart mitochondria from mice treated with nitrite isolated 5, 15 and 60 minutes post-CPR exhibited reduced state 3 respiration using the complex I substrate pyruvate (Figure 6A) at 5 and 15 minutes but not one hour compared to placebo. Nitrite therapy did not reduce electron transport efficiency based on respiratory control ratio (5min: 9.5±1.7; 60 min: 13.9±4.4) which was similar to the placebo-treated group (5min: 7.5±1.0; 60min: 10.4±4.6). Complex II (succinate) dependent state 3 respiration was similar at all times (Figure 6B) and did not change with rotenone inhibition (data not shown) indicating nitrite's complex I specificity. Complex I activity measured by NADH oxidation at 5 and 60 minutes confirmed the respirometry findings (Figure 6C). Using pyruvate as substrate, we consistently found increased oxygen consumption by placebo-treated post-arrest mitochondria compared to pre-arrest despite reduced complex I (NADH consumption). This suggests pathological oxygen consumption to form ROS rather than for energy production. Complex I inhibition was reversed 60 minutes post-CPR as measured by respirometry (Figure 6A) and NADH oxidation (Figure 6C). ATP generation was similar between 60 minute post-arrest nitrite (58.1±8.1 nmol/min/mg protein) and placebo (57.1±8.7 nmol/min/mg) treated groups and pre-arrest mice (65.1±4.3 nmol/min/mg) indicating no persistent functional complex I inhibition.

Nitrite limits reperfusion ROS generation

Consistent with pathological oxygen conversion to ROS, peroxide generation by respiring mitochondria (pyruvate substrate) 5 and 15 minutes post-CPR exceeded that of pre-arrest mice but normalized by 1 hour (Figure 7A). Cardiac mitochondria from nitrite-treated mice had significantly less peak (5 minutes post-CPR) ROS production (14.0±3.2 pmol/min/mg protein) compared to placebo-treated mice (26.6±4.4 pmol/min/mg; p<0.01). The abundant mitochondrial enzyme aconitase is susceptible to oxidative modification decreasing its activity and thus useful as an indicator of oxidative damage. Cardiac arrest significantly decreased aconitase activity (Figure 7B). Compared to placebo (14.8±8.4 mU/mg protein), nitrite therapy attenuated this loss of aconitase function (61.6±11.4 mU/mg protein; p=0.05).

Discussion

We examine the effects of nitrite repletion on mitochondrial function, reperfusion ROS generation, organ function and survival in a 12-minute mouse cardiac arrest model. Cardiac arrest results in systemic nitrite depletion and low dose nitrite replacement (therapy with 50 nmol) at CPR initiation repletes these levels to near baseline and increases cardiac S-nitrothiols. Therapeutic nitrite repletion and S-nitrosation in heart is associated with transient, reversible inhibition of complex I reducing mitochondrial reperfusion ROS generation and oxidative injury. Nitrite improved pulmonary gas exchange, cardiac contractility and survival with a suggestion of neuroprotection.

Moderate NO reperfusion therapy is known to be protective[29] but NO formation is limited by NO synthase's dependence on oxygen and reduced substrates[30]. Nitrite acts as a reservoir for NO during ischemia and nitrite reduction generates NO through NOS-independent pathways [6, 31, 32]. We demonstrate that global ischemia depletes nitrite systemically reducing its availability to act as a reperfusion NO source. This profound depletion with brief global ischemia was surprising but not unprecedented[13] and explains why our nitrite dose did not achieve the "optimal" plasma levels (11.9 μM) noted after focal ischemia[8]. Nitrite repletion early in reperfusion provides a NOS-independent source of NO to ischemic tissues.

Mitochondrial complexes I and III are major sources of pathological reperfusion ROS [33] and transient, reversible inhibition of complex I has been proposed as a mechanism to achieve cardioprotection[34–36]. The protective effects of complex I inhibition have been described for nitrite[23], S-nitrosothiol donors[28] and amobarbital[34] and observed during classical ischemic preconditioning[25]. Complex I has numerous cysteine residues available for S-nitrosation with resultant inhibition of electron flow[37, 38]. Nadtochiy and colleagues S-nitrosated complex I in cardiomyocytes and isolated heart using S-nitroso-2-mercaptopropionyl glycine with associated reduction in ROS production and improved cardiac contractility after ischemia-reperfusion[28]. Similar to our findings, these authors noted reversal of S-nitrosation and complex I inhibition 30 minutes after ischemia. Shiva and colleagues provided the first evidence that nitrite S-nitrosates complex I and reduces ROS production in liver mitochondria after *in vitro* ischemia-reperfusion[23] though inhibition persisted for 5 hours and was bypassed via complex II. Sun and colleagues have shown complex I to be one of several proteins S-nitrosated in cardioprotective ischemic preconditioning [25].

Nitrite therapy is complex I specific based on the lack of effects using succinate. Complex I efficiency is uneffected therefore this isn't due to complex I damage. Complex I inhibition is reversible based on restored oxygen and NADH consumption and ATP generation by 60 minutes. The increase in complex I oxygen consumption with placebo in the absence of increased NADH oxidation implies pathological oxygen consumption to form ROS rather than ATP which is prevented with nitrite. Based on our ROS and aconitase data, nitrite is an antioxidant. This mechanism complements prior observations of reduced tissue nitrotyrosine staining[9, 39], lipid peroxidation[9] and superoxide production[9] with nitrite therapy.

Cardiac arrest's poor prognosis is driven primarily by brain and heart injury[15]. Excepting hypothermia, no beneficial post-resuscitation therapies exist since CPR's description ~50 years ago. Present post-resuscitation care is largely supportive[15]. Nitrite's role as a novel therapeutic would be of great importance in this setting.

Human myocardial dysfunction (stunning) is common cardiac arrest[40, 41], ultimately reversible[42] and strongly associated with mortality[40, 41] The molecular mechanisms of myocardial stunning after cardiac arrest remain unknown but loss of excitation-contraction coupling is believed to result from ROS injury and calcium-mediated proteolysis[43]. Nitrite, by reducing ROS, may mitigate stunning similar to other antioxidants[44]. The reduction in

myocardial dysfunction likely explains the 50% relative survival advantage we noted. Further work is needed to characterize nitrite's effects on brain injury but our results are encouraging.

We designed a mouse model of cardiac arrest with prolonged asystole to study the effects of nitrite on heart and brain injury after resuscitation. Our model utilizes hyperkalemia to induce arrest, limiting its clinical relevance and potentially causing artifacts which may be organ protective (eg cardioplegia) or injurious (endothelial damage perhaps causing RV dysfunction). In the context of these limitations, we demonstrate improvements in gas exchange, heart and brain function and survival. We demonstrate that nitrite transiently inhibits complex I resulting in an antioxidant effect. The ease in delivering IV nitrite,, its established human safety[14, 31], its reproducible cytoprotective effects in multiple organs and species[6] all suggest that nitrite represents a promising post-resuscitation therapy after cardiac arrest.

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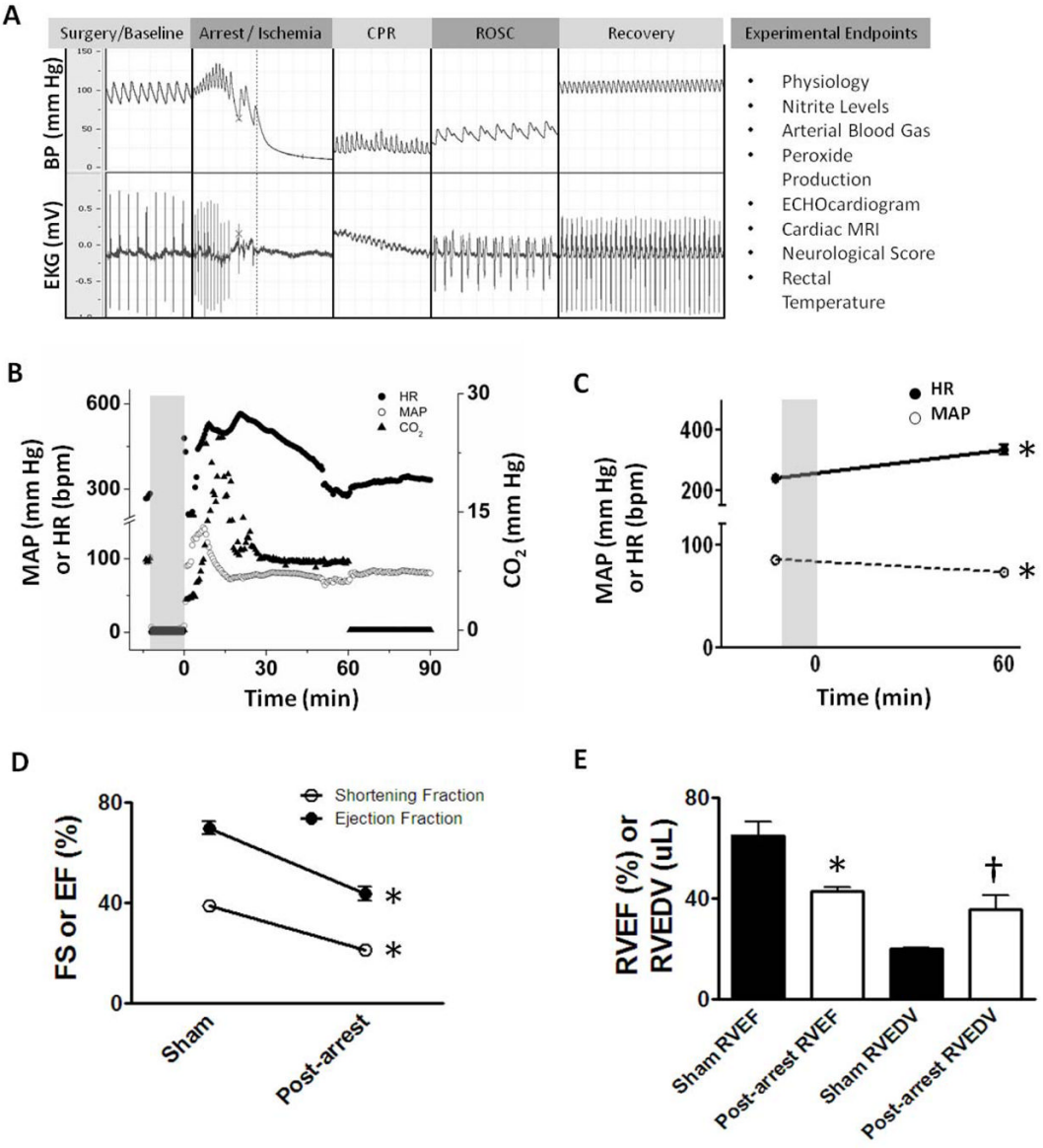

Figure 1. Cardiac arrest experiment, endpoints and cardiovascular effects

(A) Experimental outline with sample blood pressure and EKG pre-arrest, during arrest, cardiopulmonary resuscitation (CPR), return of spontaneous circulation (ROSC) and recovery phases with endpoints. (B) Physiological data from one experiment depicting heart rate (HR), mean arterial blood pressure (MAP) and exhaled carbon dioxide (CO_2). The ischemic period is shaded. (C–E) Post-arrest placebo treated mice are compared to non-arrested shams. (C) Mean HR increases and MAP decreases 60 min post-CPR vs. 1 min pre-arrest (n=21). (D) Cardiac arrest results in significant reductions in left ventricular fractional shortening (FS) and ejection fraction (EF) by echocardiography 75–90 minutes post-CPR (n=6). (E) Cardiac arrest results in diminished right ventricular ejection fraction (RVEF) and increased dilation (RVEDV: RV end-diastolic volume; n=5). Values denoted as means±SEM analyzed by paired t-test; *, $p<0.01$; †, $p=0.038$.

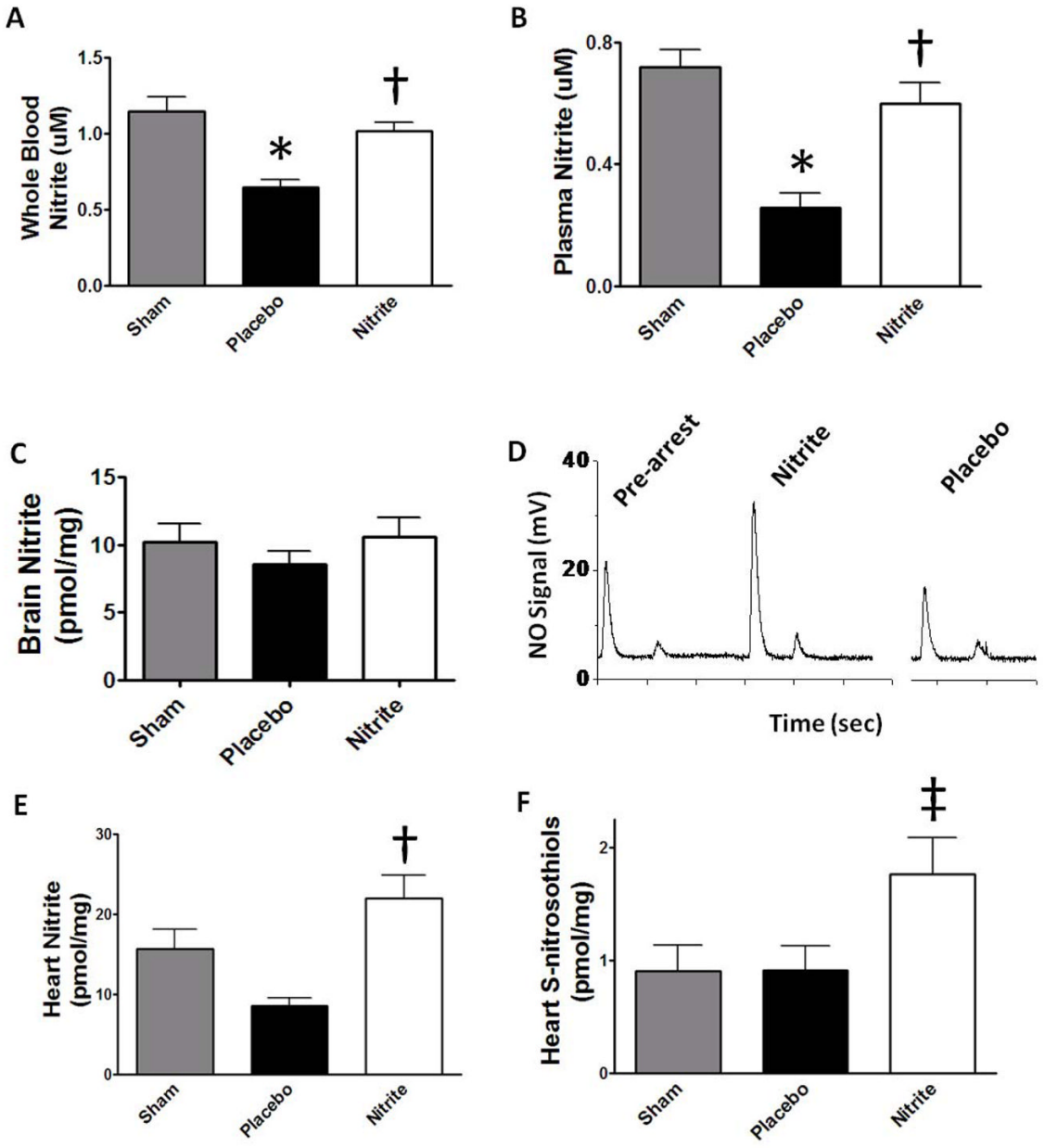

Figure 2. Systemic nitrite depletion after global ischemia and therapeutic repletion

Whole blood (A) and plasma (B) nitrite levels (n=6) measured after 12 minutes of global ischemia and 5 minutes reperfusion are depleted in placebo vs. sham, and nitrite therapy increases levels with similar trends in brain (C). (D) A sample reductive chemiluminescence tracing measuring nitrite in heart 15 min after CPR. Peaks represent total tissue nitrite (first peak), S-nitrosothiols (second peak) and the mercury stable fraction (third peak; not visible). (D) Ischemia depletes heart nitrite in placebos and nitrite therapy significantly increases total levels and (E) S-nitrosothiols (n=7). Values denoted as means±SEM analyzed by ANOVA; *, $p<0.01$ (placebo vs. sham); †, $p<0.01$ and ‡, $p<0.05$ (nitrite vs. placebo).

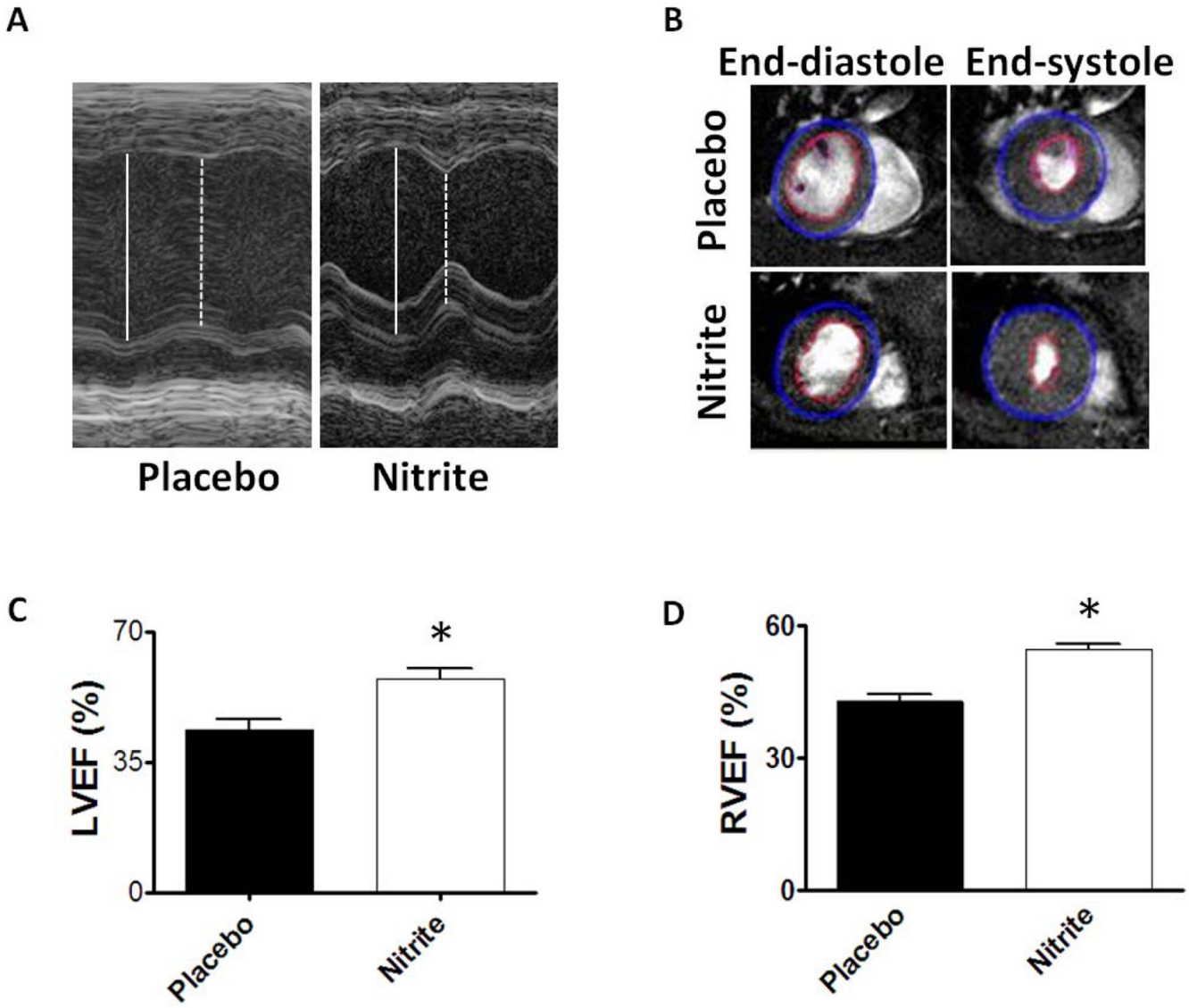

Figure 3. Improved cardiac function after nitrite therapy

(A) Sample M-mode echocardiogram images obtained 75–90 minutes post-CPR from a pair of mice randomized to placebo (n=6) or nitrite therapy (n=8). (C) Left ventricular ejection fraction (LVEF) was significantly improved with nitrite therapy. (B) Sample MRI images 24 hours after CPR demonstrate normal LVEF but a dilated right ventricle with improved right ventricular ejection fraction (RVEF) in nitrite-treated vs. placebo animals (n=5). Values denoted as means±SEM analyzed by paired t-test; *, $p<0.01$.

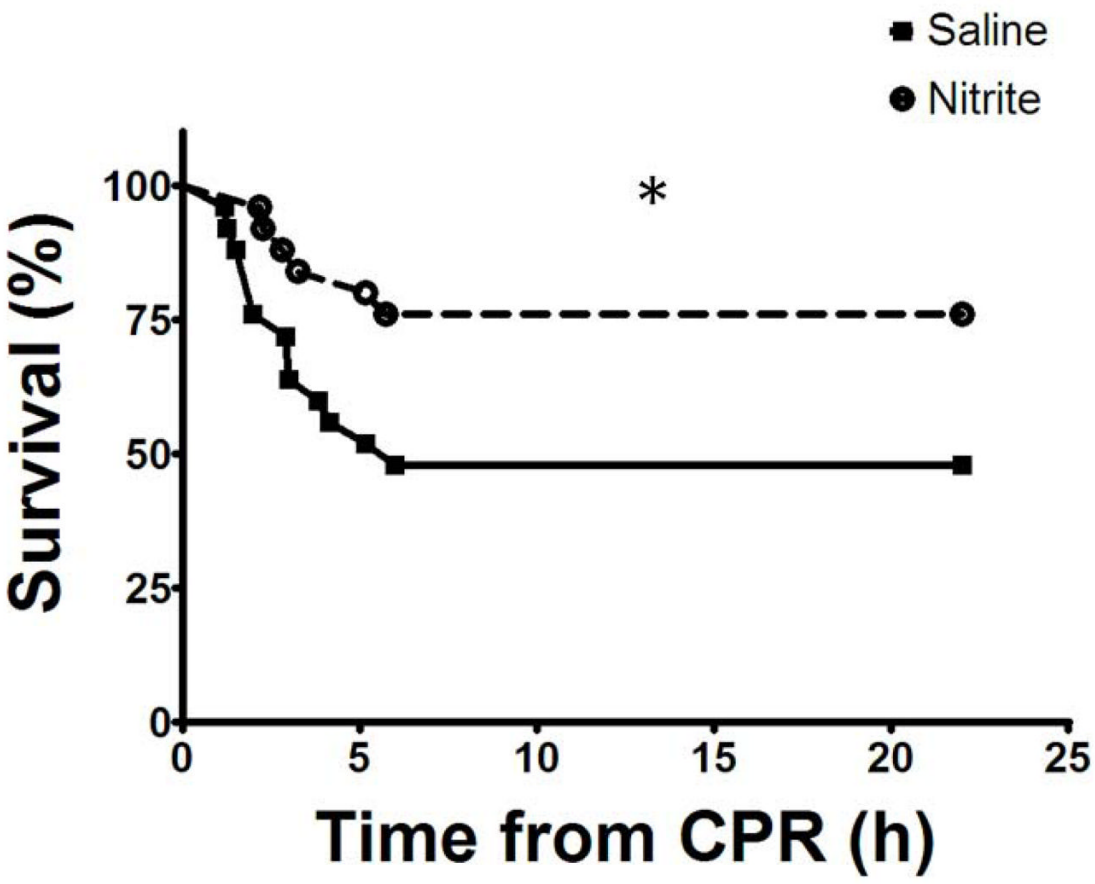

Figure 4. Nitrite therapy improves survival

After resuscitation, animals died 1–6 hours after CPR. Nitrite improved 22 hour survival compared to placebo (*, p=0.033; n=28/27 for placebo/nitrite groups).

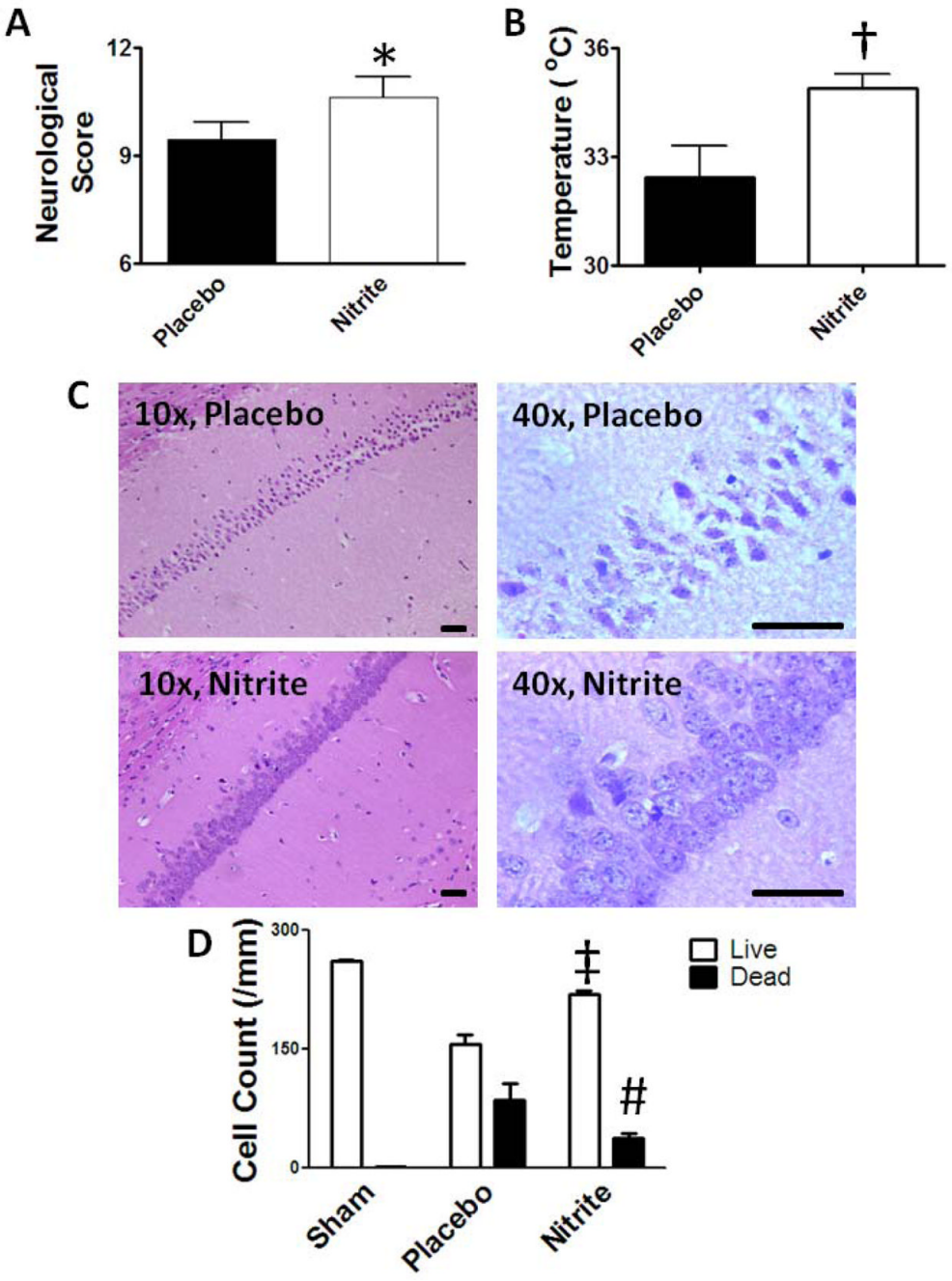

Figure 5. Nitrite neuroprotection

Neurological function in paired 22 hour survivors (n=11) was improved with nitrite based on (A) neurological scores and (B) thermoregulation (rectal temperature). (C) At 72 hours post-CPR, moderate to severe hippocampal CA1 cell death was noted in placebo-treated brains while lesser injury was noted in nitrite-treated survivors. Hematoxylin and eosin staining, bar indicates 40 μm. (D) Summary of live and dead cells (per millimeter of CA1) in serial high-powered fields. Data presented as median (A) or means±SEM (B,D); *, p=0.016; †, p=0.013; ‡, p<0.01; #, p<0.05 comparing nitrite to placebo.

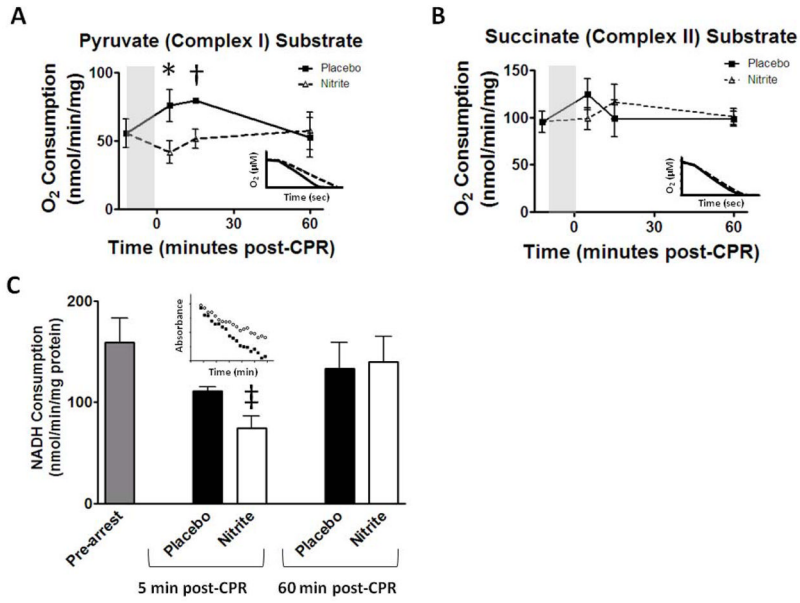

Figure 6. Nitrite therapy effects on mitochondrial function

Heart mitochondrial respiration time course where zero represents CPR start and shaded area indicates arrest. (A) Inhibition of pyruvate (complex I) mediated respiration was present at 5 (n=8) and 15 minutes (n=4) post-CPR but reversed by 1 hour. (B) Succinate (complex II) mediated respiration rates did not differ ($n \geq 4$ for each time). Inset: sample traces of mitochondrial oxygen consumption 5 minutes post-CPR. (C) Complex I activity measured in sub-mitochondrial particles by NADH oxidation. Compared to placebo, nitrite therapy significantly reduced complex I activity 5 minutes post-CPR (n=7) which was reversed by 60 minutes. Inset: sample tracing 5 minutes post-CPR. Values denoted as means±SEM, analyzed at each time by paired t-test; *, $p<0.01$; †, $p=0.021$; ‡, $p=0.014$.

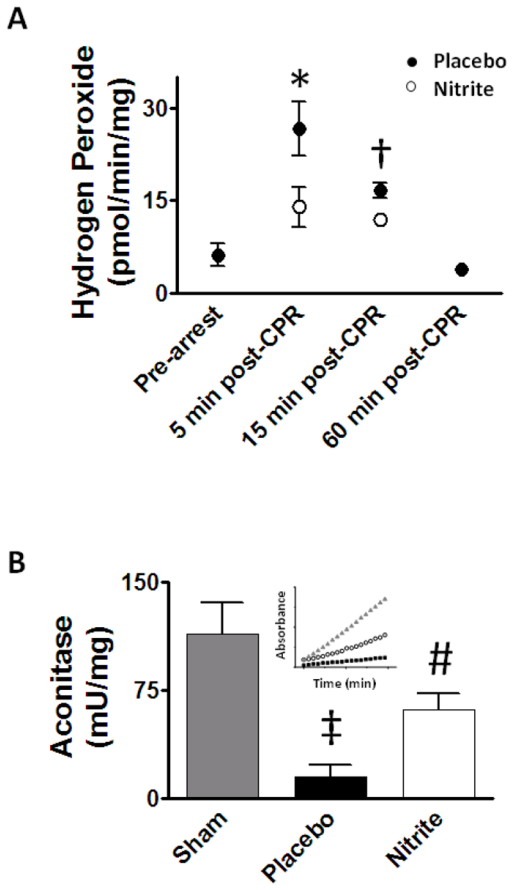

Figure 7. Nitrite therapy effects on mitochondrial oxidative burst and injury

(A) Reactive oxygen species (ROS) generation was measured as mitochondrial peroxide production using the Amplex Red assay. Cardiac arrest increased ROS at 5 and 15 minutes which resolved by 60 minutes. Compared to placebo, nitrite reduced ROS production 5 minutes (n=7) after CPR with a similar trend at 15 minutes (n=4). (B) Cardiac arrest resulted in a significant loss of heart aconitase activity which improves with nitrite therapy. Inset: sample trace from each group. Values denoted as means±SEM, analyzed by paired t-test (ROS) or ANOVA (aconitase); *, $p<0.01$; †, $p=0.071$; ‡, $p<0.01$ sham (n=11) vs. placebo(n=8); #, $p=0.05$ nitrite (n=8) vs. placebo.

Table 1

Arterial blood gas results obtained from sham mice or five minutes after CPR from mice randomized to placebo or nitrite

Characteristic	Sham	Post-Arrest Treatment	
		Placebo	Nitrite
pH	7.35±0.03	6.61±0.04 *	6.73±0.05
P_aCO_2 (mm Hg)	38.4±2.7	108.3±16.0 *	66.2±16.2 **
P_aO_2 (mm Hg)	253.7±17.6	128.2±23.8 *	210.7±25.7 **
Bicarbonate (mg/dL)	20.9±0.8	9.9±0.9 *	8.4±0.9
Lactate (mg/dL)	0.9±0.1	16.5±0.5 *	15.6±1.2

Key: All data means±SEM (n=5 per group) analyzed by ANOVA;

*, $p<0.01$ sham vs. post-arrest placebo;

**, $p<0.05$ post-arrest placebo vs. nitrite.

Table 2

Baseline and CPR characteristics of randomized animals

Characteristic	Post-Arrest Treatment	
	Placebo	Nitrite
Age at Experiment (weeks)	10±1.8	10±1.8
Weight (g)	27.0±2.3	26.5±2.3
Ischemic Time (min)	12.0±0.01	12.0±0.03
DBP 15 sec prior to CPR	4.3±0.4	4.8±0.2
DBP during 15 sec after CPR start	27.4±2.3	27.9±3.5
Successful Resuscitation	25/28 (89%)	25/27 (93%)
Time to ROSC (sec)	54.8±30.1	43.0±26.3*

Key:

*, p=0.034; DBP=diastolic blood pressure; ROSC=return of spontaneous circulation; n=21 per group