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Nitrite Anion (NO_2^-) Provides Potent Cytoprotective and Anti-apoptotic Effects as Adjunctive Therapy to Reperfusion for Acute Myocardial Infarction

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Abstract

Background: Accumulating evidence suggests that the ubiquitous anion nitrite (NO_2^-) is a physiological signaling molecule, with roles in intravascular endocrine nitric oxide (NO) transport, hypoxic vasodilation, signaling, and cytoprotection. Thus, nitrite could enhance the efficacy of reperfusion therapy for acute myocardial infarction. The specific aims of this study were: 1) to assess the efficacy of nitrite in reducing necrosis and apoptosis in canine myocardial infarction and 2) to determine the relative role of nitrite vs chemical intermediates, such as S-nitrosothiols.

Methods and Results: We evaluated infarct size, microvascular perfusion, and left ventricular function by histopathology, microspheres, and magnetic resonance imaging in 27 canines subjected to 120 minutes of coronary artery occlusion. This was a blinded, prospective study comparing a saline control group (n = 9) with intravenous nitrite during the last 60 minutes of ischemia (n = 9), and during the last 5 minutes of ischemia (n = 9). In saline treated control animals, 70±10% of the area at risk was infarcted compared with 23±5% in animals treated with a 60-minute nitrite infusion. Remarkably, a nitrite infusion in the last 5-minutes of ischemia also limited the extent of infarction (36±8% of area at risk). Nitrite improved microvascular perfusion, reduced apoptosis, and improved contractile function. S-nitrosothiol and iron-nitrosyl-protein adducts did not accumulate in the 5-minute nitrite infusion, suggesting that nitrite is the bioactive intravascular NO-species accounting for cardioprotection.

Conclusions: Nitrite has significant potential as adjunctive therapy to enhance the efficacy of reperfusion therapy for acute myocardial infarction.

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Conflicts of Interest

Dr. Gladwin and Dr. Cannon are named as co-inventors on an NIH patent application for the use of nitrite salts in cardiovascular diseases.

Introduction

The anion nitrite (NO_2^-) may represent an intravascular biological reservoir of nitric oxide (NO) [1-4]. The reductive conversion of nitrite to NO is thought to occur by a number of mechanisms including the enzymatic actions of xanthine oxidoreductase [5,6], non-enzymatic disproportionation [7,8], and a hemoglobin reductase activity that is under allosteric control [3, 9-11]. These mechanisms of nitrite reduction favor bioconversion of nitrite to NO under the hypoxic and acidic conditions present during ischemia [4].

Nitrite has vasodilatory and cytoprotective effects. Inhaled nitrite vasodilates the pulmonary vasculature of hypoxic sheep [12]. Nitrite infusions prevent middle cerebral artery vasospasm in a primate model of postaneurysmal hemorrhage [13]. Surprisingly low doses of nitrite prevent ischemia-reperfusion injury associated with acute myocardial infarction in a Langendorf heart preparation [14], as well as in the living mouse liver and heart [15]. Effectiveness in the nanomolar concentration range suggest nitrite may function as an innate physiological modulator of the ischemia stress response [4].

From a biochemical perspective, NO may be stabilized in blood by the formation of NO modified proteins, peptides and lipids, as well as by oxidation to the anion nitrite. While these concepts remain controversial, it is likely that a number of intravascular species are capable of endocrine vasodilation, including S-nitrosothiols [16,17], nitrite [1,3,12-15,18-20], N-nitrosamines [21-24], iron-nitrosyls [25], and the recently identified nitrated lipids [26-29]. It has been suggested that the vasodilatory effects of nitrite are derived from the biochemical conversion to an S-nitrosothiol [30]. In contrast, accumulating data from our laboratory and others suggest that nitrite is a direct NO-dependent signaling molecule and a major stable reservoir of NO in the circulation, that does not require intermediary conversion to an S-nitrosothiol [4].

The aim of this study was to determine if a low dose of intravenous nitrite would enhance the efficacy of reperfusion therapy for acute myocardial infarction in a protocol compatible with typical delays from onset of chest pain to acute intervention. We also aimed to understand the relative role of nitrite vs. nitrite metabolites in these experiments. We formulated two hypotheses: a) a low dose of nitrite over 60-minutes reduces infarct size; b) if the mechanism of cardioprotection involved a direct biochemical effect on the reperfusion phase of injury rather than simple vasodilation of collaterals, then a 5-minute infusion would also reduce infarct size. We also aimed to determine if the effect is mediated by intravascular nitrite or requires bioconversion to an S-nitrosothiol or iron-nitrosyl intermediate.

Methods

Animal Preparation

Experiments were approved by the NHLBI Animal Care and Use Committee. Twenty-seven 12-23 kg mongrel dogs were anaesthetized with acepromazine (0.2 mg/kg), thiopental sodium (15mg/kg), and isoflurane (0.5-2.0%). After midline sternotomy and instrumentation, a myocardial infarction was induced by occluding the LAD for 2 hours followed by 6 hours of reperfusion. Anesthetized animals were euthanized with potassium chloride following heparin administration (10,000 units).

Treatment Protocol

Three animal study groups were evaluated: a) a control group receiving a 60-minute infusion of 0.9% saline (n=9); b) a group receiving a 60-minute nitrite infusion group (n=9); and c) a group receiving a 5-minute nitrite infusion (n=9) as shown in Figure 1. The 60-min nitrite infusion group was 0.20 μmol/min/kg (1 ml/min × 20 min) followed by 0.17 μmol/min/kg (1

ml/min × 40 min) and aimed for a plasma concentration of 5-10 micromol/L. The 5-minute infusion of nitrite was 0.20 μmol/min/kg (1 ml/min × 5 min). Infusions were stopped immediately prior to reperfusion. Additional saline infusions were provided during ischemia to support systolic blood pressure on an as needed basis.

Chemical Preparation

Sterile sodium nitrite approved by the FDA for human use (IND # 70,411) was prepared on the day of the experiment by the NIH Pharmacy Development Service.

Determination of Plasma and Whole Blood Nitrite and Nitric Oxide-Hemoglobin Adducts

For plasma nitrite measurements, blood samples were collected in nitrite-free heparin and centrifuged (3000 X G for 5-minutes) immediately to avoid nitrite metabolism by erythrocytes. Plasma was removed and frozen immediately for later analysis. The nitrite in whole blood and plasma was measured using triiodide-based reductive chemiluminescence using a nitric oxide analyzer (model 280, Seivers, Boulder, Colo) as previously described and validated [31-33]. To determine the levels of specific NO adducts, each sample was separated into 3 aliquots and treated as follows: aliquot 1- no treatment (to measure total nitrite, S-nitrosothiol, and Rx-NO), aliquot 2- reaction with acidified sulfanilamide (0.5% vol:vol; to measure S-nitrosothiols and Rx-NO), and aliquot 3- reaction with acidified sulfanilamide and mercuric chloride (5 mmol/L; to measure Rx-NO). Subtraction of the signal of aliquot 2 from aliquot 1 yielded the concentration of nitrite in the sample. The signal from aliquot 3 was subtracted from aliquot 2 to calculate S-nitrosothiol concentration. The signal from aliquot 3 was representative of the total Rx-NO concentration in the sample. [31,33].

Assessment of Left Ventricular Function

Left ventricular function was evaluated by cine MRI at four time points: 1) baseline; 2) approximately 30 minutes into the first hour of occlusion; 3) approximately 30 minutes into the second hour of occlusion; and 4) at 4-6 hours into reperfusion on a 1.5 T Magnetom Avanto MRI scanner (Siemens AG Medical Solutions; Erlangen, Germany) using a segmented ECG gated steady-state free precession (TrueFISP) cine MRI sequence.

Assessment of Area at Risk

The area at risk was assessed at 30-minutes into ischemia by first-pass myocardial perfusion MRI (dual-bolus34 administration of gadopentetate dimeglumine (0.005 mmol/kg followed by 0.10 mmol/kg). The images were acquired every other heartbeat to allow volumetric coverage.

Myocardial Blood Flow by Fluorescent Microsphere

Microspheres were injected for three reasons: 1) to verify that an ischemic period was induced; 2) to observe whether the 60-minute nitrite infusion improved perfusion during the occlusion via collateral vessels; and 3) to assess reperfusion in all three groups. Approximately 5-million fluorescently-labeled microspheres 15 μm in diameter (IMT Laboratories, Irvine, Calif) were injected. Two adjacent pathological slices were aligned and treated as a single slice for microsphere analysis (8 circumferential sectors further subdivided into epicardial and endocardial portions).

Histopathology Analysis

Infarct size was measured with 1% triphenyltetrazolium chloride (TTC) staining at 37-40°C then rinsed with 0.9% saline (~ ten 3-4 mm-thick slices). Tissue was submerged in isotonic saline and photographed.

Apoptosis Analysis

A transmural section of anterior left ventricular myocardium was used for TUNEL staining (Histoserv, Inc. Germantown, MD) in an area with area at risk and infarct. Five-high power fields evenly spaced from the endocardium to the epicardium were photographed. To aid in differentiating red apoptotic nuclei from blue or purplish nuclei, a gray scale image was calculated as the ratio of the red channel divided by the blue channel. In this ratio image, the apoptotic nuclei appear white or light gray versus the normal nuclei which are black or dark gray. The apoptotic nuclei were manually counted by two readers blinded to treatment group (inter-observer correlation: r= 0.92, y = 0.81x + 1.30).

Microvascular Obstruction Analysis

The amount of microvascular obstruction was measured on first pass perfusion images acquired approximately one-hour before sacrifice. The peak intensity of normal myocardium was estimated with histogram analysis. A threshold 50% below peak normal intensity defined dark pixels[35]

Statistical Analysis

One-way and two-way repeated measures ANOVA were performed with the SigmaStat (SAS Institute Inc., Cary, North Carolina) followed by sequential Bonferroni procedures. To minimize loss of statistical power due to multiple Bonferroni corrections, the sequential correction method worked from largest to smallest differences until a non-significant comparison was found after which no further testing was performed. The Kruskal-Wallis test was used if data was not normally distributed or had unequal variance. Results are mean ± SEM unless specifically indicated. $P < 0.05$ was considered significant.

Results

Nitrite Levels in Whole Blood and Plasma

In the 60-minute nitrite infusion group, arterial plasma nitrite levels peaked after 60 minutes of nitrite infusion and remained significantly elevated until 30 minutes after reperfusion (Figure 2A). Significant arterial-to-venous gradients in plasma nitrite were observed during infusions consistent with systemic nitrite consumption (data not shown). Changes in whole blood nitrite followed a similar course with peak levels of 5 micromol/L at 30 and 60 minutes (Figure 2A), with appreciable artery-to-vein gradients (data not shown).

During the 5-minute nitrite infusions, plasma nitrite levels increased to a maximum at 5 minutes ($p<0.001$) and returned to baseline levels by 90 minutes into the reperfusion period (Figure 2B). With the five-minute infusion protocol we observed minimal changes in whole blood nitrite (Figure 2B).

In the control group, the nitrite levels remained within the baseline range described for the treated groups and remained unchanged throughout the experiment (data not shown).

Nitrite Anion Infusion Prior to Reperfusion Limits Myocardial Infarction Size

The primary study endpoint was infarct size normalized to the area at risk. As shown in Figure 3c, a 60-minute and 5-minute infusion of nitrite dramatically reduced the infarct size (by TTC) relative to the area at risk (by the myocardial perfusion scan).

In group analysis, we observed a significant reduction of the ratio of the infarct size normalized to the area at risk (MI/AAR) in the 9 animals receiving a 60-minute nitrite infusion compared with saline-treated controls (23 ± 5% vs 70 ± 10%, $p<0.001$, Figure 4). Remarkably, the 5-

minute nitrite infusion reduced infarct size to a comparable degree despite the brief infusion time, a lower cumulative dose of nitrite and a lower peak concentration of nitrite (36 ± 8% vs 70 ± 10%; $p<0.05$; Figure 4). The MI/AAR was not statistically different between the 5-minute and 60-minute nitrite infusion groups. With the exception of one animal in the 5-minute nitrite infusion group and one control animal, there was no overlap in MI/AAR between the nitrite treated groups and controls. Although the size of the area at risk was not significantly different between the control and 60-minute nitrite infusion groups (17.5 ± 1.4 % vs 19.3 ± 3.3 %, $p<0.001$), the 5-minute nitrite group had a significantly larger area at risk than either of the other groups (30.4 ± 2.7 %, $p=0.012$ and $p=0.013$, respectively).

Nitrite Reduces Cardiomyocyte Apoptosis

Prior studies in mice demonstrated an effect of low dose nitrite on inhibiting apoptosis after ischemia reperfusion in the liver [15], but these studies have not been performed in the heart or in a larger mammal. We therefore evaluated transmyocardial cardiomyocyte apoptosis using Tunnel staining at five transmural locations from endocardium to epicardium and the degree of apoptosis was defined as the number of apoptotic nuclei per high power field (Figure 5). We observed a significant effect of both 60-minutes and 5-minutes of nitrite infusion on apoptosis compared with control across all anatomical locations (Kruska Wallis Test of ranks, $p=0.001$ and $p=0.002$ at transmural layers 3 and 4 respectively).

Cardioprotective Effects of Nitrite are not Mediated by Hemodynamics

The enhanced myocardial salvage associated with the 5-min nitrite therapy was not explainable by changes in preload (inversely related to end diastolic wall thickness Figure 6, left panel), afterload (systolic wall stress Figure 6, middle panel), or rate pressure product (Figure 6, right panel). The beneficial effects of the 60-min nitrite infusion can not be separated from hemodynamic effects since the preload, afterload, and rate pressure product deviate from the control group in directions that could reduce infarct size. However, the 5-min nitrite group tracks closely with the control group indicating that myocardial salvage is more likely explained by the biochemical mechanisms than hemodynamic factors during ischemia.

Nitrite Improves Global Left Ventricular Function

The left ventricular ejection fraction (LVEF) was significantly reduced below baseline values after 30-minutes of LAD coronary artery occlusion (Figure 7) in all three groups ($p<0.001$). Trends for change in LVEF during the second hour of occlusion were not significant in any group. However, both groups receiving nitrite displayed a significant recovery of LVEF at 4-6 hours into reperfusion relative to occlusion (60-minute nitrite infusion $p = 0.01$, and 5-minute nitrite infusion $p<0.001$), whereas the control group did not significantly recover LVEF.

Effects of Nitrite on Perfusion During Ischemia and Microvascular Obstruction During Reperfusion

Myocardial perfusion, measured by microspheres, showed severely reduced perfusion 30-minutes into the occlusion and during the second hour of occlusion in all three groups (Figure 8). Thus, the 60-minute nitrite treatment did not recruit enough collateral blood flow to explain marked reductions in infarct size. At reperfusion, both nitrite treatment groups demonstrated better recovery of endocardial microsphere blood flow than the control group, a finding consistent with less severe microvascular obstruction in the nitrite treated animals. Epicardial and transmural microsphere blood flow was not significantly different between the three groups – a result that verifies that macrovascular reperfusion was achieved in all three groups.

There was more MRI evidence of microvascular obstruction in the control group (11±6.1% of the left ventricle) than either of the nitrite treatment groups (Figure 8b and 8c) and that the

microvascular obstruction was mostly localized within the endocardium. These results indicate that nitrite limits the endocardial "no-reflow phenomenon".

Nature of the NO Store: Nitrite or S-Nitrosothiol?

Because nitrite may undergo facile bioconversion to S-nitrosothiols, iron-nitrosyl complexes and possibly nitrated lipids, we tested whether the vasodilatory effects and ischemia-reperfusion effects of nitrite occur secondary to intravascular S-nitrosothiol, N-nitrosamine or iron-nitrosyl formation. We therefore directly measured plasma and red cell S-nitrosothiols and mercury stable NO adducts (which include the iron-nitrosyl and N-nitrosamine complexes and referred to as RxNO) in blood using reductive chemiluminescence during the nitrite infusion protocols.

At baseline, the concentration of whole blood (red cell and plasma) S-nitrosothiols was below 10nmol/L in all groups and remained relatively unchanged in the control group over the course of the experiment. In the group receiving the 60-minute nitrite infusion, the S-nitrosothiol levels and RxNO levels (mercury stable NO adducts consistent with iron-nitrosyls, N-nitrosamines or nitrated lipids) peaked 60-minutes into the nitrite infusion to 54.5 ± 21.2 nmol/L and 24.3 ± 12.5 nmol/L, respectively, and then decreased following reperfusion (Figure 2c and 2d). Importantly, there was no statistically significant increase in S-nitrosothiol levels during or following the 5-minute infusion of nitrite (data not shown). The appreciation of robust cardiomyocyte cytoprotection during the 5-minute nitrite infusion protocol with no change in intravascular S-nitrosothiol levels supports the thesis that nitrite is the primary mediator of these biological effects. The cytoprotection afforded by nitrite does not require measurable NO equivalent (NO+) transfer to form a secondary S-nitrosothiol in blood.

Discussion

This study demonstrates that the anion nitrite (NO_2^-) potently limits myocardial infarction and apoptosis in the reperfusion phase of injury. The mechanism of myocardial protection is independent of the time-ischemia severity integral since a brief 5-minute infusion of nitrite during the end of a two-hour occlusion reduced infarct size and apoptosis almost as much as a 60-minute infusion and the short infusion caused virtually no hemodynamic perturbations. The improved myocardial salvage associated with the 5-minute nitrite infusion was not explainable on simple hemodynamic factors such as preload, afterload, rate pressure product, or the area at risk. Both nitrite infusion protocols had beneficial effects on global left ventricular function and minimized endocardial "no-reflow" phenomenon, characterized by microvascular obstruction in the infarct core. Therefore, we conclude that nitrite provides a direct cellular cardioprotective mechanism in the reperfusion phase of injury. Furthermore, nitrite can provide this remarkable degree of cardioprotection on a time scale compatible with intravenous adjunctive therapy to acute percutaneous interventions for acute myocardial infarction.

Two recent studies suggest that nitrite potently limits ischemia-reperfusion cytotoxicity with a maximal effect observed at low concentrations [14,15]. While the protective effect was maximal at blood concentrations of 10 micromol/L (48 nmole dose for a mouse), even doses as low as 1.2 nmoles - which were associated with increases in blood levels of nitrite from 700 nmol/L to only 900 nmol/L, reduced the infarction size by 50% [15]. The cytoprotective effect of nitrite reduced apoptosis and was associated with intracellular reduction of nitrite to NO, independent of the NO synthase and hemeoxygenase 1 enzymes. In the current study, this cytoprotective effect is recapitulated in a large mammal exposed to a longer ischemic time and more extensive infarction relative to area at risk. Remarkably, a five-minute infusion of nitrite in the current study increased plasma levels of nitrite in dogs from a ~1 umol/kg at baseline up to 5 umol/L, with no associated increases in plasma or red cell S-nitrosothiols. These near-

physiological increases in nitrite decreased myocardial infarction size from 70 to 20% of the area at risk, and improved cardiac contractile function.

Nitric oxide that diffuses into blood rapidly reacts with both oxy- and deoxyhemoglobin to form methemoglobin/nitrate and iron-nitrosyl-hemoglobin ($HbFe^{II}$-NO), respectively [25,36]. These reactions shorten half-life of NO in blood to less than 2 milliseconds and thus maintain endothelial-derived NO as a paracrine vasoregulator [37,38].

While NO per se is inactivated by reactions with hemoglobin, it may be stabilized in blood by the formation of NO modified proteins, peptides and lipids, and oxidation to the anion nitrite. It is increasingly clear that a number of intravascular chemical NO-modified species are capable of mediating vasodilation, including S-nitrosothiols [16,17], nitrite [1,3,18,19], N-nitrosamines [21-24], iron-nitrosyls [25], and recently identified nitrated lipids [26-29].

Both human blood flow experiments and studies of ischemia-reperfusion over the last two years suggest that nitrite is one of the major endocrine NO species in blood. In earlier physiological studies, we observed artery-to-vein gradients in nitrite across the human forearm, with increased consumption of nitrite during exercise stress, suggesting that nitrite is metabolized across the peripheral circulation [1]. While nitrite was considered biologically inert, we found that nitrite induced concentration-dependent vasodilation healthy human volunteers [3]. Nitrite levels even as low as 900 nmol/L produced vasodilation in humans during exercise stress with concurrent NO synthase inhibition with L-NMMA suggesting a physiological role for nitrite in vascular homeostasis [3]. The potent vasodilating effects of nitrite have been verified in a number of models [12,14,19,20,39].

The degree to which nitrite-induced vasodilation and coronary collaterals contribute to myocardial protection warrants consideration. It would require a very large sample size to determine the extent to which the statistically insignificant increase in microsphere blood flow (60-minute nitritre group) contributes to myocardial protection since the magnitude of effect is very small. In the 5-minute nitrite group, nitrite-induced vasodilation cannot significantly alter the net deficit in the time-blood flow integral and thus is biologically unlikely to confer protection by a mechanism related to reduced ischemia as a result of collateral blood flow. However, collateral blood flow may provide a route for nitrite to reach into the ischemic myocardium and thus indirectly facilitate protection afforded by mechanisms that directly modulate the biochemical mechanisms underlying ischemia reperfusion injury.

The cardioprotective effects of nitrite infusion in the current study were associated with specific increases in plasma nitrite at near physiological concentrations. Although the 60-minute infusion of nitrite was associated with increases in both plasma nitrite and blood S-nitrosothiols, only nitrite levels increased during the five-minute infusion protocol. These data support the thesis that nitrite is an endocrine intravascular NO-species that modulates systemic response to hypoxic/ischemic injury.

During cardiac ischemia and reperfusion, nitrite in tissue is reduced to NO and forms iron-nitrosylated (Fe+2-NO) and S-nitrosated modified proteins, via reactions with deoxymyoglobin and other cellular heme proteins[3,10,40,41]. The rapid, facile metabolism of nitrite to NO with subsequent modification of target proteins has been documented in the heart and liver during both regional and global IR injury[15,42,43]. The formation of NO in the heart during ischemia has been documented using electron paramagnetic resonance and liquid and gas phase chemiluminescence. We have recently found that nitrite will specifically post-translationally S-nitrosate complex I of the mitochondrial electron transport chain (METC); this effectively reduces electron flow through the METC and reduces reactive oxygen species formation during reperfusion[44]. This damping or tuning of electron transport inhibits opening of the mitochondrial permeability transition pore, decreases cytochrome C release, and limits

apoptosis. The nitrite-dependent decrease in TUNNEL staining is consistent with this mechanism of cytoprotection. Other intracellular targets for S-nitrosation by nitrite during IR exposure could include the L-type calcium receptor[45]. In addition, stabilization of myglobin as iron-nitrosylated myoglobin may limit heme based oxidation reactions in the cardiomyocyte[15].

In this study, we have shown an increase in iron-nitrosylation with nitrite treatment. While this increase reflects nitrosylation of heme proteins, such as myoglobin, it may also indicate nitrosylation of non-heme iron. Cellular non-heme iron content plays a role in determining the sensitivity of cells to NO-mediated apoptosis[46], with increasing concentrations of non-heme iron rendering cells less susceptible to NO-mediated apoptosis. Non-heme iron is able to bind NO (forming Fe-NO) which decreases the bioavailability of NO, as well as oxidizes NO to NO + to promote S-nitrosothiol formation (including the S-nitrosation of caspases). In the case of nitrite, if nitrite is reduced to NO, which then mediates S-nitrosation of tissue components to illicit cytoprotection, tissue non-heme Fe would catalyze S-nitrosothiol formation and promote the anti-apoptotic effects of nitrite. This may be consistent with the increase in Fe-NO seen in tissues after nitrite administration during ischemia-reperfusion.

While current reperfusion therapies are efficacious in the treatment of acute MI, intrinsic and practical delays between symptom presentation and intervention compromise the amount of myocardial salvage. Despite great advances in percutaneous coronary interventions that result in excellent restoration of coronary blood flow, the mortality after MI remains at 7% and virtually all patients suffer some degree of myocardial necrosis. The extent of the myocardial infarction predicts future cardiac function. Post MI heart failure represents a huge burden on our health care system. Adjunctive pharmacological therapies that improve the amount of myocardial salvage following reperfusion of an acute MI could positively impact cardiac function and possibly prognosis. Such adjunctive therapies should possess the following characteristics: a) significant cardioprotection after prolonged ischemia; b) simple administration; c) low expense; d) low dose required for pharmacological action; e) short half-life and rapid onset of action; f) minimum associated regional and systemic side effects; and g) a cardioprotective mechanism that is not dependent on vasodilation or changing rate pressure product. Nitrite satisfies these requirements.

There are limits to the current study. While it would have been desirable to also study cardioprotection with 2 doses of nitroglycerin, this was not practical for sample size considerations due to the large number of potential comparisons. Nitrite did provide better cardioprotection than nitroglycerin in a mouse model[15] and inhaled NO was more potent than nitroglycerin in a swine model[47]. Even in the current set of experiments, there is limited power to detect differences between groups. Thus, one must interpret statistics showing no change between groups with caution. However, the key findings that nitrite provides cardioprotection and reduces infarct size are supported with statistical confidence. Furthermore, biological factors that modulate infarct size such as rate pressure product, residual perfusion during ischemia, and systolic wall stress all indicate that the 5-minute nitrite group faced as challenges that directionally should have lead to larger infarcts than the control group.

In conclusion, nitrite (NO_2^-) possesses the characteristics of an ideal adjunctive therapy for acute MI. From a feasibility perspective, nitrite can be administered intravenously and the 5-minute dose does not significantly alter hemodynamics. In patients with acute MI, the 5-minute infusion of nitrite could be initiated on arrival to the catheterization laboratory shortly prior to percutaneous coronary intervention.

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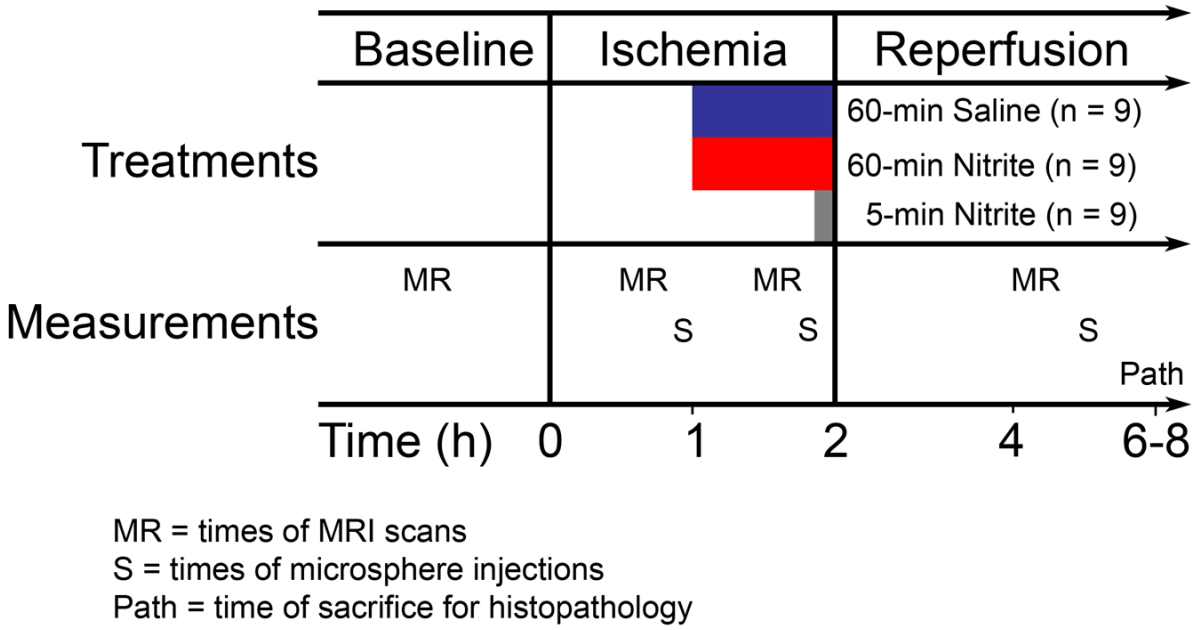

Figure 1.
Schematic diagram of experimental protocol and major measurements. MR imaging was performed at baseline, between approximately 30 and 50 minutes during the first hour of ischemia, was repeated with similar timing during the second hour of ischemia, and between 4 and 6 hours after reperfusion. Microspheres were injected after each MR scans except the baseline scan. Histopathology was performed at least 6 hours after reperfusion for optimal triphenyltetrazolium chloride (TTC) staining.

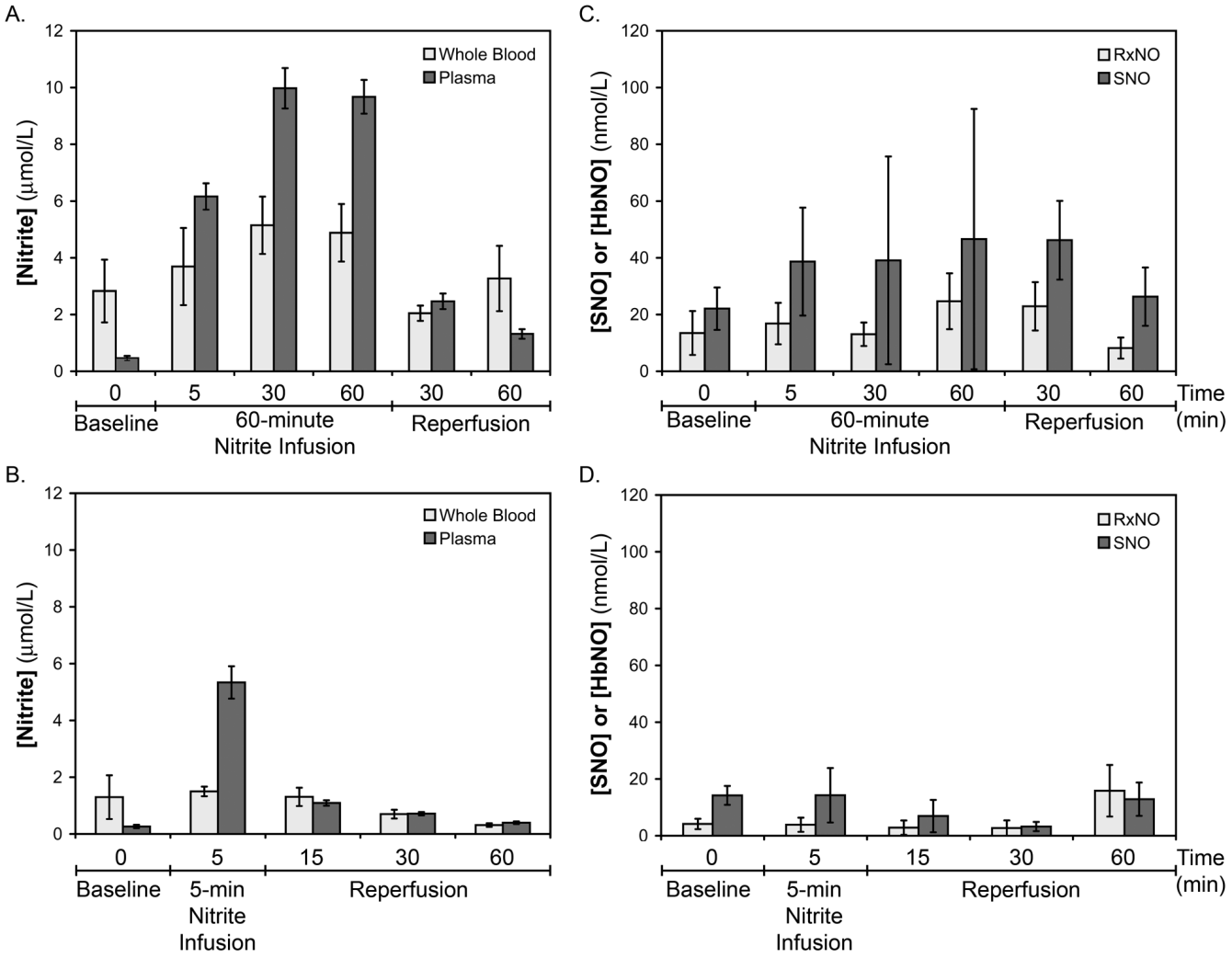

Figure 2.

Nitrite, S-nitrosothiol (SNO), and nitrosyl-hemoglobin (RxNO) concentrations. Nitrite significantly increased ($p<0.001$) in the 60-minute group (A) and the 5-minute nitrite group (B) while nitrite concentrations did not change significantly in control experiments (data not shown). Although SNO did accumulate significantly during the 60-minute nitrite infusion (C), SNO levels did not increase significantly during or following the 5-minute infusion of nitrite (D).

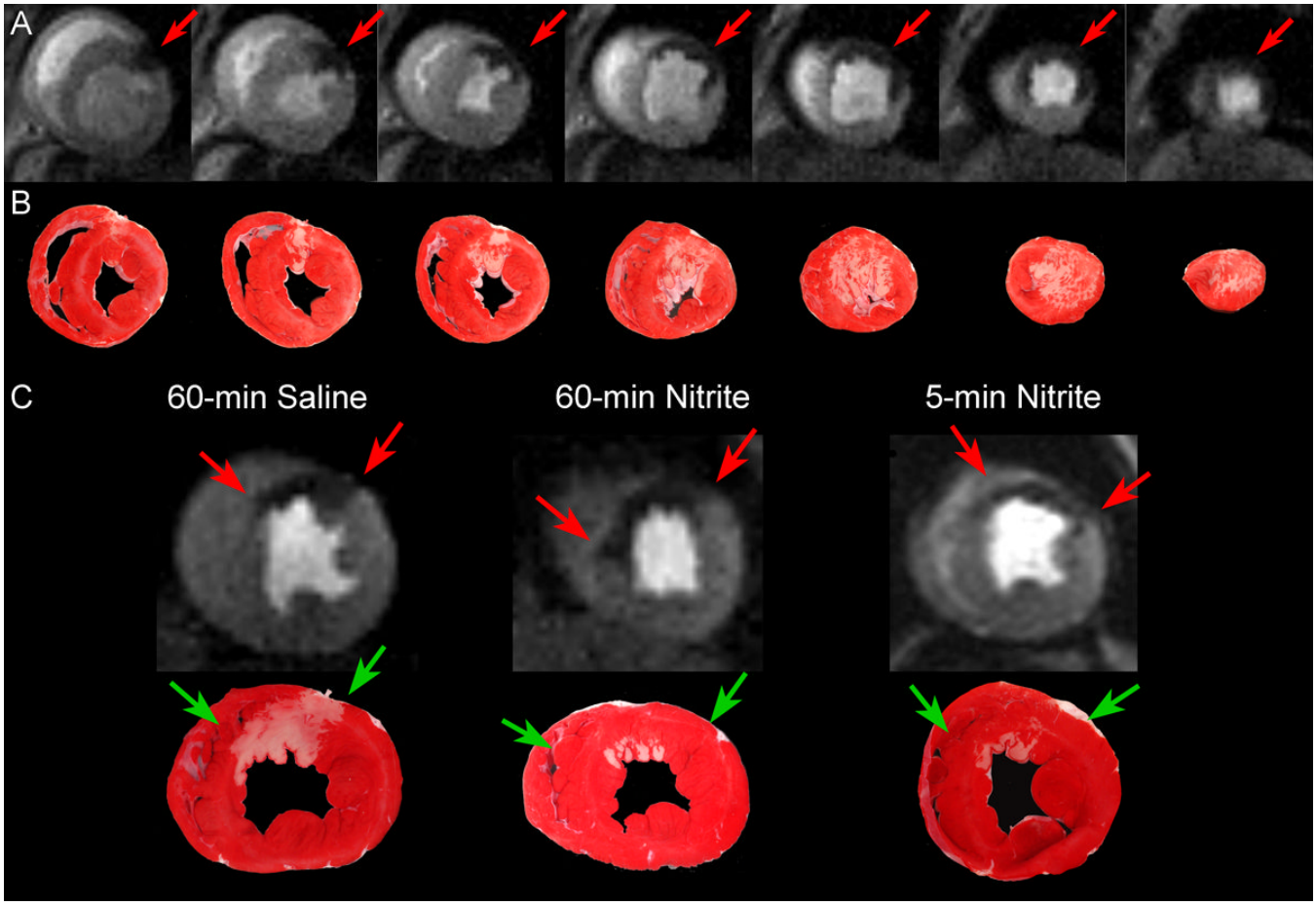

Figure 3.

Area at risk was measured volumetrically based on the hypointense zone (red arrows) on the perfusion images from a single animal from the papillary muscle level to the apex (A). Infarct size was measured as the size of the pale zone on the TTC stained myocardium (B). The background was masked to better delineate the endocardial borders. For similar sized perfusion defects (C), both nitrite infusions resulted in small subendocardial infarcts while the saline group tended to have nearly transmural infarcts. The red arrows on the perfusion images (C) delineate the epicardial extent of the area at risk. The green arrows delineate corresponding points on the TTC stained myocardium. Note the TTC negative zone encompasses a much smaller percent of the area at risk in the nitrite treated animals.

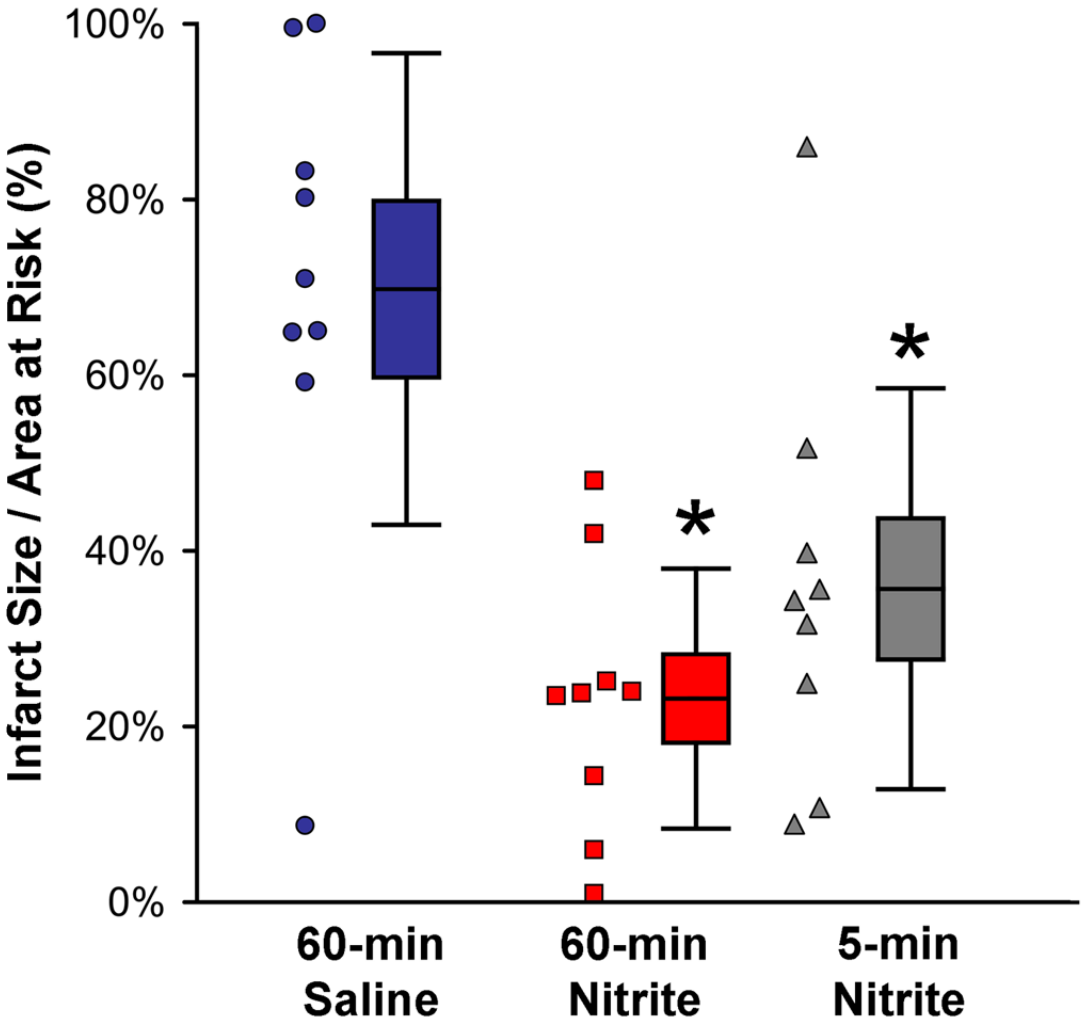

Figure 4.
Infarct size normalized to area at risk was significantly reduced by both the 60-minute nitrite infusion ($p<0.001$) and the 5-minute nitrite infusion ($p<0.05$) relative to the saline control infusion. Results for individual animals (symbols) indicate there is almost no overlap between groups other than 1 outlier in the saline and the 5-minute nitrite groups. The box and whisker plot shows mean value ± standard error of the mean (box) and ± standard deviation (whiskers).

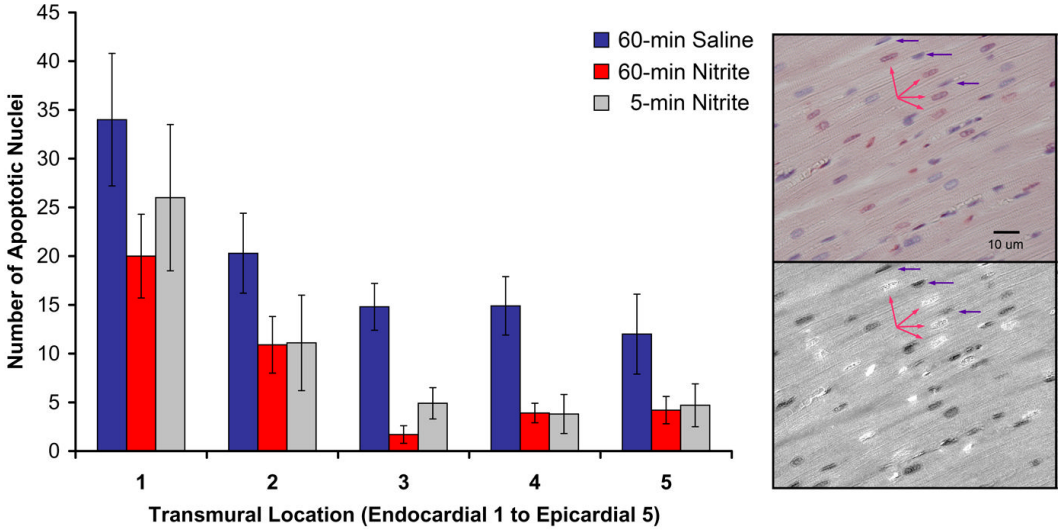

Figure 5.

Both nitrite infusions significantly reduced apoptosis relative to the saline control infusion, particularly in layers 3 and 4 (60-min $p<0.001$ and 5-min $p<0.002$). Apoptotic nuclei appear red (red arrows) on the color images and appear whitish in the gray scale ratio image.

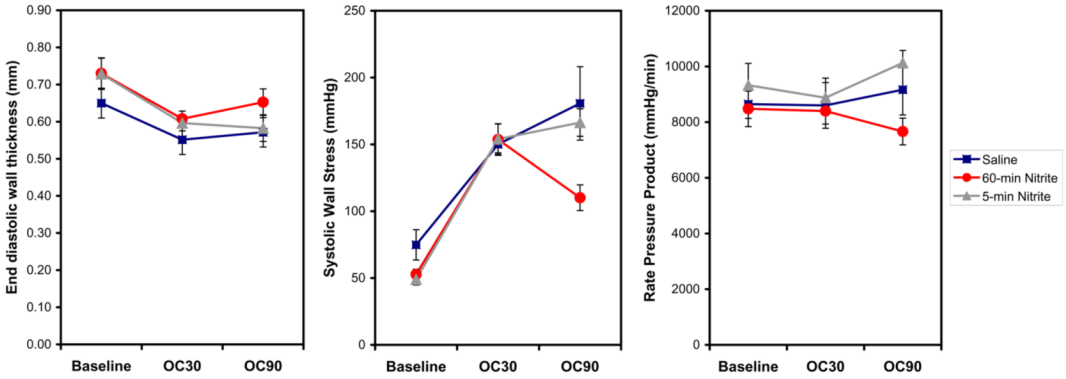

Figure 6.

Although variations in preload, afterload, and rate pressure product confound interpretation of myocardial salvage associated with the 60-min nitrite infusion, there were no significant differences in preload (inversely related to ischemic zone end diastolic wall thickness), afterload (as measured by systolic wall stress), or rate pressure product (RPP) when comparing saline control and the 5-min nitrite group. OC30 = 30 minutes into occlusion (ischemia); OC90 = 90 minutes into occlusion (ischemia).

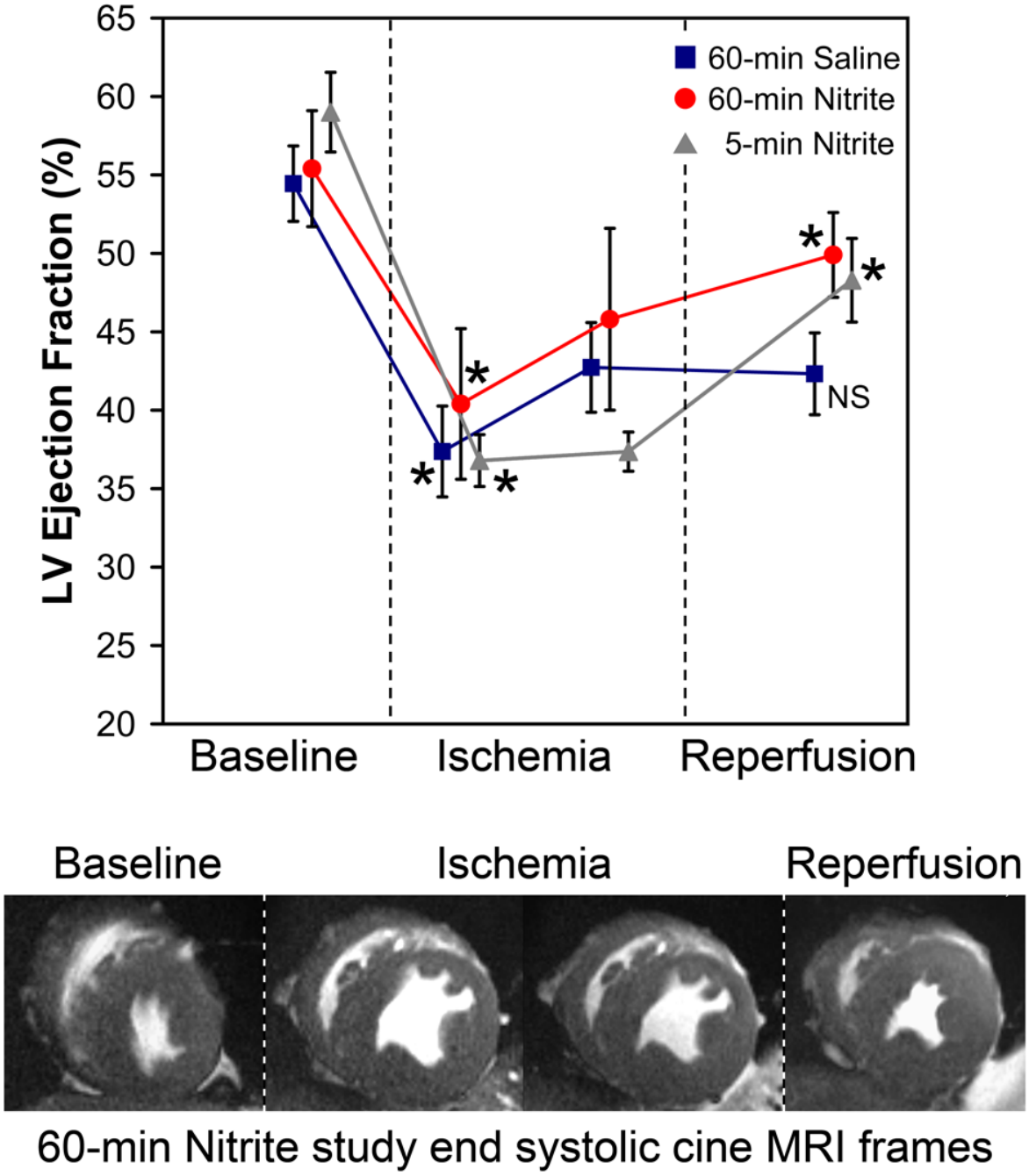

Figure 7.

Left ventricular ejection fraction (LVEF) decreased to similar extent in all three groups ($p<0.001$) during ischemia and recovered significantly by the end of the experiment in both nitrite treated groups (60-min $p<0.01$ and 5-min $p<0.001$). LVEF did not rebound significantly in the saline group. Single end systolic short axis images from the 4 MRI scans in one nitrite treated animal show the significantly larger LV cavity during ischemia and similar extent of akinesis in the anterior and anteroseptal wall. There is minimal residual wall motion abnormality after reperfusion.

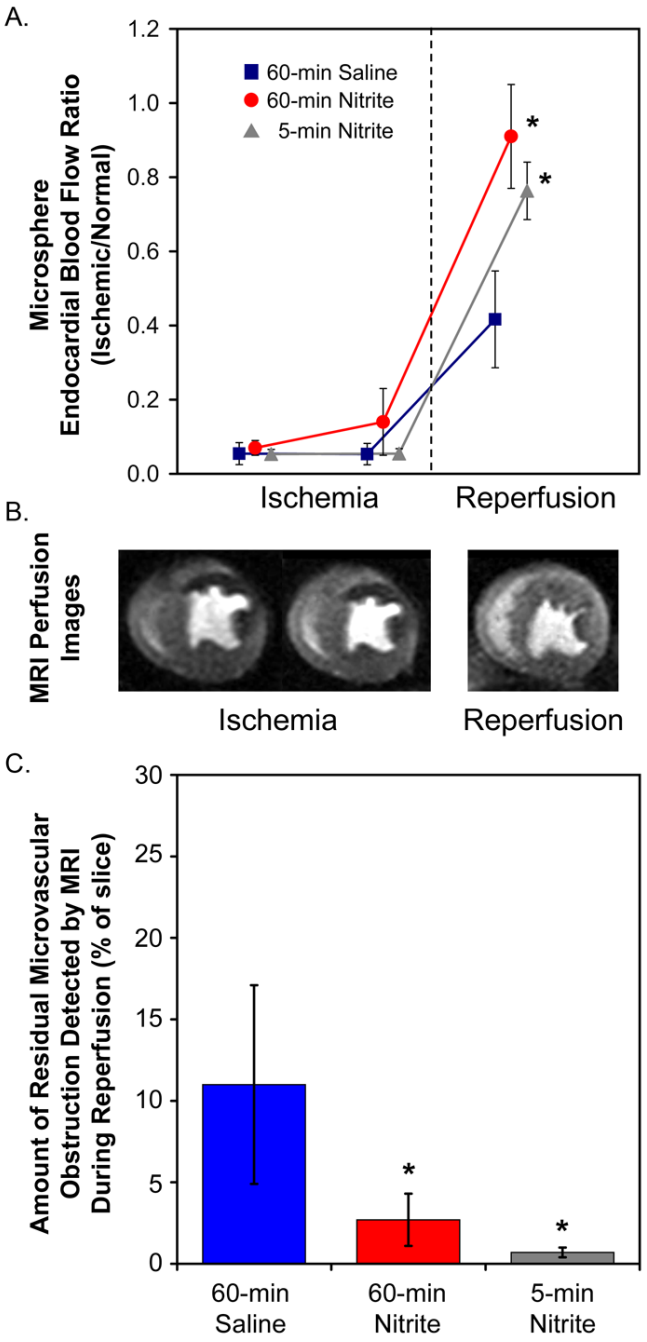

Figure 8.

Effects of nitrite on perfusion during ischemia and microvascular obstruction after reperfusion. Microsphere blood flow (panel A) and qualitative perfusion abnormalities on MRI (inset) remained severely abnormal during ischemia despite potential vasodilation related to nitrite. After reperfusion, there was less microvascular obstruction in the two nitrite treated groups (*) relative to control. This was visible on serial MR perfusion images as mild or patchy residual hypointense zones relative to the dark defects seen during ischemia (example shows a single slice at the three time points from one animal treated with nitrite).