Randomized Phase 2 Trial of Intra-Coronary Nitrite During Acute Myocardial Infarction

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ABSTRACT

**Rationale:** Pre-clinical evidence demonstrates that inorganic nitrite, following its in situ conversion to nitric oxide, attenuates consequent myocardial reperfusion injury.

**Objective:** We investigated whether intra-coronary injection of nitrite during primary percutaneous coronary intervention (PCI) might improve infarct size in ST-elevated myocardial infarction (STEMI).

**Methods and Results:** Patients undergoing primary PCI (n=80) were randomised to receive intracoronary (10mL) sodium nitrite (1.8μmol) or NaCl (placebo) before balloon inflation. The primary endpoint was infarct size assessed by measuring creatine kinase (CK) release. Secondary outcomes included infarct size assessed by troponin T release and by cardiac magnetic resonance imaging (CMR) on day 2.

Baseline characteristics were similar between the groups. No evidence of differences in CK release (p=0.92), troponin T (p=0.85) or CMR-assessed infarct size (p=0.254) were evident. In contrast there was a reduction in myocardial salvage index (p=0.05) and MACE at 1 year (2.6% vs 15.8%, p=0.04) in the nitrite group. In a 66-patient sub-group with TIMI≤1 flow there was reduced serum CK (p=0.030) and a 19% reduction in CMR-determined infarct size (p=0.034) with nitrite. No adverse effects of nitrite were detected.

**Conclusions:** In this phase II study intra-coronary nitrite infusion did not alter infarct size although a trend to improved myocardial salvage index and a significant reduction in MACE was evident. In a sub-group of patients with TIMI flow≤1 nitrite reduced infarct size and MACE and improved myocardial salvage index indicating that a phase III clinical trial assessing intra-coronary nitrite administration as an adjunct to PCI in STEMI patients is warranted.

**Clinical Trial Registration.**

**Keywords:**
Acute myocardial infarction, nitric oxide, cardiac magnetic resonance imaging, primary percutaneous coronary intervention.

**Nonstandard Abbreviations and Acronyms:**
AAR  Area at risk  
AUC  Area under the curve  
CMR  Cardiac magnetic resonance imaging  
MACE  Major Adverse Cardiac Event  
MVO  Microvascular obstruction  
NO  Nitric oxide  
NO₂⁻  Nitrite  
PCI  Percutaneous coronary intervention  
STEMI  ST-segment elevation myocardial infarction  
TIMI  Thrombolysis In Myocardial Infarction
INTRODUCTION

ST-segment elevation myocardial infarction (STEMI) is thought to account for ~25-47% of all acute myocardial infarctions (AMI)\(^1\), \(^2\). Presently, timely and effective reperfusion with primary percutaneous coronary intervention (PCI) is the treatment of choice for reducing infarct size, preserving left ventricular ejection fraction, and preventing the onset of heart failure\(^3\), \(^4\). Myocardial reperfusion injury may account for up to 50% of final myocardial infarct size and is a major determinant of prognosis\(^5\), and this underlies interest in targeting reperfusing injury using adjunctive pharmacotherapy.

Whilst several therapeutic interventions have been tested in this regard and failed, in more recent years an improved understanding of the pathophysiological mechanisms underlying ischaemia/reperfusion injury has resulted in identification of some promising mechanical (ischaemic post-conditioning\(^6\), remote ischaemic pre-conditioning\(^7\)), and pharmacological (cyclosporine\(^8\), exenatide\(^9\)) strategies\(^10\). More recently an additional possibility has emerged in the form of inorganic nitrite (NO\(_2\)). The activity of nitrite resides in its conversion to nitric oxide (NO) under the optimal conditions of low pO\(_2\) and pH\(^11\), conditions that prevail during ischaemic episodes. NO exerts a number of beneficial effects including anti-inflammatory and anti-platelet actions and prevents the opening of the mitochondrial permeability transition pore which is a critical and final common step in reperfusion\(^12\), \(^13\).

We first demonstrated the cardioprotective effects of intra-coronary nitrite in isolated rat hearts\(^14\), an observation likewise demonstrated by others with intra-ventricular and intra-coronary nitrite administration both in vitro and in vivo\(^15\), \(^16\), \(^17\). In all of these studies the beneficial effects were attributed to NO and were more often associated with local rather than systemic application of high local concentrations of nitrite (3-12 \(\mu\)mol/L), prior to reperfusion. Together, these observations provide the rationale for the investigation of the therapeutic potential of nitrite in the treatment of acute STEMI, where nitrite is delivered locally before balloon inflation at the time of primary PCI.

METHODS

Study design and participants.

This study was a double-blind, randomized, single-centre, placebo-controlled trial to determine whether intra-coronary injection of sodium nitrite reduces infarct size in patients with acute STEMI undergoing primary PCI. The trial was approved by an independent ethics committee, the Medicines and Healthcare Products Regulatory Agency, registered in approved registries (NCT01584453, EudraCT nr. 2011-000721-77) and performed in accordance with the Declaration of Helsinki (1996) and the principles of the International Conference on Harmonization–Good Clinical Practice (ICH-GCP) guidelines. Full details of the trial protocol have been published\(^19\). All appropriate subjects gave written informed consent before being included in the study (see supplement for further details). Consent in the emergency situation is challenging and thus patients who were unconscious, critically unstable (cardiogenic shock) or deemed unable to consent (pain, distress, language) were excluded. Consent was a 2 stage process where initially a study summary sheet consisting of a one-page sheet with diagrams, explaining the procedure and events, was given to the patient prior to randomization and full written consent taken at this time. A more detailed patient information sheet (PIS) was given following the procedure for reading. A second stage of consent was required for agreement to subsequent cardiac magnetic resonance imaging (CMR) analyses (see below).

All consecutive patients presenting to Barts Health Heart Attack Centre, based at The London Chest Hospital, suspected of an acute STEMI and candidates for primary PCI were considered eligible for participation. Inclusion criteria were symptoms of chest pain suggestive of myocardial ischaemia, time
from onset of symptoms of ≤ 6 h, aged between 18 and 80 years of age and an ECG showing ST-segment elevation of 0.1 mV in two or more limb leads or 0.2 mV in two or more contiguous precordial leads, or presumed new left bundle branch block.

Patients with cardiac arrest, cardiogenic shock, previous AMI or CABG were not included in the study. Patients with known congenital methaemoglobinemia, left ventricular systolic dysfunction due to pre-existing heart failure, chronic renal failure (i.e. with an estimated glomerular filtration rate <30mls/min) and women who were pregnant were not included. Finally, patients on pre-existing treatment with organic nitrate therapy (Noricrandil, isosorbide mononitrate), or had active malignancy, a life-threatening condition or had participated in any investigational drug or device study within the past 30 days were excluded.

**Randomization and intervention.**

After coronary angiography, patients were randomised (1:1) to a high-dose bolus injection of intracoronary sodium nitrite (1.8 μmol in 10 mL of 0.9% NaCl) or placebo (10 mL of 0.9% NaCl) administered prior to balloon inflation. After crossing the obstruction of the infarct-related coronary artery with a guide wire, an over-the-wire balloon (Emerge, Boston Scientific, Natick, MA, USA) was positioned beyond the obstruction. The guide wire was removed and the study drug solution injected by hand through the central lumen of the balloon catheter into the distal vascular bed over 30 seconds, irrespective of TIMI flow beyond the occlusion point. The guide wire was then reinserted through the balloon catheter and advanced to a distal position. The procedure was then continued as per operator preference with no restriction placed on vascular access route, type of stent or method of stenting (predilatation or direct).

The dose of nitrite administered was chosen since studies in the forearm of healthy volunteers demonstrate bioactivity of local concentrations of 2.5-10 μmol/L following bolus administration\(^\text{20-22}\): a range broadly associated with cardioprotection in reperfusion injury achieved though bolus dose administration in pre-clinical studies\(^\text{15, 18}\). Manufacture of the interventions, blinding, coding and randomisation were conducted by the Pharmacy Manufacturing Unit at Ipswich Hospital prior to transfer to the London Chest Hospital Pharmacy. The randomisation list was computer-generated based on blocks of ten and kept in a sealed opaque envelope in the hospital pharmacy. No stratification factors were used. A total of 80 indistinguishable vials of sodium nitrite and placebo were provided. All study personnel were blind to treatment allocation until the study and all analyses had been completed. All patients underwent standard UK and Barts Health Trust care protocols prior to and post-primary PCI (Table 1).

**Endpoints.**

Primary End-Point: The primary end point was infarct size assessed by measurement of area under the curve (AUC) for creatine kinase (CK) in line with previous studies\(^\text{8}\).

Secondary end-points: The principal secondary end point was infarct size assessed by troponin T AUC and the area of delayed hyperenhancement evident by CMR, assessed on day 2 and 6 months after infarction.

**Assessment of infarct size.**

Blood samples were obtained at admission and repeatedly over the next two days following treatment as per Barts Health Trust protocols. The AUC (expressed in arbitrary units) for CK and troponin T was measured in each patient by computerised planimetry (GraphPad prism v5.0, California).

Infarct size was also assessed using CMR. A CMR scans was offered as a sub-study with separate consent to participants and was were conducted according to standard protocols (see online supplement for detail). CMR related measures in addition to the above-mentioned secondary endpoints were area at risk (AAR),
myocardial salvage index, microvascular obstruction (MVO), left ventricular volumes and ejection fraction. The latter two are conventional measures of cardiac function the rest of the measures provide some insight into the potential mechanisms involved in any beneficial effects that might be seen.

**Coronary angiography and sub-group.**

Coronary angiograms obtained before and after primary PCI were used to make an assessment of Thrombolysis In Myocardial Infarction (TIMI) flow grade and AAR using standard (BARI and APPROACH) validated approaches (see online supplement for detail). The TIMI flow assessments were then utilized to make an assessment of inclusion for a single sub-study analysis of the effect of nitrite in only patients with TIMI flow \( \leq 1 \) at revascularisation. This sub-group was specifically assessed since evidence demonstrates that cardioprotective strategies are most effective in such cohorts of STEMI patients\(^7\) and since the biochemistry of nitrite reduction indicates that activity of the anion is greatest in hypoxic environments.

**Safety and tolerability.**

Following 6 months and 1 year after AMI, major adverse cardiac events (MACE) (defined as death, myocardial infarction, recurrent revascularization, stroke and heart failure) were recorded. MACE was assessed at clinic follow-up at 6 months and by telephone follow-up by trained research co-ordinators at 1 year. All events were verified with source documentation. Further safety measures included assessment of the acute safety and tolerability of intra-coronary nitrite (haemodyamics and level of methaemoglobin), and the incidence of major adverse events occurring within the first 48 hours after reperfusion, including death, heart failure, AMI, stroke, recurrent ischaemia, need for repeat revascularization, renal/hepatic insufficiency, vascular complications, and bleeding (see on line supplement for details).

**Measurement of platelet reactivity and assessment of nitrite.**

Since nitrite has been shown to have important anti-platelet effects additional hypothesis generating biochemical and functional assessments of platelet function were made\(^{23, 24}\). These included assessments of including platelet aggregation and P-selectin expression at baseline, 30 minutes post delivery of nitrite/placebo, 4, 24 hours and 6 months after infarction (see online supplement for details). In addition, to confirm successful administration of nitrite circulating plasma nitrite/nitrate levels (collectively termed NOx) were measured at baseline and 30 minutes post delivery of the study drug (see online supplement for details). Analysis of local coronary concentration following intervention administration was not possible due to the nature of the PCI procedure.

**Statistical analysis.**

Primary endpoint infarct size CK analysis: We hypothesised that nitrite would reduce the CK AUC by 30%, as per previous cardioprotective strategies namely cyclosporine\(^8\) and postconditioning\(^6\). We chose to assess CK rather than CK-MB as this matches previous studies\(^{6, 8}\) and since CK measurement is part of the routine clinical assessments made in the UK following PCI. Moreover, whilst CK-MB might be considered more specific for cardiac injury recent evidence suggests that CK AUC is comparable to CK-MB, Trop T or Trop I for the assessment of infarct size\(^{25}\). For a statistical power of 80% and a probability of a type I error of 0.05 using a two-sided test, we calculated that the sample size should be 70 subjects (35 per group). Since 4-8% of patients will die by the time of the endpoint at 6 months and 10% will either not tolerate or fail to attend the CMR at 6 months an additional 10 patients were recruited to account for these eventualities, giving a total of 80 patients.

Secondary endpoint CMR: Based upon the assumption of a predicted relative decrease of CMR-determined infarct size of 20% (as per previous studies\(^{8, 29}\)) calculations determined that 31 patients were needed in each patient group (statistical power of 80% and a probability of a type I error of 0.05 using a two-sided test assuming mean infarct of 25G and a SD of 7G).
Analysis was based on the intention-to-treat principle. Baseline demographic and clinical variables were summarised for each arm of the study. Descriptive summaries of the distributions of continuous baseline variables are presented in terms of percentiles (e.g., median, 25th and 75th percentile), while discrete variables are summarised in terms of frequencies and percentages.

Comparisons are between the sodium nitrite-treated and placebo control-treated group for the primary and secondary outcomes. The statistical comparison between the treatment groups for the primary endpoint of CK AUC was performed using the Wilcoxon rank-sum test for non-parametric data since previous studies clearly demonstrate a non Normal distribution for this biomarker\(^8\).

For all other hypothesis-generating outcome measures statistical comparisons between the groups were performed using unpaired Student’s t test for data with a Normal distribution or Wilcoxon rank sum tests for data with a non Normal distribution. For comparisons between treatment groups assessing platelet reactivity data are expressed as mean ± standard error and analysis performed using two-way repeated measures ANOVA.

To explore mechanisms associations between indices were measured using Pearson’s correlation coefficient with 95% confidence intervals to ascertain whether the CMR measures of infarct size were associated with biochemical measures of infarct size, to determine whether angiographic measures of AAR were associated with the CMR assessment of AAR and whether infarct size was associated with platelet reactivity. Statistical significance was established at p<0.05 (2-tailed) for all tests and performed using SPSS version 19, (SPSS Inc, Chicago, Ill).

RESULTS

Characteristics of study population.

Between April 2012 and December 2012, 430 patients were hospitalised for management of AMI at The Barts Health Heart Attack Centre. Of these patients 353 underwent PCI. Among these 353 patients, whilst 13 were not evaluated for enrollment because study personnel were not available. Another 251 were evaluated and excluded as depicted in Figure 1. This left 89 suitable patients, of which 9 declined. Data are thus presented for 80 patients (40 in the control group and 40 in the nitrite group, Figure 1).

All baseline and procedural characteristics were similar between the treatment groups except ischaemia time (Table 1 and 2). The mean age of the trial participants was 57 years, with 84% male. 25% of the cohort had anterior infarcts with similar numbers in both treatment groups. Stenting of the culprit lesion was performed in 97.5% of all patients. In five patients, TIMI 3 flow was not achieved after PCI (3 in the nitrite group and 2 in placebo).

Infarct size.

There was no evidence of a difference in the CK AUC between the nitrite and control groups, with a median of 56,398 arbitrary units (IQR: 31,185 to 83,531) in the nitrite group versus 48,195 (IQR: 27,726 to 82,841) in the control group (p=0.92). The median AUC for troponin T release was 140,782 arbitrary units (IQR, 84,949 to 218,133) in the nitrite group and 136,412 arbitrary units (IQR: 70,045 to 239,483) in the control group. This difference was not statistically different (p=0.85).
Of the 80 patients recruited 12 declined consent for the CMR protocols. In the remaining 68 patients no evidence of a difference in left ventricular volumes, mass or ejection fraction between the nitrite and placebo-treated groups were evident (Table 3). However, myocardial salvage index was improved in the nitrite group compared to placebo, although this difference fell on the borders of conventional statistical significance (p=0.05; Figure 2A). There was also a trend to smaller infarct size, incidence and MVO in the nitrite compared to placebo-treated group. CMR-assessed infarct size was positively associated with cardiac biomarkers (CK, r=0.770, p<0.01; troponin T, r=0.787, p=0.01, Online Figure I). The CMR-assessed AAR was associated with both angiographic risk scores (APPROACH: r=0.678, p<0.01, BARI: r=0.541, p<0.01, Online Figure II).

Coronary angiography and sub-group analysis.

Since nitrite bioactivity is thought to occur to a greater extent under ischaemic conditions we assessed the effect of nitrite on infarct size according to whether the culprit vessel was occluded or not at the time of drug administration. Angiographic analysis indicated that 66 of the 80 patients had TIMI flow ≤1 pre-procedure and successful drug delivery (i.e. unsuccessful procedures excluded). In this sub-group ischaemia time was the same between the two groups as were all other baseline characteristics (Online Table I). Importantly, in this sub-group there was a significant reduction in myocardial infarct size assessed by CK AUC between the nitrite and control groups, with a median of 44,608 arbitrary units (IQR: 27,535 to 64,848) in the nitrite group versus 55,666 (IQR: 41,591 to 93,659) in the control group (p=0.030). This represents a 19% reduction in infarct size (Figure 3A). The median AUC for troponin T release was 131,410 (IQR: 71,337 to 183,452) in the nitrite group and 176,492 (IQR: 89,831 to 245,094) in the control group (p=0.16) (Figure 3B).

In the nine patients with TIMI flow >1 at time of infusion, baseline characteristics were similar between the groups aside from a significantly longer ischaemia time in the nitrite group (supplement Table S2). No evidence of a difference in infarct size assessed by CK or troponin T AUC was seen in the patients with TIMI flow >1 treated with nitrite compared to placebo, although there was a trend to increased values in the nitrite group (Online Table II).

In the TIMI flow ≤1 group there was a significant decrease in CMR-determined myocardial infarct size (15.31 (12.36-18.27) vs 20.08 (16.72-23.43), p=0.03) associated with an increased myocardial salvage index (0.56 (0.50-0.62) vs 0.43 (0.37-0.49), p=0.002) (Figure 2B) associated with a reduction in MVO (37% vs 72.4%) in the nitrite-treated patients (Table 3). No evidence of difference in infarct size or AAR was seen in patients with TIMI flow >1 treated with nitrite compared to placebo (Table 3).

Safety and tolerability of nitrite.

Following administration of nitrite 45% of patients developed a >10% decrease in systolic blood pressure (within 10 minutes) however the magnitude and incidence was similar to the control group and did not alter clinical management. There was a small (but clinically insignificant) rise in met-Hb in the patients receiving nitrite however, the levels were not different to the control group (Table 2).

During the first 48 hours after reperfusion, 7 adverse clinical events were recorded in the control group compared to 3 in the nitrite group (Table 2). 1 year after infarction, 6 MACE events were recorded in the control group compared to 1 in the nitrite group (p=0.04, see Table 2). There were no differences in the prescription of prognostic medication between the treatment groups at discharge or at 1 year of follow-up (Table 2).
**Plasma NOx levels.**

Similar nitrate and nitrite levels between the groups were evident at baseline, but an increase in circulating nitrite, but not nitrate, levels was evident at 30 minutes following sodium nitrite administration indicating successful administration.

**Platelet reactivity.**

Platelet aggregation and P-selectin expression changed substantially over time (Figure 5 and Online Figure III, IV), in both groups. In all conditions, platelet reactivity was greatest at baseline with no differences between the treatment groups in the whole cohort (e.g. unstimulated mean ± SD P-selectin expression in the whole cohort was 8.9±4.6% and 7.9±4.8% and ADP-induced P-selectin expression was 47.2±18.9 and 46.9±18.2 in the placebo and nitrite-treated groups respectively). In both groups there was a decrease at 4 hours, followed by a slight elevation at 24 hours and a further decrease by 6 months. However, these changes post-baseline were all suppressed in the nitrite group versus placebo in both the whole cohort (p<0.05 or 0.01, Figure 5, Online Figure III, IV) and in the TIMI ≤1 sub-group (p<0.01, Figure 5, Online Figure III, IV). Post-hoc analyses demonstrated that the reactivity of platelets to activating stimuli (only ADP shown for clarity) appears directly associated to CMR-determined infarct size (Figure 5) with 6 month CMR infarct size positively associated with both P-selectin expression (r=0.401, p=0.002, Figure 5E) and platelet aggregation (r=0.344, p=0.007, Figure 5F) at 6 months in response to ADP.

**DISCUSSION**

In this proof-of-concept phase 2 study intra-coronary administration of nitrite at the time of reperfusion in patients with AMI was not associated with a reduction compared to placebo in the primary outcome measure of infarct size as assessed by cardiac biomarkers. Although there was greater myocardial salvage index with an 18% increase in the nitrite group compared to placebo (this was on the boundaries of conventional statistical significance (p=0.05)), and significant reductions in MACE at 6 months and 1 year. In a single retrospective sub-group analysis of patients with TIMI flow ≤1, at the time of primary PCI, treatment with nitrite was associated with a 20% reduction in infarct size compared to placebo as assessed by cardiac biomarkers (AUC for CK). This effect was replicated by the CMR analyses demonstrating a reduction of infarct size of 25% associated with a greater myocardial salvage index and reduced platelet reactivity.

In this study we show statistically significant reductions of infarct size in the sub-group of patients with TIMI flow ≤1 but not in the whole cohort. Important determinants of infarct size after primary PCI include AAR and the duration of ischaemia, both of which may be confounding variables in the present study. Despite the use of best practice through randomisation and double-blinding nitrite-treated patients had a longer mean ischaemia time compared to the placebo treated group, which is known to adversely affect myocardial salvage and infarct size. Importantly, in the sub-group analysis in those with TIMI flow ≤1 there were no differences in ischaemia time or any other baseline values between the groups. This result suggests that for nitrite to be most effective in reducing infarct size in STEMI patients it needs to be administered while the culprit artery is still occluded. The mean ischaemia time in the whole cohort (~189 minutes) reflects well when compared to other studies assessing potential cardioprotective strategies e.g. cyclosporine with values ranging from 331-252 minutes. The comparatively reduced ischaemia time in the present study likely underlies the smaller
infarct sizes seen herein in comparison to other published studies (e.g. 6, 8), a fact corroborated by the CMR analyses. It is noteworthy that, although not statistically different, the AAR assessed by angiographic scores displayed a trend to be larger in the nitrite-treated group in the whole cohort with increases of 5-14% depending on the method used: this could confound the results with theoretically larger infarcts in the nitrite group.

A recent study using intravenous nitrite in STEMI patients, with a pre-specified recruitment criteria of TIMI flow $\leq 1$ prior to reperfusion, showed no reduction in infarct size 30. These findings contrast directly with our sub-group assessment of TIMI flow $\leq 1$ patients where a substantial cardioprotective effect, in almost all measures of cardioprotection, was evident. This difference may relate to differences in the route of administration and dose. In the Frenneaux study nitrite was administered intravenously using a dose shown previously to achieve circulating levels of 6 μmol/L, in dogs, that was associated with profound cardioprotection 18. This concentration sits within the previously demonstrated efficacious levels of 3-12 μmol/L in numerous pre-clinical studies in vivo in various species 15-17. Unfortunately in the Frenneaux study 30 this dose increased circulating levels of nitrite from 0.76 to 1.4 μmol/L only, suggesting that the pharmacokinetics of intravenously administered nitrite in humans is different from dogs. In our study we gave a bolus dose of nitrite directly into the coronary artery providing a local estimated concentration of ~10 μmol/L and at the very least 3 μmol/L. We believe that achieving this high local concentration prior to reperfusion is the key factor underlying the efficacy in the TIMI flow $\leq 1$ patients in our cohort.

In patients with significant coronary flow (TIMI flow $>1$) prior to infusion a lack of benefit is not unexpected, although the numbers are small. Extensive pre-clinical evidence demonstrates that the cytoprotective properties of nitrite in models of myocardial infarction 14-18 is most evident with application into or on the ischaemic organ with the culprit vessel occluded at time of drug delivery i.e. zero flow. Bioactivation of nitrite to nitric oxide within the circulation does occur under physiological conditions in humans 20, 22, however, this phenomenon is enhanced with decreasing oxygen tension and this underlies its improved bioactivity under hypoxic/ischaemic conditions 11, 22. Standard care pathways for STEMI patients presenting to hospitals for primary PCI include early and efficient administration of anti-platelet and anti-thrombotic therapies. Indeed, studies suggest that >40% of patients will have spontaneously reperfused in the infarct-related territory resulting in significant coronary flow (TIMI flow $>1$) within the culprit coronary artery prior to revascularisation 31. Assessing whether nitrite would benefit, or indeed cause no harm, to a representative group of patients including those with TIMI flow $>1$ is important.

The mechanisms underlying the beneficial effects of nitrite have been attributed to its conversion to nitric oxide which improves mitochondrial function but also exerts anti-inflammatory and anti-platelet effects 12, 13. Interestingly, MVO was reduced in patients treated with nitrite. MVO has been implicated in worse clinical outcomes due to poor myocardial perfusion despite epicardial coronary artery revascularization 32. In our study, the incidence of MVO at the initial CMR scan was reduced by ~20% and 35% in the nitrite-treated group in the whole cohort and the TIMI flow $<1$ subgroup respectively. It is worth noting that factors that impact on MVO, such as comorbid conditions and the use of antiplatelet and anticoagulant therapy, were similar between the two treatment groups. In addition, it is important to appreciate that whilst statistical differences were shown with two group statistical comparisons that we did not perform statistical correction for multiple comparisons in this study. Thus, further prospective studies powered for statistical significance across all of the CMR-related measures would be required to confirm the validity of our observations.

Our exploratory mechanistic analyses assessing platelet function suggest that the underlying reason for the difference in MVO may relate to reductions in platelet reactivity. Post-hoc analyses show direct correlation between platelet reactivity and infarct size, and we suspect that the reduced infarct size and as a consequence a reduced systemic inflammation might underlie this improvement with nitrite.
Further analyses assessing levels of systemic inflammation and whether they correlate with infarct size/platelet reactivity are additional secondary outcome measures that should inform on this possibility. However, further prospective studies powered for assessment of platelet reactivity are warranted.

Although small this study also shows no indication that intra-coronary nitrite administration has adverse effects in the cohort as a whole or within the sub-group. Specifically, we assessed blood pressure due to the known vasodilator and blood pressure lowering effects of raised circulating nitrite levels. The data demonstrate that blood pressure did drop in some patients although this was equally evident in both arms and likely due to bradycardia and hypotension which are a common feature of reperfusing occluded coronary arteries (Bezold-Jarisch reflex), particularly the right coronary artery. We also assessed methaemoglobinemia, due to the known interaction of nitrite with oxyhaemoglobin to generate methaemoglobin particularly occurring with systemic nitrite infusions, and here also no adverse effect was noted. Pre-clinical evidence indicates that nitrite is cytoprotective against ischaemia-reperfusion injury only when given at concentrations (3-12 μmol/L) that far exceed physiological (0.1-0.4 μmol/L) levels. We suggest that the lack of adverse effect despite the use of high (supra-physiological) levels of nitrite in this study reflects the advantage of intra-coronary nitrite administration i.e. achieving high local concentrations within the myocardium only. An additional advantage of the intra-coronary route is that it provides an option causing no delay in reperfusion. This is compared to other therapies including cyclosporine and exenatide where intra-venous administration may result in both greater side-effects and a delay in reperfusion whilst administered.

Study limitations.

Despite the same method of drug delivery used in both randomised patient groups, there was a longer ischaemia time in the nitrite group, which will have limited the potential effect of the therapy seen in the study cohort. The study was powered based on the enrolment of all-comers to prevent any treatment delay and to test the therapy in as broad a group as possible. Despite this sufficient numbers of patients with TIMI flow ≤1 were available to conduct powered statistical analyses.

Further studies powered for assessing safety in TIMI flow >1 patients are essential to determine the generalised safety of intra-coronary nitrite administration in patients presenting with an AMI, and could be incorporated into a larger phase 3 study assessing the therapeutic utility of nitrite in AMI.

For the CMR measures, our study was powered for single statistical comparison for the secondary outcome measure of infarct size, we did not conduct multiple testing. However, CMR provides information regarding several other features of cardiac structure and function as detailed in the tables. A further study powered sufficiently for statistical comparison of multiple CMR-derived measures of infarct size, MVO and other indices providing valuable information regarding cardiac function such as ejection fraction may be of value to confirm the observations in this study.

Infarct size is an intermediate outcome measure that is commonly used to assess cardioprotective strategies in STEMI patients. However, as an intermediate outcome measure this does not provide clear understanding on hard outcomes such as MACE. Our study was not powered to detect changes in MACE and although we saw evidence of benefit the low number of events prevent drawing of any reliable conclusion. We suggest that our data provide strong support for conducting a phase 3 study in patients with TIMI ≤1 flow at point of revascularisation assessing the therapeutic potential of intra-coronary nitrite administration with MACE as the primary outcome measure.
Conclusion.

This study demonstrates that intra-coronary nitrite infusion should be added to the list of ‘promising’ cardioprotective agents for potential use in AMI when administered intra-coronary at the point of revascularisation. Further investigation of this potential in a larger Phase 3 clinical trial is warranted.

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DISCLOSURES

AA is a director of Heartbeet Ltd. We declare that we have no other conflicts of interest.

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FIGURE LEGENDS

Figure 1. Trial profile.

Figure 2. Effect of intracoronary nitrite on cardiac magnetic resonance imaging (CMR)-determined myocardial salvage index. The myocardial salvage index on CMR is presented for (A) 35 TIMI flow=0-3 patients in the control and 33 patients in the nitrite group. (B) Myocardial salvage index is reduced in the nitrite-treated group of 27 TIMI≤1 patients vs 28 in the control. Significance evaluated using unpaired t test and data shown as mean±SEM.

Figure 3. Intracoronary nitrite lowers infarct size by biomarker assessment in the TIMI flow ≤1 subgroup. Serum CK was measured at baseline and between 4-48 hours after coronary reperfusion. Curves for the nitrite and control group are shown in Panel A. Serum troponin T was measured at the same time points as CK and curves shown in Panel B. T bars denote standard errors of the mean (SEM).

Figure 4. Plasma NO2⁻ and NO3⁻ levels pre and post intervention. Plasma NO2⁻ and NO3⁻ levels measured at baseline and 30 minutes after delivery of either intra-coronary nitrite or placebo in all patients. Each line representing the difference between baseline and 30 minute plasma NO2⁻ and NO3⁻ levels shown for each patient in the nitrite group in panel A and placebo in panel B. Error bars represent mean ± SD for each group. ***P<0.0001 using paired t-test. (NO2⁻ = Nitrite, NO3⁻ = Nitrate)

Figure 5. Platelet reactivity post-intervention. Platelet reactivity measured at baseline, 30 minutes, 4 hours, 24 hours and 6 months after coronary reperfusion. Platelet P-Selectin expression assessed in whole blood in response to ADP (10 μmol/L) is shown for nitrite versus placebo for all patients in panel A. Panel B shows whole blood impedance aggregometry in response to the same ADP stimulus in all patients. Panel C shows P-selectin expression in response to ADP in patients with TIMI flow <1. Panel D shows aggregation in response to ADP in the TIMI <1 subgroup. All panels show nitrite treated versus placebo. Data expressed as mean ± SEM. # =P<0.05, ##=P<0.01, for two-way repeated measures ANOVA (ADP: adenosine diphosphate). (E) There was a positive association between platelet P-selectin expression in response to ADP and LGE (late gadolinium enhancement) assessed infarct size on CMR at 6 months. Panel F depicts a similar positive association between platelet aggregation in response to ADP and LGE CMR infarct size at 6 months. Correlations determined using Pearson’s correlation coefficient.
### TABLE 1. Baseline characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Nitrite (n=40)</th>
<th>Placebo (n=40)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (yr) (Mean±SD)</strong></td>
<td>56.35±11.16</td>
<td>57.60±13.20</td>
</tr>
<tr>
<td><strong>Sex (M/F)</strong></td>
<td>36/4</td>
<td>31/9</td>
</tr>
<tr>
<td><strong>Diabetes mellitus</strong></td>
<td>3 (7.5%)</td>
<td>3 (7.5%)</td>
</tr>
<tr>
<td><strong>Body-mass index (kg/m²) (Mean±SD)</strong></td>
<td>28.97±5.14</td>
<td>28.58±5.17</td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
<td>20 (50.0%)</td>
<td>14 (35.0%)</td>
</tr>
<tr>
<td><strong>Hypercholesterolaemia</strong></td>
<td>16 (40.0%)</td>
<td>12 (30.0%)</td>
</tr>
<tr>
<td><strong>Heart rate (BPM) (Mean±SD)</strong></td>
<td>72.68±18.62</td>
<td>77.35±21.31</td>
</tr>
<tr>
<td><strong>Systolic BP (mmHg) (Mean±SD)</strong></td>
<td>124.48±29.92</td>
<td>136.13±26.99</td>
</tr>
<tr>
<td><strong>Ischaemia time (min) (Mean±SD)</strong></td>
<td>207.05±76.34</td>
<td>171.63±67.72</td>
</tr>
<tr>
<td><strong>Door to Balloon time (min) (Mean±SD)</strong></td>
<td>46.45±13.76</td>
<td>42.35±11.94</td>
</tr>
</tbody>
</table>

**Culprit Vessel**
- **Left anterior descending**: 9 (22.5%) vs 12 (30%)
- **Circumflex**: 5 (12.5%) vs 5 (12.5%)
- **Right coronary**: 26 (65.0%) vs 23 (57.5%)

**TIMI flow before PCI**
- **0**: 30 (75.0%) vs 31 (77.5%)
- **1**: 6 (15.0%) vs 3 (7.5%)
- **2**: 2 (5.0%) vs 5 (12.5%)
- **3**: 2 (5.0%) vs 1 (2.5%)
- **0/1**: 36 (90.0%) vs 34 (85.0%)

**Syntax score (Mean±SD)**
- 13.41±5.50 vs 13.58±6.20

**DES use**
- 33 (82.5%) vs 30 (78.9%)

**Treatment before PCI**
- **Morphine**: 26 (43.3%) vs 34 (56.7%)

**Treatment at time of PCI**
- **Heparin**: 40 (100%) vs 40 (100%)
- **Aspirin**: 40 (100%) vs 40 (100%)
- **Clopidogrel/Prasugrel (No/No)**: 35/5 vs 37/3
- **Glycoprotein IIb/IIIa inhibitor**: 40 (100%) vs 40 (100%)

Values shown as number (%) unless otherwise stated.

Abbreviations: PCI, percutaneous coronary intervention; TIMI, Thrombolysis in myocardial infarction; DES drug-eluting stent.

*a* The body-mass index is the weight in kilograms divided by the square of the height in meters.

*b* Ischaemia time determined from symptom to balloon times for each patient.
<table>
<thead>
<tr>
<th>TABLE 2. Delivery of IMP, procedural and clinical outcomes</th>
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</thead>
<tbody>
<tr>
<td>IMP Delivery</td>
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<tr>
<td>Systolic BP drop (Median (IQR))</td>
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<tr>
<td>Systolic BP drop &gt;10%</td>
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<tr>
<td>MetHb (Median (IQR))</td>
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<tr>
<td>Angiographic AAR</td>
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<tr>
<td>APPROACH (Mean (95% CI))</td>
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<tr>
<td>BARI (Mean (95% CI))</td>
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<tr>
<td>Contrast</td>
</tr>
<tr>
<td>ST segment resolution (&gt;70%)</td>
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<tr>
<td>Manual Thrombectomy</td>
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<tr>
<td>Procedural Success</td>
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<tr>
<td>Clinical events</td>
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<tr>
<td>48 hour</td>
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<tr>
<td>Death</td>
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<tr>
<td>Recurrent Ischaemia</td>
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<tr>
<td>Heart failure</td>
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<tr>
<td>CIN</td>
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<tr>
<td>6 month</td>
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<td>Repeat revascularisation</td>
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<tr>
<td>Clinical events</td>
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<tr>
<td>1 year</td>
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<tr>
<td>MACE</td>
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<tr>
<td>Death</td>
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<tr>
<td>Repeat revascularisation</td>
</tr>
<tr>
<td>Recurrent myocardial infarction</td>
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<tr>
<td>Recurrent myocardial infarction</td>
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<tr>
<td>Hospitalisation for heart failure</td>
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<tr>
<td>Medication at 1 year</td>
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<tr>
<td>Beta-blocker</td>
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<tr>
<td>ACE-i</td>
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<tr>
<td>ARB</td>
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<tr>
<td>Statin</td>
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<tr>
<td>Aspirin</td>
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<tr>
<td>ADP antagonist</td>
</tr>
</tbody>
</table>
Values shown as number (%) unless otherwise stated.
Abbreviations: AAR, area at risk; MACE, major adverse cardiac events; CIN, contrast-induced nephropathy; IMP, investigational medicinal product.
## Table 3. CMR data for study population split by TIMI flow at presentation

<table>
<thead>
<tr>
<th></th>
<th>Whole Cohort (n=33)</th>
<th>Nitrite (n=27)</th>
<th>Placebo (n=27)</th>
<th>P value</th>
<th>TIMI ≤1 (n=30)</th>
<th>Nitrite (n=19)</th>
<th>Placebo (n=13)</th>
<th>P value</th>
<th>TIMI &gt;1 (n=4)</th>
<th>Nitrite (n=2)</th>
<th>Placebo (n=5)</th>
<th>P value</th>
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<tbody>
<tr>
<td><strong>Baseline CMR</strong></td>
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<tr>
<td>LVEDVi (ml/m²)</td>
<td>76.13 (71.11-81.14)</td>
<td>70.58 (65.22-75.94)</td>
<td>75.69 (69.93-81.44)</td>
<td>0.13</td>
<td>70.33 (64.31-76.34)</td>
<td>78.01 (51.65-104.40)</td>
<td>0.19</td>
<td>71.99 (54.30-89.68)</td>
<td>0.58</td>
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<tr>
<td>LVESVi (ml/m²)</td>
<td>36.16 (32.56-39.75)</td>
<td>35.71 (30.94-40.48)</td>
<td>35.16 (31.20-39.12)</td>
<td>0.88</td>
<td>35.41 (30.62-40.20)</td>
<td>39.90 (18.85-60.94)</td>
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<td>37.37 (12.14-62.80)</td>
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<tr>
<td>LVMi (g/m²)</td>
<td>63.02 (58.65-67.40)</td>
<td>58.23 (54.67-61.79)</td>
<td>61.07 (56.27-65.88)</td>
<td>0.09</td>
<td>58.07 (54.44-61.71)</td>
<td>74.11 (58.19-90.03)</td>
<td>0.31</td>
<td>59.12 (40.75-77.49)</td>
<td>0.13</td>
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<tr>
<td>LVEF (%)</td>
<td>52.87 (49.88-55.86)</td>
<td>50.07 (46.46-53.67)</td>
<td>53.86 (50.40-57.32)</td>
<td>0.23</td>
<td>50.00 (46.43-53.72)</td>
<td>49.55 (40.32-58.78)</td>
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<td>50.50 (29.96-71.04)</td>
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<td>IS (%) LV</td>
<td>17.10 (14.12-20.08)</td>
<td>19.55 (16.40-22.70)</td>
<td>15.31 (12.36-18.27)</td>
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<td>20.08 (16.72-23.43)</td>
<td>21.43 (9.62-33.23)</td>
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<td>15.86 (4.42-31.30)</td>
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<tr>
<td>AAR (%) LV</td>
<td>34.58 (31.62-37.55)</td>
<td>33.05 (29.40-36.72)</td>
<td>33.89 (30.54-37.24)</td>
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<td>33.27 (29.12-37.42)</td>
<td>35.71 (21.15-50.28)</td>
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<td>31.74 (21.62-41.87)</td>
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<td>MSI</td>
<td>0.52 (0.46-0.58)</td>
<td>0.44 (0.39-0.49)</td>
<td>0.56 (0.50-0.62)</td>
<td>0.05</td>
<td>0.43 (0.37-0.49)</td>
<td>0.41 (0.31-0.51)</td>
<td>0.002</td>
<td>0.54 (0.30-0.78)</td>
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<td>MVO No. (%)</td>
<td>16 (48.5%)</td>
<td>23 (69.7%)</td>
<td>10 (37.0%)</td>
<td>0.13</td>
<td>21 (72.4%)</td>
<td>3 (75.0%)</td>
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<td>1 (25.0%)</td>
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<tr>
<td>MVO Amount (g)</td>
<td>2.98 (0-6.25)</td>
<td>3.47 (0-4.75)</td>
<td>1.00 (0.80-5.87)</td>
<td>0.34</td>
<td>4.50 (1-7.50)</td>
<td>1 (0-8.25)</td>
<td>0.003</td>
<td>1 (0-1)</td>
<td>0.09</td>
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<table>
<thead>
<tr>
<th></th>
<th>6 month CMR (n=29)</th>
<th>Nitrite (n=25)</th>
<th>Placebo (n=25)</th>
<th>P value</th>
<th>6 month CMR (n=29)</th>
<th>Nitrite (n=20)</th>
<th>Placebo (n=19)</th>
<th>P value</th>
<th>6 month CMR (n=2)</th>
<th>Nitrite (n=1)</th>
<th>Placebo (n=1)</th>
<th>P value</th>
<th>6 month CMR (n=4)</th>
<th>Nitrite (n=2)</th>
<th>Placebo (n=2)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDVi (ml/m²)</td>
<td>82.13 (74.80-89.46)</td>
<td>75.62 (69.90-81.34)</td>
<td>79.27 (72.01-86.54)</td>
<td>0.15</td>
<td>75.37 (69.20-81.53)</td>
<td>109.94 (-184.6-204.5)</td>
<td>0.40</td>
<td>80.57 (60.05-101.1)</td>
<td>0.16</td>
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<tr>
<td>LVESVi (ml/m²)</td>
<td>36.50 (31.48-41.52)</td>
<td>34.85 (30.53-39.17)</td>
<td>33.80 (30.08-37.54)</td>
<td>0.61</td>
<td>34.94 (30.16-39.73)</td>
<td>64.97 (-216.3-346.2)</td>
<td>0.71</td>
<td>37.42 (25.62-49.21)</td>
<td>0.13</td>
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<td></td>
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</tr>
<tr>
<td>LVMi (g/m²)</td>
<td>55.67 (51.66-59.68)</td>
<td>51.20 (48.17-54.24)</td>
<td>54.21 (50.35-58.07)</td>
<td>0.07</td>
<td>51.09 (47.75-54.44)</td>
<td>73.36 (-80.60-227.3)</td>
<td>0.21</td>
<td>52.14 (46.98-57.30)</td>
<td>0.05</td>
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</tr>
<tr>
<td>LVEF (%)</td>
<td>55.93 (52.71-59.15)</td>
<td>54.75 (51.62-57.87)</td>
<td>57.19 (54.12-60.27)</td>
<td>0.59</td>
<td>54.51 (50.96-58.05)</td>
<td>42.43 (-61.45-146.30)</td>
<td>0.25</td>
<td>53.51 (43.38-63.65)</td>
<td>0.18</td>
<td></td>
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</tr>
<tr>
<td>IS (%)</td>
<td>11.88 (9.52-14.24)</td>
<td>13.15 (10.75-15.56)</td>
<td>10.69 (8.38-13.02)</td>
<td>0.45</td>
<td>13.70 (11.16-16.24)</td>
<td>16.33 (-9.40-42.06)</td>
<td>0.08</td>
<td>10.59 (0.24-20.93)</td>
<td>0.32</td>
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</tbody>
</table>

Values shown as mean (95% CI). Abbreviations: LVEDVi, Indexed left ventricle end-diastolic volume; LVESVi, Indexed left ventricle end-systolic volume; LVMi, Indexed left ventricle mass; LVEF, left ventricle ejection fraction; AAR, area at risk; MSI, myocardial salvage index; MVO, microvascular obstruction.
Novelty and Significance

What Is Known?

- Primary percutaneous coronary intervention (PCI) is used currently for the treatment of acute myocardial infarction (AMI), but significant morbidity and mortality rates remain, in part due to the damaging effects of reperfusion following revascularization.
- Reducing reperfusion injury could improve outcomes.
- Extensive pre-clinical data suggest that local delivery of sodium nitrite reduces reperfusion injury.

What New Information Does This Article Contribute?

- This phase 2 double-blind randomized placebo controlled clinical trial assessed the efficacy of intra-coronary nitrite infusion during primary PCI post-AMI.
- There was a significant reduction in infarct size in a subgroup of patients with occluded culprit arteries at the time of PCI.
- This effect was associated with an apparent decrease in platelet reactivity over the 6 months following PCI and a reduction in major adverse cardiovascular events at 6 months and 1 year.

The results of this clinical trial demonstrate that local intra-coronary administration of sodium nitrite reduces infarct size as assessed by the measurement of cardiac enzyme release and scar size determined using cardiac MRI, in patients with an occluded artery (TIMI flow≤1) at the time of PCI. The intra-coronary administration of nitrite prior to balloon inflation offers a potential cardioprotective strategy that causes no significant delay in delivery of the primary angioplasty procedure. In addition, this procedure enables high dose delivery of nitrite that is not associated with significant methaemoglobinemia or a decrease in blood pressure, the two potential concerns with nitrite delivery. The results of this study suggest that intra-coronary nitrite administration to the culprit vessel of select patients presenting with AMI has no adverse safety profile, and may provide a new therapeutic option as an adjunct to primary angioplasty. These findings warrant further investigation in larger outcome studies.
Assessed for eligibility  
$n=430$

348 Excluded
- 77 not ST-elevation myocardial infarction
- 53 age > 80
- 39 Previous AMI
- 34 angiographically unsuitable
- 32 recruitment to other research studies
- 30 Chest pain history greater than 6 hours
- 28 No informed consent
- 16 other reasons (malignancy, pregnancy, ESRF)
- 15 Previous CABG
- 13 cardiac arrest or cardiogenic shock
- 13 study team unavailable

Randomised  
$n=82$

40 randomised to sodium nitrite
40 received sodium nitrite

- 40 included in primary endpoint analysis at 48 hours
- 1 Lost to follow-up
- 39 included in clinical endpoint at 6 months
- 1 Lost to follow-up
- 38 included in clinical endpoint at 1 year

42 randomised to placebo
42 received placebo

- 2 excluded due to exclusion criteria
- 40 included in primary endpoint analysis at 48 hours
- 1 Lost to follow-up
- 39 included in clinical endpoint at 6 months
- 1 Lost to follow-up
- 38 included in clinical endpoint at 1 year
Figure 2

A

Whole cohort

Myocardial salvage index

Placebo  Nitrile

P = 0.051

B

TIMI ≤ 1

Myocardial salvage index

Placebo  Nitrile

P = 0.002
Figure 3

A

**TIMI≤1**

- **Placebo**
- **Nitrite**

Serum CK release (AU)

Time (hours)

P = 0.03

B

**TIMI≤1**

- **Placebo**
- **Nitrite**

Serum Troponin T release (AU)

Time (hours)

P = 0.158
Figure 4

(A) Nitrite

(B) Placebo

Plasma [NO₂⁻] (µM)

Baseline 30 minute

Baseline 30 minute
Figure 5

Whole Cohort

A

% P-Selectin Expression

B

Aggregation (AUC)

TIMI ≤ 1

C

% P-Selectin Expression

D

Aggregation (AUC)

E

Six month P-Selectin expression (%) vs Six month LGE infarct size (%)

F

Six month Aggregation (AUC) vs Six month LGE infarct size (%)
Randomized Phase 2 Trial of Intra-Coronary Nitrite During Acute Myocardial Infarction
Daniel A Jones, Cyril Pellaton, Shanti Velmurugan, Mervyn Andiapen, Sotiris Antoniou, Sven van Eijl, Andrew J Webb, Mark Westwood, Mahesh Parmar, Anthony Mathur and Amrita Ahluwalia

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Data Supplement (unedited) at:
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Supplemental material
Randomised phase 2 trial of intra-coronary nitrite during acute myocardial infarction

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Krishnaraj Sinha Rathod, Mervyn Andiapen, Sotiris Antoniou BPharmS, Sven van Eijl PhD, Andrew J Webb FRCP PhD, Mark A Westwood MRCP MD, Mahesh K Parmar, Anthony Mathur FRCP PhD, Amrita Ahluwalia PhD

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Supplemental Methods

Informed consent

Seeking informed consent for clinical research from patients suffering acute myocardial infarction (AMI) is an ethical challenge owing to the medical condition of the patients, the emergency situation, and the limited time available. There was no guaranteed solution to the particular difficulties of informed consent in this situation. It is extremely important that patients are provided with information, which is as concise and simple as possible, although sufficient for them to make an informed decision. To achieve this we firstly excluded patients who were unconscious, critically unstable (cardiogenic shock) or deemed unable to consent (pain, distress, language) and then followed a two step process of consent that was based upon our extensive investigations to identify the most efficient and considerate mechanism of gaining consent from patients in this emergency setting. A clear concise study summary sheet was shown to the patient during the consent process, this was a one-page sheet with diagrams, clearly explaining the procedure and events. A more detailed patient information sheet (PIS) was given following the procedure for reading. Secondly the oral information provided during the consent process had been discussed and planned with members of the public who agreed that they explain the trial effectively. There was a clear algorithm to follow with rehearsed statements. These were designed to match the summary PIS. Patients then signed an approved consent form in addition to the standard primary PCI consent form. This process and all forms were approved by the local research ethics committee.

Thrombolysis In Myocardial Infarction (TIMI)

Coronary angiograms obtained before and after primary PCI were reviewed by two experienced observers blinded to treatment allocation and clinical data. From these angiograms an assessment of TIMI flow grade and AAR using standard (BARI and APPROACH) validated approaches (see online supplement for further detail), both the modified Bypass Angioplasty Revascularisation Investigation [BARI]\(^1\), \(^2\) and modified Alberta Provincial Project for Outcome Assessment in Coronary Heart Disease [APPROACH]\(^3\) jeopardy scores were made.
Cardiac imaging protocols

Cardiac magnetic resonance (CMR) imaging was performed on a 1.5 T Philips Achieva scanner with a cardiac 32-channel phased array coil. Balanced steady-state free precession cine imaging was used to acquire 10-12 short axis slices (8 mm slice thickness, 2 mm gap) with one slice per breath-hold. Sequence parameters were 1.5 ms echo time (TE), 3.1 ms repetition time (TR), and acquired voxel size was 1.8 x 1.86 mm with a typical FOV of 360 mm in the phase encode direction. We acquired 45 phases with 25% phase sharing. Parallel imaging (SENSE) was used with an acceleration factor of 2.0.

Delayed enhancement images were acquired ten minutes after injection of a dose of 0.2 mmol/kg of gadoterate meglumine (Dotarem) for late gadolinium enhancement. A T1-weighted segmented inversion-recovery gradient echo pulse sequence (TR 3.9 ms, TE 1.9 ms, flip angle 15°, voxel size of 2 x 2 mm, typical FOV 360 mm) was used to obtain 10-12 short axis slices (matched with short-axis cine images) with one slice per breath-hold. The inversion time was adjusted individually according to a T1 scout sequence (Look-Locker). Images were acquired every other heart beat with 2 signal averages.

Myocardial oedema was assessed using fat suppressed T2-weighted triple inversion turbo spin echo STIR (Short tau inversion recovery) imaging (TE 80 ms, TR 2 heart beats, TSE factor 31, voxel size 1.8 x 1.8 mm). 10-12 slices were obtained (8 mm per slice, 2 mm gap matched to DE/cine slices) with one slice per breath-hold. This sequence has previously been used and validated for assessment of myocardial oedema and MSI4-7.

Images were anonymised, batched and analyzed in blinded fashion by two experienced operators. Scar and oedema volumes were calculated by manually drawing endocardial and epicardial contours followed by semi-automated selection of normal remote myocardium per slice. Myocardial oedema was described as >2SD in signal intensity from remote normal myocardium. Infarct size was calculated using the full-width half maximum method as previously described8. In case of discordance between operators, blinded review by a level III accredited CMR reader was
performed. Analysis was performed using dedicated software (CVI\textsuperscript{42}, Circle Cardiovascular Imaging Inc, Calgary, Alberta, Canada). Interobserver variability was calculated.

**Platelet reactivity**

Platelet reactivity was assessed by determining platelet aggregation and P-selectin expression at baseline, 30 minutes post delivery of nitrite/placebo, 4, 24 hours and 6 months after infarction. These were assessed in whole blood in response to adenosine diphosphate (ADP) (10 μmol/L), collagen (3 μg/mL), and PBS (as control) using an impedance aggregometer (MultiplateRanalyzer, Dyabyte Medical, Germany) and flow cytometry respectively using previously published protocols\textsuperscript{9}. These measures were conducted at baseline, 30 minutes post delivery of nitrite/placebo, 4, 24 hours and 6 months after infarction.

**Whole-blood aggregometry**

Platelet aggregation was assessed in whole blood in response to ADP (10μmol/L), collagen (3μg/ml) and PBS (as control) using an impedance aggregometer (Multiplate Ranalyzer, Dyabyte Medical, Germany) measured over a six-minute period. Aggregation is quantified as AUC giving a measure of total resistance Ω*time. Briefly 300mL of citrated whole blood was added to 300 mL of normal saline with 3 mmol/L CaCl\textsubscript{2} (Sigma, UK) and equilibrated with constant magnetic stirring for three minutes prior to the addition of agonist and platelet aggregation measurement.

**P-Selectin expression**

Two-colour whole blood flow cytometry was used to measure platelet P-selectin using a previously published protocol\textsuperscript{10}. Whole blood was collected from individuals at the specified time-points. The samples were immediately incubated with selective antibodies, at room temperature for 20 minutes, and then fixed using 1% paraformaldehyde (Sigma,UK) stored at 4°C and then analysed using a Becton Dickinson FACS Calibur flow cytometer (Becton Dickinson, SanJose, CA). The platelet population was identified preliminarily based on forward and side-scatter...
properties, then further delineated via labeling with CD42b monoclonal antibody conjugated to allophycocyanin (APC). Gates were used to isolate this population, and CD62 (P-selectin) monoclonal antibody conjugated to (fluorosce-nisothiocyanate) FITC was used to determine P-selectin expression. Populations were further confirmed by use of antibody negative iso-types to P-selectin and CD42b. 10,000 platelets were acquired in the CD42b region, and results were expressed as the percentage of platelets positive for P-selectin.

**Ozone chemiluminescence**

Briefly, to determine total nitrate and nitrite levels (collectively termed [NOx]), samples were added to 0.1 mmol/L vanadium (III) chloride in 1 mmol/L hydrochloric acid refluxing at 95 °C under nitrogen. Nitrite concentrations were determined by addition of samples to 0.09 mmol/L potassium iodide in glacial acetic acid under nitrogen at room temperature. [Nitrate] were calculated by subtraction of [nitrite] from [NOx] as previously described11.

**Major adverse events**

Major adverse events at 48 h included including death, heart failure, acute myocardial infarction, stroke, recurrent ischaemia, need for repeat revascularization, renal/hepatic insufficiency, vascular complications, and bleeding. Heart failure was defined as dyspnea (either new-onset or persisting) accompanied by both physical signs of heart failure (pulmonary crackles/rales, peripheral oedema, jugular venous distension, S3 gallop, radiological evidence of pulmonary oedema) and a need for increased heart failure therapy (diuretic or other oral heart failure therapies e.g. ACEi or mechanical/surgical intervention).
Supplemental References


Supplemental Results

Tables

Online Table I. Baseline characteristics of the TIMI <1 subgroup

<table>
<thead>
<tr>
<th></th>
<th>Nitrite (n=33)</th>
<th>Placebo (n = 33)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs) (mean±SD)</td>
<td>57.30±11.29</td>
<td>56.94±13.48</td>
<td>0.90</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>29/4</td>
<td>28/5</td>
<td>0.99</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>2 (6.1)</td>
<td>1 (3.0)</td>
<td>0.99</td>
</tr>
<tr>
<td>Body-mass index (kg/m²) (mean±SD)(^a)</td>
<td>29.27±5.30</td>
<td>29.06±5.17</td>
<td>0.87</td>
</tr>
<tr>
<td>Hypertension</td>
<td>16 (48.5%)</td>
<td>8 (24.2%)</td>
<td>0.07</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>13 (39.4%)</td>
<td>10 (30.3%)</td>
<td>0.61</td>
</tr>
<tr>
<td>Heart rate (mean±SD)</td>
<td>70.94±18.97</td>
<td>79.06±22.10</td>
<td>0.11</td>
</tr>
<tr>
<td>Systolic Blood pressure (mean±SD)</td>
<td>120.76±29.64</td>
<td>132.94±23.44</td>
<td>0.07</td>
</tr>
<tr>
<td>Ischaemia time (minutes) (mean±SD)(^b)</td>
<td>194.45±69.05</td>
<td>168.63±69.94</td>
<td>0.11</td>
</tr>
<tr>
<td>Culprit Vessel</td>
<td></td>
<td></td>
<td>0.57</td>
</tr>
<tr>
<td>Left anterior descending</td>
<td>8 (24.2%)</td>
<td>9 (27.3%)</td>
<td></td>
</tr>
<tr>
<td>Circumflex</td>
<td>3 (9.1%)</td>
<td>5 (12.5%)</td>
<td></td>
</tr>
<tr>
<td>Right coronary</td>
<td>22 (66.7%)</td>
<td>19 (57.6%)</td>
<td></td>
</tr>
<tr>
<td>Syntax score (mean±SD)</td>
<td>13.29±5.42</td>
<td>13.68±5.42</td>
<td>0.77</td>
</tr>
<tr>
<td>DES use</td>
<td>29 (87.9%)</td>
<td>26 (81.3%)</td>
<td>0.51</td>
</tr>
<tr>
<td>Angiographic AAR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APPROACH (mean±SD)</td>
<td>30.72±7.75</td>
<td>26.94±10.73</td>
<td>0.11</td>
</tr>
<tr>
<td>BARI (mean±SD)</td>
<td>27.32±10.88</td>
<td>24.75±9.26</td>
<td>0.31</td>
</tr>
<tr>
<td>Treatment before PCI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morphine</td>
<td>22 (66.7)</td>
<td>29 (87.9)</td>
<td>0.08</td>
</tr>
<tr>
<td>Treatment at time of PCI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>33 (100)</td>
<td>33 (100)</td>
<td>0.99</td>
</tr>
<tr>
<td>Aspirin</td>
<td>33 (100)</td>
<td>33 (100)</td>
<td>0.99</td>
</tr>
<tr>
<td>Clopidogrel/Prasugrel (no/no)</td>
<td>29/4</td>
<td>30/3</td>
<td>0.99</td>
</tr>
<tr>
<td>Glycoprotein IIb/Illa inhibitor</td>
<td>33 (100)</td>
<td>33 (100)</td>
<td>0.99</td>
</tr>
<tr>
<td>ST segment resolution &gt;70%</td>
<td>33 (100%)</td>
<td>28 (84.8%)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Values shown as number (%) unless otherwise stated

Abbreviations: PCI, percutaneous coronary intervention; TIMI, Thrombolysis in myocardial infarction; DES, drug-eluting stent.

\(^a\) The body-mass index is the weight in kilograms divided by the square of the height in meters.

\(^b\) Ischaemia time determined from symptom to balloon times for each patient.
## Online Table II. Baseline characteristics of the TIMI >1 subgroup

<table>
<thead>
<tr>
<th></th>
<th>Nitrite (n=4)</th>
<th>Placebo (n = 5)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (yrs) (mean±SD)</strong></td>
<td>54.75±5.50</td>
<td>60.00±13.87</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>Sex (male/female)</strong></td>
<td>4/0</td>
<td>2/3</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Diabetes mellitus</strong></td>
<td>1 (25.0)</td>
<td>2 (40.0)</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>Body-mass index (kg/m²)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mean±SD)a</td>
<td>28.53±5.43</td>
<td>25.95±5.85</td>
<td>0.54</td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
<td>3 (75.0%)</td>
<td>4 (80.0%)</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>Hypercholesterolaemia</strong></td>
<td>3 (75.0%)</td>
<td>1 (25.0%)</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>Heart rate (mean±SD)</strong></td>
<td>74.00±17.38</td>
<td>72.80±13.41</td>
<td>0.91</td>
</tr>
<tr>
<td><strong>Systolic Blood pressure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mean±SD)</td>
<td>131.50±26.90</td>
<td>135.80±33.82</td>
<td>0.84</td>
</tr>
<tr>
<td><strong>Ischaemia time (minutes)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mean±SD)b</td>
<td>286.80±96.70</td>
<td>174.33±55.19</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Culprit Vessel</strong></td>
<td></td>
<td></td>
<td>0.49</td>
</tr>
<tr>
<td>Left anterior descending</td>
<td>1 (25.0%)</td>
<td>2 (40.0%)</td>
<td></td>
</tr>
<tr>
<td>Circumflex</td>
<td>1 (25.0%)</td>
<td>0 (0.0%)</td>
<td></td>
</tr>
<tr>
<td>Right coronary</td>
<td>2 (50.0%)</td>
<td>3 (60.0%)</td>
<td></td>
</tr>
<tr>
<td><strong>Syntax score (mean±SD)</strong></td>
<td>15.63±7.91</td>
<td>13.30±10.79</td>
<td>0.73</td>
</tr>
<tr>
<td><strong>DES use</strong></td>
<td>1 (25.0%)</td>
<td>2 (40.0%)</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>Angiographic AAR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APPROACH (mean±SD)</td>
<td>31.68±10.15</td>
<td>25.18±9.98</td>
<td>0.30</td>
</tr>
<tr>
<td>BARI (mean±SD)</td>
<td>26.35±6.06</td>
<td>24.24±4.69</td>
<td>0.57</td>
</tr>
<tr>
<td><strong>Treatment before PCI</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Morphine</td>
<td>3 (75.0)</td>
<td>4 (80.0)</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>Treatment at time of PCI</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>4 (100)</td>
<td>5 (100)</td>
<td>0.99</td>
</tr>
<tr>
<td>Aspirin</td>
<td>4 (100)</td>
<td>5 (100)</td>
<td>0.99</td>
</tr>
<tr>
<td>Clopidogrel/Prasugrel</td>
<td>3/1</td>
<td>5/0</td>
<td>0.44</td>
</tr>
<tr>
<td>Glycoprotein IIb/Illa inhibitor</td>
<td>4 (100)</td>
<td>5 (100)</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>Infarct size (Median (IQR))</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatine Kinase</td>
<td>78398 (30945-104752)</td>
<td>21196 (13864-73887)</td>
<td>0.413</td>
</tr>
<tr>
<td>Troponin T</td>
<td>144798 (59452-207770)</td>
<td>83796 (55473-182372)</td>
<td>0.413</td>
</tr>
</tbody>
</table>

Values shown as number (%) unless otherwise stated

Abbreviations: PCI, percutaneous coronary intervention; TIMI, Thrombolysis in myocardial infarction; DES, drug-eluting stent.

a The body-mass index is the weight in kilograms divided by the square of the height in meters.
b Ischaemia time determined from symptom to balloon times for each patient.
Online Figure I. Associations between cardiac biomarker assessment of infarct size and infarct size on cardiac magnetic resonance imaging

There was a significant positive correlation between infarct size assessed by creatine kinase area under the curve (AUC) and LGE (late gadolinium enhancement) assessed infarct size on CMR (late gadolinium enhancement) ($r=0.770$), as shown in panel A. Panel B depicts a similar positive association between troponin T AUC and LGE CMR infarct size ($r=0.787$). Associations determined using Pearson’s correlation coefficient assessment.
Online Figure II. Associations between angiographic area at risk scores and area at risk assessed by cardiac magnetic resonance imaging (CMR)

There was a significant positive correlation between the angiographic area at risk as assessed by the modified APPROACH score and the area at risk assessed by T2 oedema imaging on CMR ($r=0.678$), as shown in panel A. Panel B depicts a similar positive association between the modified BARI score and the area at risk assessed by T2 oedema imaging on CMR ($r=0.541$). Associations determined using Pearson's correlation coefficient assessment.
Online Figure III. Intracoronary nitrite lowers ex vivo assessed platelet reactivity to collagen

Platelet reactivity measured at baseline, 30 minutes, 4 hours, 24 hours and 6 months after coronary reperfusion. Platelet P-Selectin expression assessed in whole blood in response to collagen (3 μmol/L) is shown for nitrite versus placebo for all patients in panel A. Panel B show using whole blood impedance aggregometry in response to the same collagen stimulus in all patients. Panel C shows P-selectin expression in response to collagen in patients with TIMI flow <1. Panel D shows aggregation in response to collagen in the TIMI <1 subgroup. All panels show nitrite treated versus placebo. Data expressed as mean ± SEM. =P<0.05, =P<0.01, for two-way repeated measures ANOVA.
Online Figure IV. Intracoronary nitrite lowers baseline ex vivo assessed platelet reactivity
Platelet reactivity measured at baseline, 30 minutes, 4 hours, 24 hours and 6 months after coronary reperfusion. Platelet P-Selectin expression assessed in whole blood following incubation with phosphate buffered saline control (PBS) is shown for nitrite versus placebo for all patients in panel A. Panel B show whole blood impedance aggregometry in response to the same PBS stimulus in all patients. Panel C shows P-selectin expression in response to PBS in patients with TIMI flow <1. Panel D shows aggregation in response to PBS in the TIMI <1 subgroup. All panels show nitrite treated versus placebo. Data expressed as mean ± SEM. =P<0.05, =P<0.01, for two-way repeated measures ANOVA. PBS=Phosphate buffered saline.