Impact of chronic congestive heart failure on pharmacokinetics and vasomotor effects of infused nitrite

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Running Title: The Vascular Effects of Intra-Arterial Nitrite in Heart Failure Patients.

Abbreviations:

Nitrite (NO_2^-)

Nitric Oxide (NO)

Sodium nitrite ($NaNO_2$)

Forearm blood flow (FBF)

Unstressed Venous Volume (UVV)

Forearm venous volume (FVV)

Protein-bound Nitric Oxide (RXNO)

Glyceryl Trinitrate (GTN)

Half Maximal Effective Concentration (EC_{50})

Ethylenediamine Tetraacetic Acid (EDTA)

Aldehyde Dehydrogenases (ALDH)

SUMMARY

Background and Purpose: Nitrite (NO_2^-) has recently been shown to represent a potential source of nitric oxide (NO), in particular under hypoxic conditions. The aim of the current study was to compare the hemodynamic effects of nitrite in healthy volunteers and patients with stable congestive heart failure (CHF).

Experimental Approach: The acute hemodynamic effects of brachial artery infusion of nitrite (0.31 to 7.8μmoles/min) was assessed in normal subjects (n=20) and CHF patients (n=21).

Key Results: NO_2^- infusion was well tolerated in all subjects. Forearm blood flow (FBF) increased markedly in CHF-patients at NO_2^- infusion rates which induced no changes in normal subjects (ANOVA: F=5.5; p=0.02). Unstressed venous volume (UVV) increased even with the lowest NO_2^- infusion rate in all subjects (indicating venodilation), with CHF patients being relatively hyporesponsive compared with normal subjects (ANOVA: F=6.2; p=0.01). There were no differences in venous blood pH or oxygen concentration between groups or during NO_2^- infusion. Venous plasma NO_2^- concentrations were lower in CHF-patients at baseline, and rose substantially less with NO_2^- infusion, without incremental oxidative generation of nitrate, consistent with accelerated clearance in these patients. Plasma protein-bound NO concentrations were lower in CHF-patients than normal subjects at baseline. This difference was attenuated during NO_2^- infusion. Prolonged nitrite exposure *in-vivo* did not induce oxidative stress, nor did it induce tolerance *in vitro.*

Conclusions and Implications: The findings of arterial hyper-responsiveness to infused NO_2^- in CHF-patients, with evidence of accelerated transvascular NO_2^- clearance

(presumably with concomitant NO release) suggests that NO_2^- effects may be accentuated in such patients. These findings provide a stimulus for the clinical exploration of NO_2^- as a therapeutic modality in CHF.

Keywords: Sodium Nitrite, vascular effects, heart failure

INTRODUCTION

Nitrite (NO_2^-), present in plasma at submicromolar concentrations, has been regarded in the past as a relatively inert product of nitric oxide (NO) catabolism and dietary nitrate/nitrite ingestion. Nitrite is also generated as a component of organic nitrate metabolism. It is now clear that NO_2^- can indeed exert vasodilator effects, possibly via reduction to NO (Cosby *et al*., 2003; Crawford *et al*., 2006).

Although the mechanism(s) of bioactivation remain incompletely understood, a number of reductases have been shown to "reactivate" NO from NO_2^-, independent of endothelial nitric oxide synthase (eNOS) activity (Baker *et al.,* 2007;Webb *et al.,* 2008). This process appears to be markedly potentiated in hypoxia, although once again it is not clear what mechanism(s) underlie this (Maher *et al.*, 2008). The implication is that exogenous administration of NO_2^- represents a means of selective release of NO (and hence vasodilatation) to hypoxic (and presumably ischaemic) tissues, with minimal risk of inducing the "steal" phenomenon and no dependence on intact endothelial function. Thus NO_2^- represents a potential treatment for conditions such as limb and myocardial ischaemia, and congestive heart failure.

A desirable characteristic of a vasodilator agent for potential use in the management of acutely decompensated heart failure is that it exerts marked venodilator, rather than arteriolar dilating effects. Salutary consequences of selective venodilatation include relief of congestive symptoms without significant risk of precipitating symptomatic hypotension. Specifically, venodilator agents ameliorate the phenomenon of diastolic ventricular interaction, thus increasing cardiac output and thereby organ perfusion (Atherton *et al,*. 1997;Dupuis *et al.*, 1990; Williams and Frenneaux, 2006).

In this regard, organic nitrates such as glyceryl trinitrate (GTN) are frequently utilized in the management of acute heart failure with pulmonary congestion. Although organic nitrate-based therapy exerts prominent venodilator effects (Manyari *et al.*, 1993; Muir and Nolan, 1991) and appears to have a number of advantages over a diuretic-based treatment regimen in such patients, there is no evidence that NO release from GTN is hypoxia-selective. Furthermore, therapy with GTN and other organic nitrates not only suffers from the problem of attenuation of effect during prolonged therapy (nitrate tolerance and pseudo-tolerance) (Daiber *et al*., 2005; Munzel *et al.*, 2005), but also exhibits the phenomenon of NO resistance (de novo hyporesponsiveness to all sources of NO) in the presence of CHF, despite infusion at very high rates (Anderson *et al*., 2004; Chirkov *et al.*, 1999; Chirkov *et al*., 2001). Moreover, the arteriolar vasodilation induced by organic nitrates can lead to deleterious reductions in systemic blood pressure, often accompanied by throbbing headache.

We have previously described the local forearm vascular responses to infused NO_2^- in normal subjects, documenting a marked venodilation and modest arteriolar vasodilation under normoxic conditions. However, during hypoxia, there was selective potentiation of arterial vasodilation (Maher et al., 2008).

In the current study, we have compared vasomotor responsiveness to NO_2^- in stable CHF patients and in normal subjects, relating response to concomitant plasma NO_2^- concentrations. We theorised that CHF patients might largely circumvent the problem of NO resistance at the arterial level by virtue of the potentiating effect of tissue hypoxia on NO_2^- bioactivation. As such, the primary null hypothesis was that the vasomotor effects of nitrite would not vary between patients with CHF and normal subjects. The secondary hypothesis was that the pharmacokinetics of nitrite would not differ significantly between the two groups. Additional

experiments were performed to determine whether prolonged nitrite exposure might result in incremental oxidative stress and/or induce the development of tolerance.

METHODS

Subject selection:

The study involved a comparison between patients with stable NYHA Class II-III CHF (n=21) and healthy volunteers (n=20). CHF patients were recruited from an 'advanced heart failure and cardiomyopathy' outpatient clinic. Among CHF patients, contraindications to study entry were long-acting nitrate therapy, symptomatic hypotension and clinically significant hepatic or renal dysfunction. None of the normal subjects had any known coronary risk factors, and none was taking cardioactive medications or vitamin supplements. The study was approved by the Local Research Ethics Committee and all patients gave written informed consent. The study conformed to the principles of the Declaration of Helsinki. Subjects had consumed a light breakfast and abstained from caffeine drink intake for at least 6 hours. Pre-study dietary nitrate/nitrite intake was not modified.

Experimental Protocol:

(a) Instrumentation:

Subjects rested supine in a dedicated vascular laboratory and brachial artery cannulation was performed as previously described (Maher *et al.*, 2008).

(b) Hemodynamic assessment:

The principal hemodynamic investigations performed were serial determination of unstressed forearm venous volume (UVV) as an index of venodilator response, and forearm blood flow

(FBF) as an index of arteriolar response to infused NO_2^-. Forearm venous volume (FVV) was assessed utilising radionuclide plethysmography as previously described (Schmitt *et al.*, 2002) and FBF was measured utilizing strain-gauge-plethysmography, as previously described (Gunaruwan *et al.*, 2002). Results were expressed relative to baseline values and those in the infused arm, corrected for changes in the non-infused arm.

(c) NO_2^- infusion:

Figure 1 is a schematic of the overall experimental design. After determination of baseline data, nitrite was infused into the non-dominant brachial artery. Infusion rates were 0.31µmoles/min for 30 minutes, thereafter increasing to 0.78µmoles/min, 3.1µmoles/min and 7.8 µmoles/min, each for further 30-min infusion periods. Changes in hemodynamic parameters were measured 5, 12 and 20 minutes after initiation of each infusion rate.

(d) Blood sampling/analysis:

Blood was withdrawn via venous cannulae in both arms at baseline and after the conclusion of each infusion. Blood gas analysis was performed for pH, oxygen and methaemoglobin concentrations. (Bayer Rapidlab 865). Blood for determination of venous plasma NO_2^-, nitrate and protein-bound NO (RXNO) concentrations was taken into EDTA tubes and immediately centrifuged (200rpm for 10 minutes at 4°C). Samples were stored at -80°C prior to assay. Plasma NO_2^-, nitrate and protein-bound NO content were determined via ozone-based chemiluminescence (Pinder *et al.*, 2009) or HPLC (Rassaf *et al.*, 2002) as previously described.

(e) Assessment of Nitrite Clearance in Human Plasma *in vitro*:

To ensure that nitrite clearance *in vitro* did not vary between normal subjects and patients, experiments were performed in which fresh venous blood (EDTA) from normal and CHF subjects was spiked with sodium nitrite *in vitro* to final concentrations of 2 and 20μM; after spiking samples were incubated under gentle agitation at 37ºC with aliquots being removed after 1, 2, 5, 10, 20 and 60 minutes prior to addition of N-ethylmaleimide (10 mM), centrifugation and assay.

(f) Reagents:

Sodium nitrite was purchased from Martindale Pharmaceuticals, UK. HPLC-grade nitrite-free water (Fisher Scientific) was utilised for extractions and dilutions.

(g) *In Vitro* Tolerance induction experiments:

In vitro studies were performed to address the possibility that prolonged infusion of nitrite might induce tolerance to itself and/or cross-tolerance to GTN. Segments of saphenous veins discarded after bypass grafting were collected from patients undergoing non-emergent coronary artery bypass grafting who had not received long-acting nitrates for at least 24 hours, placed in ice-cold Krebs solution, cleaned and cut into 2-3 mm segments. For vascular reactivity studies, venous segments were suspended under tension in 15-ml organ baths containing Krebs solution at 37˚C. Resting tension was set at 1gm, as previously described (Sage *et al*., 2000) and segments were equilibrated for 60 minutes before being constricted with 120 mmol/L KCl; vessel segments in which constriction was less than 1g were discarded. After a further 30 minutes of washout, the segments were pre-constricted with phenylephrine to produce 70% of maximal tension. Once contractile response had reached a

plateau, each segment was exposed to increasing concentrations of sodium nitrite ($NaNO_2$) ($4x10^{-9}$ to $1.2x10^{-2}$ M) and GTN ($4x10^{-9}$ to $1.2x10^{-5}$ M) in order to obtain cumulative vasodilator concentration-response curves. The order of administration of $NaNO_2$ and GTN was randomised. A washout period of 30 minutes was allowed between vasodilator response curves.

Tolerance induction experiments were performed via incubation of venous segments in 10^{-2} M $NaNO_2$ under resting tension for 45 minutes. After a further washout period of 30 minutes, $NaNO_2$ and GTN concentration-response curves were repeated, again in random order. In each experiment, control vessels were utilised in order to exclude spontaneous changes in responsiveness to either vasodilator.

Vascular relaxation responses to $NaNO_2$ and GTN were compared before and after prolonged $NaNO_2$ exposure, via curve-fitting of individual concentration response data to obtain EC_{50} values for each curve. In the case of $NaNO_2$ administration, which yielded bi-sigmoidal concentration-response curves, EC_{50} values were calculated from the low-affinity, high-capacity component of the response. As EC_{50} data were normally distributed, these were compared via paired t tests.

(h) Assessment of nitrite infusion upon levels of oxidative stress:

Heart failure patients (n=15) were exposed to saline infusion (20min), followed by two incremental doses of nitrite (7.84 nM and 7.84 μM; 20 min intravenous infusion for each dose) under normoxic conditions. The patients were then exposed to 12% hypoxia for 20 min and infused with 7.84 μM nitrite. At the end of each infusion, blood samples were taken for plasma 8-isoprostane analysis.

(i) Assessment of oxidative stress:

Total plasma 8-iso Prostaglandin F2α (8-isoprostane) was measured using a commercial 8-Isoprostane EIA assay (Cayman Chemical). Briefly, plasma samples were collected in vacutainers containing EDTA that was supplemented with 0.005% BHT to prevent spontaneous oxidative formation of 8-isoprostane. Total 8-isoprostane was determined by first hydrolyzing the samples, followed by affinity sorbent/column purification step. Total 8-isoprostane content was then measured according to the assay kit protocol. The assay of both free and bound isoprostanes was used as a substantial proportion of 8-isoprostanes, which are esterified in lipids, would not be detected by measurement of free isoprostane alone.

(j) Analysis of results:

The current studies had >80% power to detect 20% differences in both FBF and UVV responses between groups at $P<0.05$ level.

Clinical characteristics of normal subjects and CHF patients were compared utilising non-paired t-tests (2-sided) for normally distributed parameters, and Wilcoxon tests for skewed data. Categorical data were compared using a Fishers exact test. Serial changes in FBF and UVV, pH and venous O_2 saturation, and concentrations of NO_2^- and protein-bound NO in both the infused and non-infused forearms were compared in normal and CHF subjects by two-way ANOVA with repeated measures, utilizing Dunnett's t-test to assess for significance of changes at individual time points. Logit transformation of data was utilized to detect possible disparity of concentration-response relationships between groups of subjects. All results are expressed as mean +/- SEM unless otherwise stated. p-value of <0.05 was taken as statistically significant.

RESULTS

Subject/Patient Characteristics

The characteristics of the patients and healthy controls are detailed in Table 1 and the drug therapy in patients in Table 2. It should be noted that although no patient had calculated creatinine clearance values less than 30 mls/minute, mean plasma creatinine levels were marginally abnormal in the CHF group, typical of populations with heart failure.

Hemodynamic effects of nitrite infusion

Sodium nitrite infusion was well-tolerated. Mean arterial blood pressure and heart rate did not vary significantly during the experiment: mean arterial pressure values for normal subjects were 88 ± 4 mmHg and 88 ± 15 at baseline and peak nitrite infusion, respectively. For CHF patients the mean arterial pressure values were 73 ± 4 and 70 ± 8 mmHg at baseline and peak nitrite infusion, respectively, while the corresponding values for heart rate were 62 ± 5 and 65 ± 4 beats/minute in normal subjects and 66 ± 4 and 69 ± 4 beats/minute in CHF patients. Maximal venous methemoglobin concentrations in the infused arm were less than 2% of total haemoglobin at all nitrite infusion rates.

FBF changes are shown in Figure 2. In the non-infused forearm, both in normal subjects and CHF patients, there was significant vasoconstriction during the course of the study (ANOVA: $F=2.6$; $p=0.04$) which was attenuated at the highest nitrite infusion rate, raising the possibility of the onset of NO_2^- induced vasodilatation due to recirculation (Inset, Figure 2) . In the infused arm (main graph, Figure 2), the relationship between infusion rate and effect varied markedly between groups. For normal subjects, there was a progressive increase in FBF with infusion rates of 3.14 µmoles/min and higher. In CHF patients, vasodilator responses were

induced already at the lowest infusion rates of nitrite, but with similar responses at doses >3.14 μmoles/min. Logit transformation of concentration-response data confirmed that the gradients of the linearised relationships were significantly steeper (p=0.017) in normal subjects than in CHF patients; partitioned ANOVA methodology was therefore utilized for analysis of these data. At lower, but not higher, infusion rates there were considerably greater FBF responses (F=5.5; p=0.02) than those in normal subjects, in whom the threshold for increases in FBF was NO_2^- infusion rate of 3.1μmoles/min.

Unstressed venous volume (UVV) changes are summarised in Figure 3. In the non-infused arm (inset), both in normal subjects and patients, there was venoconstriction (F=9.6; p<0.0001) which was more marked in normal subjects (F= 15.4;p<0.001)). In the infused arm (main graph, Figure 3), both groups exhibited evidence of increases in UVV commencing with the lowest infusion rate of sodium nitrite infusion, with a monophasic and progressive dose-related increase in UVV. However, overall responses in CHF patients were substantially lower than those in normal subjects (F=6.3; p=0.01).

Changes in venous pH and O_2 saturations

pH in venous blood did not vary significantly between normal subjects and CHF patients, nor did it fluctuate significantly (Figure 4) during nitrite infusion in either arm. Venous oxygen saturation also did not vary significantly between patient groups (p= 0.27 and p=0.35 for changes in the CHF group versus healthy controls in the non infused and infused arms, respectively). With nitrite infusion there was a trend to lower venous oxygen saturation with time in the non infused arm (p=0.07) which may be explained by prolonged immobility and consecutive decreases in blood flow with time. In comparison in the infused arm there was no significant change in venous oxygen saturation with time (p= 0.53). At peak dose venous

oxygen saturations were 79.5% ± 7.2 and 83.4 ± 1.8 in the CHF and healthy volunteers, respectively.

Plasma NO_2^- and nitrate concentrations

Changes in NO_2^- concentrations in venous blood during nitrite infusion are summarised in Figure 5A and 5B. Resting plasma NO_2^- concentrations did not differ statistically between groups (0.60 ±.0.07 vs 0.41 ± 0.08 μM in control vs CHF patients: p=0.08). NO_2^- concentrations did not change much in venous blood in the non-infused arm in both normal subjects and CHF patients (Figure 5A), but revealed a trend towards greater levels (F=4.3;p=0.04) in the normal subject group.

In the infused arm (Figure 5B), NO_2^- concentrations increased approximately 70-fold during nitrite infusion (F=32.7;p<0.0001). In normal subjects, venous nitrite concentrations increased from 1.2 ± 0.10 to 54.9 ± 16.5 μM, while in CHF patients the increase was from 0.39 ± 0.07 to 29.0 ± 9.3 μM. Thus, the increase in venous NO_2^- levels was greater in normal subjects than in CHF patients (F=10.6;p=0.002). The increase in venous NO_2^- concentrations per 10-fold increase in nitrite infusion rate was disproportionately small: approximately 7-fold for normal subjects and 3-4 fold for CHF patients.

Consistent with previous observations (Usui *et al.*, 1998) basal nitrate levels in the venous effluent were found to be higher in the CHF patients compared to healthy volunteers (Figure 6B; p=0.0034). Venous nitrate levels rose similarly (p= 0.96 and p=0.67 for the non infused and infused arms respectively) in the two groups of subjects (Figure 6A and 6B) suggesting that the degree of oxidation of nitrite to nitrate did not differ between groups.

Protein-bound NO concentrations

In the non-infused arm (Figure 7A), protein-bound NO concentrations were significantly greater in normal subjects than in patients with CHF (F=8.6; p=0.04). In the infused arm, this difference was attenuated during nitrite infusion, becoming non-significant. Furthermore, protein-bound NO tended to increase with the highest nitrite infusion rate.

Assessment of *in vitro* Nitrite Clearance in Human Plasma

Following spiking of whole blood with nitrite *in vitro* plasma nitrite decayed similarly (p= 0.66) in the two groups, with an apparent half-life of 5-6 min, irrespective of the initial concentration of nitrite achieved, as summarised in Figure 8. Although basal levels of nitrate were higher in heart failure patients, the accompanying rate of nitrate formation was similar (p=0.99) in the two groups (not shown). Fitting attempts revealed that the reaction obeyed neither first nor second order kinetics suggesting the involvement of multiple processes including uptake, oxidation, reduction and redistribution between plasma and erythrocytes.

***In vitro* tolerance/cross-tolerance study:**

In pre-constricted saphenous vein rings *in vitro*, nitrite induced vasorelaxation with a bi-sigmoidal concentration-response characteristic (Figure 9). After exposure to very high concentrations of nitrite (10^{-2}M) for 45 minutes followed by 30 minutes washout, there was no significant shift in the nitrite concentration-response curve (log EC_{50} for the low-affinity components -3.7±0.10 vs -4.0±0.08 M (n=7; p=NS, for before and after attempted tolerance induction, respectively). Furthermore, there was no cross-tolerance to GTN (log EC_{50} -7.9±0.09 vs -7.9±0.07 M; n=7, p=NS for before and after nitrite tolerance induction, respectively).

Assessment of nitrite infusion on oxidative stress

Plasma total isoprostane levels (Figure 10) did not increase significantly with nitrite infusion in both normoxia and hypoxia (8.99±1.71, 7.07±0.93, 11.11±2.11 and 9.09±1.48 at baseline, "low-dose nitrite", "higher-dose nitrite" and during hypoxia respectively. (P=0.44)).

DISCUSSION

Previous studies, have suggested that infused nitrite exerts vasomotor responses that are similar to those of the organic nitrates, with marked venodilatation, and moderate dose-dependent arteriolar dilatation, but in the case of nitrite these effects are augmented by hypoxia or exercise. (Cosby *et al.*, 2003; Maher *et al.*, 2008). On the other hand, infused NO_2^- represents in many ways a particularly attractive agent for treatment of CHF complicated by fluid overload. Not only is it a potent venodilator, but its effects appear to be (somewhat surprisingly) devoid of tolerance development (Dejam *et al*., 2007). Furthermore, NO_2^- provides a "needs-based" vasodilator effect, with effects accentuated by hypoxia in many systems (Ingram *et al*., 2010; Milsom *et al*., 2010; Shiva *et al*., 2007). The precise mechanism(s) underlying this accentuated effect remain incompletely understood.

In the present study, effects of nitrite infusion were compared in normal subjects and patients with stable CHF. The previously documented (Maher *et al*., 2008) selective venodilator effects of NO_2^- at low infusion rates was again documented in normal subjects: the lowest two infusion rates of nitrite induced significant increases in UVV, without increasing FBF. On the other hand, in CHF patients marked and selective hyper-responsiveness of forearm resistance vessels to lower rates of NO_2^- infusion was observed, with substantially reduced venodilator responses in CHF patients. The latter finding may represent NO resistance at the level of the capacitance bed. Alternatively it may be a consequence of increased clearance of NO_2^- across the vascular bed, resulting in lower concentrations in the capacitance vasculature at any given infusion rate (see below). Thus the main implication of the current findings is that in the presence of CHF the spectrum of vasomotor responses to nitrite is altered, with enhancement of arteriolar dilatation, but attenuation of venodilator responses.

The finding that CHF-patients had lower protein-bound NO and marginally lower nitrite concentrations than normal subjects is consistent with previous findings in subjects with endothelial dysfunction (Heiss *et al*., 2006; Kleinbongard *et al*., 2003; Lauer *et al*., 2001). Steady-state NO_2^- concentrations (in the absence of dietary or intravenous NO_2^- supplementation) may be regarded as being indicative of NO generation and therefore indicative of diminished eNOS activity in CHF (Lauer *et al*., 2001), whereas protein-bound NO concentrations reflect the generation of S-nitrosoproteins from either NO or nitrite (Bryan *et al*., 2005), as well as a measure of prevalent redox stress (Ng *et al*., 2004). With sodium nitrite infusion, venous NO_2^- concentrations increased, but not in proportion to the increases in infusion rates, suggesting some selective loss of NO_2^- at higher infusion rates. These changes were noted both in the infused and non-infused arms, indicating that the non-infused arm cannot be regarded as a simple "control" site: indeed the FBF changes (Figure 2, Inset) suggest the onset of some dilator effect with the highest nitrite infusion rate. Furthermore, NO_2^- levels increased far less markedly with increasing sodium nitrite infusion rates in CHF patients compared to normal subjects (Figure 5B) indicating greater rates of NO_2^- clearance in these patients. Although NO_2^- may be reduced to NO (bio-activation), a major clearance mechanism for NO_2^- is oxidation to nitrate. Therefore, venous nitrate concentrations were measured in both infused and non-infused arms, revealing that, in the presence of predominant oxidation of nitrite to nitrate, there was nevertheless a similar increase in plasma nitrate levels in the two groups upon infusion of nitrite, making enhanced clearance through oxidation to nitrate less likely to account for these differences. Similarly, our *in vitro* results suggest that there is no systematic difference in the rate of nitrite disappearance in the two groups, thus excluding sampling artefacts as a possible explanation for the differences in nitrite concentrations between normal subjects and CHF-patients.

As regards the additional potential formation of S-nitrosoproteins via NO_2^- metabolism, venous protein-bound NO concentrations tended to increase in the infused arm in the CHF patients (coupled with a relative decrease in protein-bound NO concentrations in the controls), attenuating the difference between baseline levels in the two groups. These findings are also consistent with selective bio-activation of NO_2^- in the CHF patients, and might have been accounted for by the presence of tissue hypoxia and/or acidosis in these patients, given the previously described incremental bio-activation of NO_2^- in the presence of hypoxia (Maher *et al.,* 2008). However, neither venous blood pH nor oxygen concentrations differed significantly between groups. There was a trivially (non- significantly) lower venous oxygen saturation in the infused arm of the heart failure patients, but this seems unlikely to provide an explanation for the difference in clearance. It therefore appears either that a component of subcellular/microvascular hypoxia was present in the CHF patients without detection in venous samples (a possible but somewhat unlikely event) or that these results indicate that, apart from hypoxia, NO_2^- bioactivation to NO can be induced by factors other than hypoxia, such as changes in redox state. This issue is worthy of further investigation.

Taken together, these results show that CHF patients are hyper-responsive to the arteriolar dilating effects of infused sodium nitrite, presumably due to increased release of NO (despite the absence of arterial hypoxaemia). This finding may imply the need for some caution in the use of infused nitrite preparations in patients with decompensated CHF, for fear of precipitating falls in systolic blood pressure. On the other hand, symptomatic hypotension was not observed in the currently studied patient cohort. However, CHF patients were hyporesponsive both to the effects of infused sodium nitrite on venous capacitance and, to a lesser extent, to the effects on FBF at high infusion rates. These findings may imply the existence of NO resistance, as previously documented in both arteries and platelets of such

patients (Anderson *et al*.,2004). Although platelet NO resistance exhibits partial responsiveness to ACE inhibitor therapy (Chirkov *et al.*, 2004), which was utilized in the majority of the CHF group, its interaction with vascular NO resistance is less certain. Alternatively, the apparent resistance of the capacitance vessels may in whole or in part be due to a lower concentration of nitrite in venous blood due to the increased transvascular clearance. This would potentially result in higher doses being required when treating heart failure patients acutely. As net responsiveness to NO_2^- reflects both accelerated bioactivation and diminished tissue responses to NO, it is likely that individual responses vary markedly, according to each of these component factors.

Nitrate therapy is associated with the development of tolerance and increased oxidative stress. It is now accepted that a central component of nitrate tolerance induction is progressive failure of enzymatic bioactivation (Sage *et al.*, 2000), and although the nature of the relevant nitrate reductase remains controversial (D'Souza *et al*., 2011), it is clear that GTN inhibits aldehyde dehydrogenases (ALDH) (Chen *et al.*, 2002 ; D'Souza *et al.,* 2011; Towell., *et al.* 1986). In particular, the possibility that inhibition of the predominantly mitochondrial form of ALDH may contribute to nitrate tolerance development (and possibly also to accentuation of redox stress) has been the subject of considerable recent investigation (Chen *et al*., 2002; Mackenzie *et al,*. 2005). Given that the bioactivation of nitrite is catalysed by a large number of nitrite reductases, which may include ALDH (Feelisch *et al.*, 2008), there is a theoretical potential both for the induction of tolerance after prolonged administration of nitrite and for the associated induction of redox stress. We therefore evaluated the potential for nitrite tolerance induction utilising cross-tolerance to GTN, a relevant issue given that this may theoretically develop after prolonged exposure to the agent (Henry *et al,*. 1990). Under the conditions of this experiment, nitrite did not induce tolerance

to itself (consistent with the findings of Dejam *et al*., 2007) or to GTN. Furthermore, prolonged systemic administration of nitrite in patients with CHF was not associated with aggravation of redox stress, as measured by plasma concentrations of total isoprostanes. On the other hand, it is possible that oxidative modification of proteins may have occurred.

A number of potential limitations and caveats apply to the current findings. Most importantly, the biochemical basis of the observed arterial hyper-responsiveness to NO_2^- was not determined, and indeed the possible relevance of tissue hypoxia to this phenomenon cannot be completely excluded with the methodology utilised in this study. Furthermore, changes in tissue redox stress were not documented: this remains a major priority for future investigations. While the observation of attenuation of the increase in plasma NO_2^- in association with increasing rates of sodium nitrite infusion implies accelerated transvascular clearance of NO_2^- in such patients, the release of NO was not measured and nitrate generation data were incomplete for normal subjects. Therefore, it cannot be absolutely certain that there was a close relationship between such accelerated clearance and incremental rates of NO generation. Indeed, there is recent evidence that nitrite may exhibit vasoactivity independent of bioconversion and that nitrite may exert a component of its effects independent of the formation of NO or NO adduct (Bryan *et al*., 2005; Shiva and Gladwin, 2009). Quantitation of heme-nitrosyl adducts in red blood cells and/or identification of specific NO adducts in plasma might have provided an additional, more direct index of NO formation, However, as observed by Dejam *et al* (2007), increases in concentrations of such adducts are likely to parallel those of plasma nitrite concentrations. Furthermore, the precise proportion of nitrite clearance via oxidation to nitrate cannot be assessed completely from the current results, although it was possible to exclude the possibility that transvascular nitrate generation occurred selectively in CHF patients given the similar rise in nitrate levels.

Oxidative stress is associated with many chronic diseases and oxidative damage markers can be measured in the plasma. However, plasma measurement of oxidative stress remains a challenging area of research because of the highly reactive nature of these molecules. Several studies have focused on measuring either the total antioxidant capacity of plasma or specific measures of free radical-mediated damage such as F2-isoprostane or oxidised-LDL (Ox-LDL). While we acknowledge that other oxidative stress markers could have been assessed in the current study, we opted to quantify total 8-isoprostane (8-iso Prostaglandin F2α). Isoprostanes are among the most reliable markers of oxidative stress that can be assessed in translational studies by specific immunoassay-based techniques. Moreover, these products are not only byproducts of oxidative stress, but also effector molecules involved in pathophysiology. 8-isoprostane is a potent pulmonary and renal vasoconstrictor and has been implicated as a causative mediator of hepato-renal syndrome and pulmonary oxygen toxicity. Finally, the findings of the current investigation, while relevant to the potential therapeutic administration of nitrite, may not be indicative of physiological modulation of NO_2^- at far lower concentrations.

While NO_2^- infusion has the potential to lead to the generation of methemoglobin, (a possible concern) we did not observe a significant increase in venous methemoglobin levels in this study.

We noted a significant vasoconstriction in the non-infused arm during the study. In theory this could have been secondary to hypotension, however no significant drop in blood pressure was observed; in fact, there appeared to be a trend towards an increase. It has been our experience in prolonged intrabrachial infusion studies with other agents that forearm blood flow falls in the contralateral arm over time despite no significant changes in blood pressure. We suspect this result from the effects of slight discomfort and often a full bladder during

these long studies. It is because of such changes in flow in the non infused arm during prolonged studies that bilateral plethysmography with correction for the non infused arm is recommended for intrabrachial infusion studies (Benjamin *et al*., 1995).

With regards to the medication received by the CHF patients, while there are data to suggest that angiotensin converting enzyme inhibitors and angiotensin receptor blockers improve NO production/ endothelial function we could not find any evidence to suggest any impact upon nitrite conversion. The possibility that the medication taken by the CHF patients may account for some of the differences observed remains a limitation of this study.

In the current study, nitrite was infused intra-arterially in order to document peripheral vasomotor responsiveness with minimal changes in systemic blood pressure. This circumstance differs both from the potential mode of treatment to increase nitrite effect in chronic heart failure (for example via administration of dietary sources of nitrate) and from the intravenous infusion of nitrite as a component of management of acute heart failure. Nevertheless, the findings of the current investigation extend the question of the potential clinical utility of NO_2^- as a component of the management of patients with both acute and chronic heart failure. The major residual issue to be explored is the extent of change in vascular responsiveness to be seen in such patients, especially in decompensated CHF, where significant tissue hypoxia is more likely to be present, with resultant potentiation of NO_2^--bio-activation, perhaps counter-balanced in part by the phenomenon of tissue resistance to NO (Chirkov and Horowitz 2007). Despite the occurrence of NO resistance, treatment with organic nitrates is at least as effective as diuretic/morphine-based acute pharmacotherapy for decompensated heart failure with acute pulmonary oedema (Cotter *et al*., 1998; Sharon *et al*., 2000). However, nitrate therapy is often associated with the unpleasant side effect of headache. In this study none of the subjects who received intravenous nitrite suffered from

this symptom, potentially providing a distinct advantage over traditional nitrate therapies. A comparison with NO_2^- based therapy now seems indicated.

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Disclosures/ Conflicts of Interest

Professor Frenneaux has received a research grant from Medtronic. He has an ownership interest in a "method of use" patent held for Perhexiline in Chronic Heart Failure. He has also served on the advisory board or as a consultant to Medtronic and Biotronik.

References

Anderson RA, Ellis GR, Chirkov YY, Holmes AS, Payne N, Blackman DJ *et al* (2004). Determinants of platelet responsiveness to nitric oxide in patients with chronic heart failure. Eur.J.Heart Fail., 6, (1) 47-54.

Atherton J.J, Moore TD, Lele SS., Thomson, H.L., Galbraith, A.J., Belenkie, I, *et al*. (1997). Diastolic ventricular interaction in chronic heart failure. Lancet, 349, (9067) 1720-1724.

Baker, J.E., Su, J., Fu, X., Hsu, A., Gross, G.J., Tweddell, J.S, *et al*. (2007). Nitrite confers protection against myocardial infarction: role of xanthine oxidoreductase, NADPH oxidase and K(ATP) channels. J.Mol.Cell Cardiol., 43, (4) 437-444.

Benjamin N, Calver A, Collier J, Robinson B, Vallance P, & Webb, D. (1995). Measuring forearm blood flow and interpreting the responses to drugs and mediators. Hypertension, 25, (5) 918-923.

Bryan NS, Fernandez BO, Bauer SM, Garcia-Saura MF, Milsom AB, Rassaf T, *et al*. (2005). Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. Nat Chem Biol., 1, (5), 290-7.

Chen Z, Zhang J, & Stamler JS. (2002). Identification of the enzymatic mechanism of nitroglycerin bioactivation. Proc.Natl.Acad.Sci.U.S.A, 99, (12) 8306-8311.

Chirkov YY, Holmes AS, Chirkova LP, & Horowitz JD. (1999). Nitrate resistance in platelets from patients with stable angina pectoris. Circulation, 100, (2) 129-134.

Chirkov YY, Holmes AS, Martelli JD, & Horowitz JD. (2004). Effect of perindopril on platelet nitric oxide resistance in patients with chronic heart failure secondary to ischemic left ventricular dysfunction. Am.J.Cardiol., 93, (11) 1438-40, A10.

Chirkov YY, Holmes AS, Willoughby SR, Stewart S, Wuttke RD, Sage PR *et al* (2001). Stable angina and acute coronary syndromes are associated with nitric oxide resistance in platelets. J.Am.Coll.Cardiol., 37, (7) 1851-1857.

Chirkov YY & Horowitz JD. (2007_. Impaired tissue responsiveness to organic nitrates and nitric oxide: a new therapeutic frontier? Pharmacol.Ther., 116, (2) 287-305.

Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, *et al.* (2003). Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. Nat.Med., 9, (12) 1498-1505.

Cotter G, Metzkor E, Kaluski E, Faigenberg Z, Miller R, Simovitz A, *et al*. (1998). Randomised trial of high-dose isosorbide dinitrate plus low-dose furosemide versus high-dose furosemide plus low-dose isosorbide dinitrate in severe pulmonary oedema. Lancet, 351, (9100) 389-393.

Crawford JH, Isbell TS, Huang Z, Shiva S, Chacko BK, Schechter AN, *et al*. (2006). Hypoxia, red blood cells, and nitrite regulate NO-dependent hypoxic vasodilation. Blood, 107, (2) 566-574.

D'Souza Y, Dowlatshahi S, & Bennett BM. (2011). Changes in aldehyde dehydrogenase 2 expression in rat blood vessels during glyceryl trinitrate tolerance development and reversal. Br.J.Pharmacol., 164, (2b) 632-643.

Daiber A, Mulsch A, Hink U, Mollnau H, Warnholtz A Oelze M *et al*. (2005). The oxidative stress concept of nitrate tolerance and the antioxidant properties of hydralazine. Am.J.Cardiol., 96, (7B) 25i-36i.

Dejam A, Hunter CJ, Tremonti C, Pluta RM, Hon YY, Grimes G, *et al*. (2007). Nitrite infusion in humans and nonhuman primates: endocrine effects, pharmacokinetics, and tolerance formation. Circulation, 116, (16) 1821-1831.

Dupuis J, Lalonde G, Lebeau R, Bichet D, & Rouleau JL. (1990). Sustained beneficial effect of a seventy-two hour intravenous infusion of nitroglycerin in patients with severe chronic congestive heart failure. Am.Heart J., 120, (3) 625-637.

Feelisch M, Fernandez BO, Bryan NS, Garcia-Saura MF, Bauer S, Whitlock DR, *et al.* (2008). Tissue processing of nitrite in hypoxia: an intricate interplay of nitric oxide-generating and -scavenging systems. J.Biol.Chem., 283, (49) 33927-33934.

Gunaruwan P, Schmitt M, Taylor J, Lee L, Struthers A, & Frenneaux MP. (2002). Lack of rapid aldosterone effects on forearm resistance vasculature in health. J.Renin.Angiotensin.Aldosterone.Syst., 3, (2) 123-125.

Heiss C, Lauer T, Dejam A, Kleinbongard P, Hamada S, Rassaf T, *et al*. (2006). Plasma nitroso compounds are decreased in patients with endothelial dysfunction. J.Am.Coll.Cardiol., 47, (3) 573-579.

Henry PJ, Horowitz JD, & Louis WJ. (1990). Nitrate tolerance induced by nicorandil or nitroglycerin is associated with minimal loss of nicorandil vasodilator activity. J.Cardiovasc.Pharmacol., 15, (3) 365-370.

Ingram TE, Pinder AG, Bailey DM, Fraser AG, & James PE. (2010). Low-dose sodium nitrite vasodilates hypoxic human pulmonary vasculature by a means that is not dependent on a simultaneous elevation in plasma nitrite. Am.J.Physiol Heart Circ.Physiol, 298, (2) H331-H339.

Kleinbongard P, Dejam A, Lauer T, Rassaf T, Schindler A, Picker O, *et al*. (2003). Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. Free Radic.Biol.Med., 35, (7) 790-796.

Lauer T, Preik M, Rassaf T, Strauer BE, Deussen A, Feelisch M,*et al*. (2001). Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. Proc.Natl.Acad.Sci.U.S.A, 98, (22) 12814-12819.

Mackenzie IS, Maki-Petaja KM, McEniery CM, Bao YP, Wallace SM, Cheriyan J, *et al*. (2005). Aldehyde dehydrogenase 2 plays a role in the bioactivation of nitroglycerin in humans. Arterioscler.Thromb.Vasc.Biol., 25, (9) 1891-1895.

Maher AR, Milsom AB, Gunaruwan P, Abozguia K, Ahmed I, Weaver RA, *et al*. (2008). Hypoxic modulation of exogenous nitrite-induced vasodilation in humans. Circulation, 117, (5) 670-677.

Manyari DE, Wang Z, Cohen J, & Tyberg JV. (1993). Assessment of the human splanchnic venous volume-pressure relation using radionuclide plethysmography. Effect of nitroglycerin. Circulation, 87, (4) 1142-1151.

Milsom AB, Patel NS, Mazzon E, Tripatara P, Storey A, Mota-Filipe H, *et al.* (2010). Role for endothelial nitric oxide synthase in nitrite-induced protection against renal ischemia-reperfusion injury in mice. Nitric.Oxide., 22, (2) 141-148.

Muir AL & Nolan J (1991). Modulation of venous tone in heart failure. Am.Heart J., 121, (6 Pt 2) 1948-1950.

Munzel, T, Daiber A, & Mulsch A (2005). Explaining the phenomenon of nitrate tolerance. Circ.Res., 97, (7) 618-628.

Ng ES, Jourd'heuil D, McCord JM, Hernandez D, Yasui M, Knight D, *et al.* (2004). Enhanced S-nitroso-albumin formation from inhaled NO during ischemia/reperfusion. Circ.Res., 94, (4) 559-565.

Pinder AG, Rogers SC, Khalatbari A, Ingram TE, & James PE. (2009). The measurement of nitric oxide and its metabolites in biological samples by ozone-based chemiluminescence. Methods Mol.Biol., 476, 10-27.

Rassaf T, Bryan, NS, Kelm M, & Feelisch M. (2002). Concomitant presence of N-nitroso and S-nitroso proteins in human plasma. Free Radic.Biol.Med., 33, (11) 1590-1596.

Sage PR, de la Lande IS, Stafford I, Bennett CL, Phillipov G, Stubberfield J *et al*. (2000). Nitroglycerin tolerance in human vessels: evidence for impaired nitroglycerin bioconversion. Circulation, 102, (23) 2810-2815.

Schmitt M, Blackman DJ, Middleton GW, Cockcroft JR, & Frenneaux MP. (2002). Assessment of venous capacitance. Radionuclide plethysmography: methodology and research applications. Br.J.Clin.Pharmacol., 54, (6) 565-576.

Sharon A, Shpirer I, Kaluski E, Moshkovitz Y, Milovanov O, Polak R *et al*. (2000). High-dose intravenous isosorbide-dinitrate is safer and better than Bi-PAP ventilation combined with conventional treatment for severe pulmonary edema. J.Am.Coll.Cardiol., 36, (3) 832-837.

Shiva S & Gladwin MT (2009. Nitrite mediates cytoprotection after ischemia/reperfusion by modulating mitochondrial function. Basic Res.Cardiol., 104, (2) 113-119.

Shiva S, Huang Z, Grubina R, Sun J, Ringwood LA, MacArthur PH *et al*. (2007). Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration. Circ.Res., 100, (5) 654-661.

Towell JF, Barboriak JJ, Townsend WF, Kalbfleisch JH, & Wang RI. (1986). Erythrocyte aldehyde dehydrogenase: assay of a potential biochemical marker of alcohol abuse. Clin.Chem., 32, (5) 734-738.

Usui M, Matsuoka H, Miyazaki H, Ueda S, Okuda S, & Imaizumi T (1998). Increased endogenous nitric oxide synthase inhibitor in patients with congestive heart failure. Life Sci., 62, (26) 2425-2430.

Webb AJ, Milsom AB, Rathod KS, Chu WL, Qureshi S, Lovell MJ, *et al* (2008). Mechanisms underlying erythrocyte and endothelial nitrite reduction to nitric oxide in hypoxia: role for xanthine oxidoreductase and endothelial nitric oxide synthase. Circ.Res., 103, (9) 957-964.

Williams L & Frenneaux MP. (2006). Diastolic ventricular interaction: from physiology to clinical practice. Nat.Clin.Pract.Cardiovasc.Med., 3, (7) 368-376.

Figure Legends

Figure 1: Schematic diagram of study protocol.

Figure 2: Changes in Forearm Blood Flow (FBF) in the non-infused arm (Inset) and Infused arm in normal subjects (open symbols) and CHF patients (closed symbols). In the non-infused arm, FBF decreased significantly (F=2.6;p=0.04); in the infused arm there was a selective increase in FBF associated with lower NO_2^- infusion rates (F=5.5;p=0.02). * = p < 0.05 vs normal subjects

Figure 3: Changes in unstressed venous volume (UVV) in the non-infused arm (inset) and infused arm in normal subjects (open symbols) and CHF patients (closed symbols). In the non-infused arm, there was a decrease in UVV which was more marked in normal subjects (F=15.4;p<0.001). In the infused arm the increase in UVV with NO_2^- infusion was attenuated in CHF patients (F=6.2;p=0.01)..

*= p < 0.05 vs normal subjects

Figure 4: pH fluctuations in venous blood from (A) non-infused arm and (B) infused arm in normal subjects (open symbols) and CHF patients (closed symbols). pH did not vary significantly either with NO_2^- infusion rate or between groups.

Figure 5: Venous plasma nitrite concentration (μM) in (A) non-infused arm and (B) infused arm in normal subjects (open symbols) and CHF patients (closed symbols). Baseline nitrite concentrations were marginally lower in CHF patients than in normal subjects (p=0.08). Nitrite concentrations rose significantly (F=32.7;p<0.0001) with NO_2^- infusion in the infused arm: this increase was attenuated significantly (F=10.6;p=0.002) in CHF patients. * = p <0.05 vs normal subjects

Figure 6: Venous plasma nitrate concentrations in (A) non infused arm and (B) infused arm in normal subjects (open symbols) and CHF patients (closed symbols). Baseline nitrate concentrations were greater (p=0.003) in CHF subjects. During nitrite infusion, nitrate concentrations increased (F=16.2 P<0.0001)) in the infused arm, but without significant difference between normal subjects and CHF patients.

Figure 7: Venous plasma protein-bound NO (RXNO:nmol/L)in (A) non-infused arm and (B) infused arm in normal subjects (open symbols)and CHF patients (closed symbols). RXNO concentrations did not vary significantly with NO2- infusion in either arm (F= 0.44 p= 0.99), but were significantly lower in CHF patients than in normal subjects in the non-infused arm (p=0.04), while this difference was attenuated in the infused arm.

Figure 8: Clearance of nitrite *in vitro* from human plasma. Closed symbols represent data from normal subjects; open symbols represent data from CHF patients.

Figure 9: *In vitro* tolerance/cross-tolerance induction study. Concentration-response curves are shown for nitrite and GTN, indicating percentage relaxation of saphenous vein rings, before and after prolonged incubation with nitrite.

Figure 10: Plasma 8-isoprostane concentrations following nitrite infusion in heart failure patients. Values did not vary significantly between infused (A) and non-infused (B) arms.

Table 1: Demographic data for both groups of subjects

Table 2: Summary of the medication taken by the CHF patients.

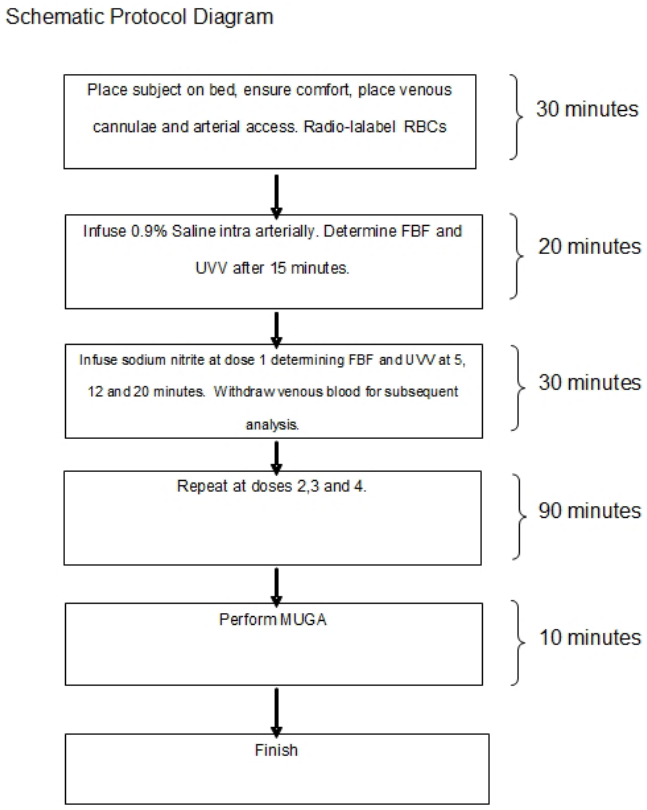
Table 1

Demographic and Clinical Features	CHF Patients (mean±SEM)	Healthy Controls (mean±SEM)	P Value
Age (y)	62.9±2.7	57.6±1.2	0.08
Sex, M/F (%)	18/3 (86/14)	15/5 (75/25)	0.70
Body mass index (kg/m^2)	30.0±1.2	26.8±0.6	0.05
Serum cholesterol (mmol/L)	5.3±0.4	5.4±0.2	0.69
Plasma glucose (mmol/L)	5.8±0.4	4.7±0.1	0.05
Serum Creatinine (µmol/l)	125±6.7	103.4±5.2	0.03
Heart rate	74±2	61.5±1.9	0.01
Blood pressure (mmHg)			
Systolic	117±3.7	128±2.0	0.03
Diastolic	74.8±1.9	73.4±1.4	0.73
Left Ventricular Ejection Fraction	34.8±2.5	56.6±1.7	0.001
Aetiology (Ischaemic/DCM)	10/11	N/A	

Table 2

Figure 2

Medication	% of patients receiving
Beta blockers	76%
ACE Inhibitors / ARB	95%
Aldosterone Antagonists	57%
Loop diuretics	100%
Perhexiline	29%
Warfarin	57%
Antiplatelets	48%
'Statins'	52%
Digoxin	19%



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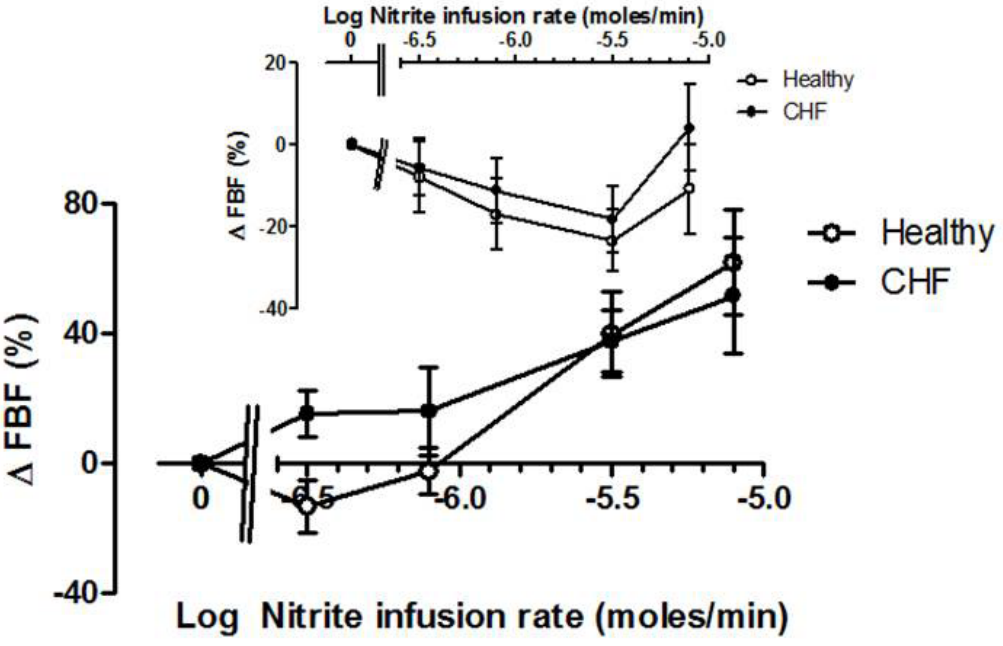

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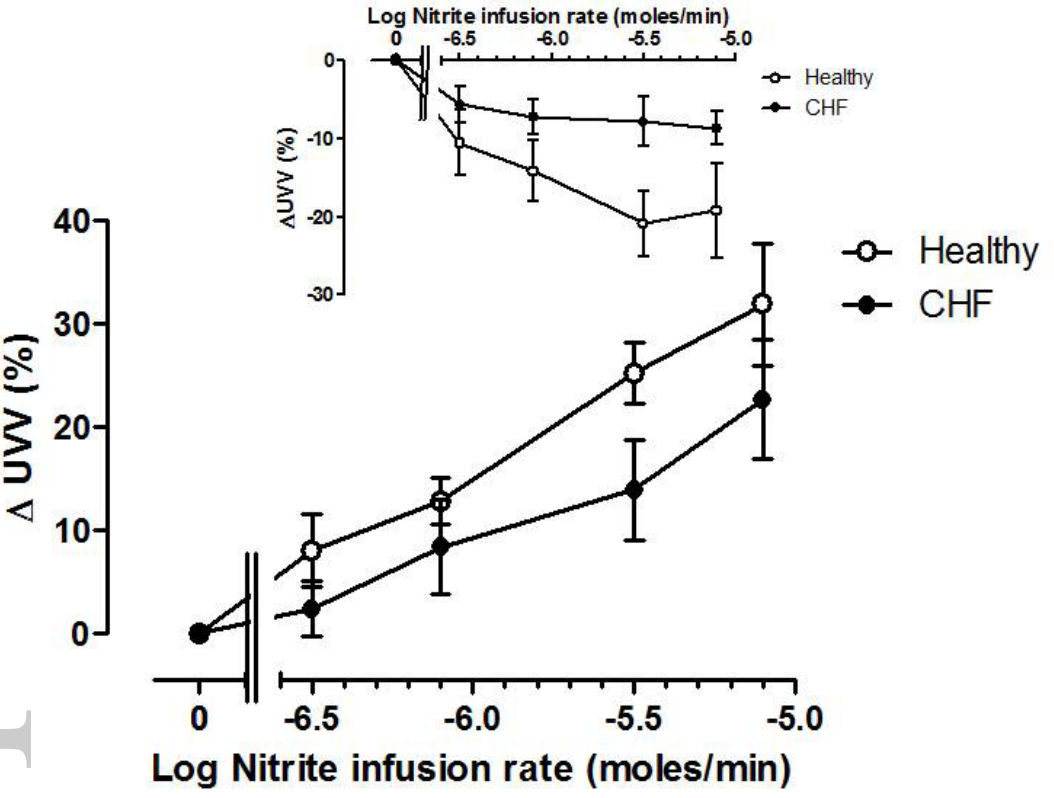

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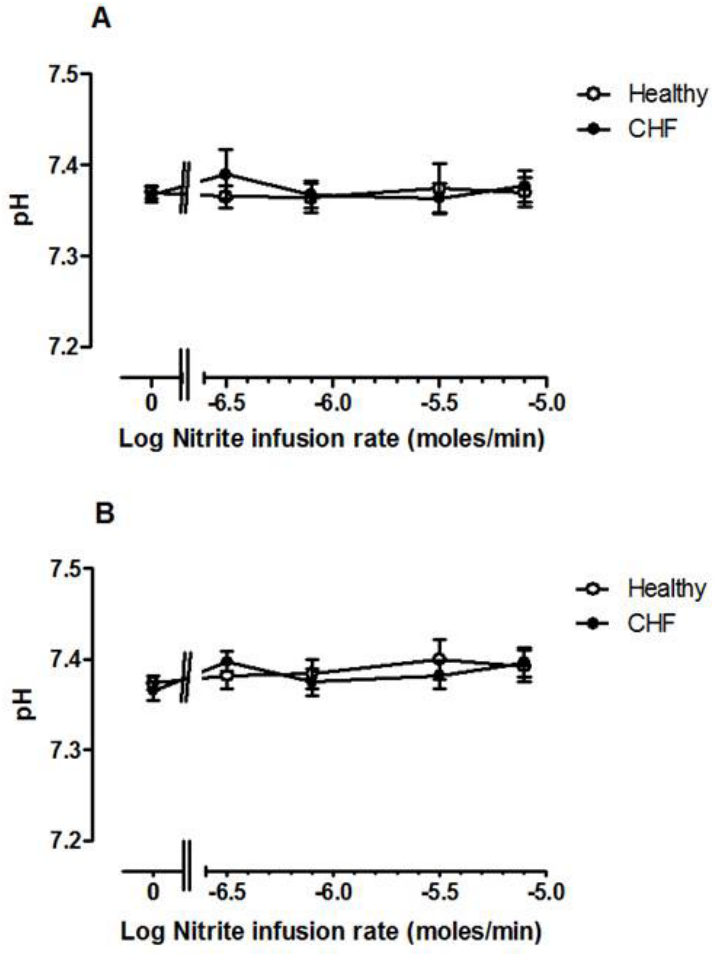

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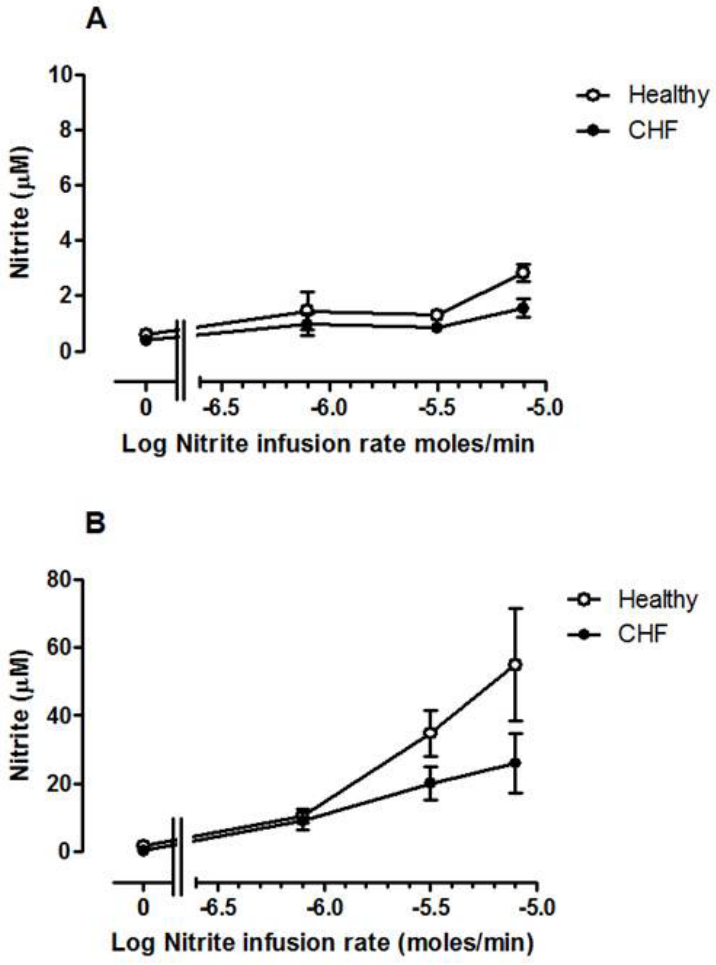

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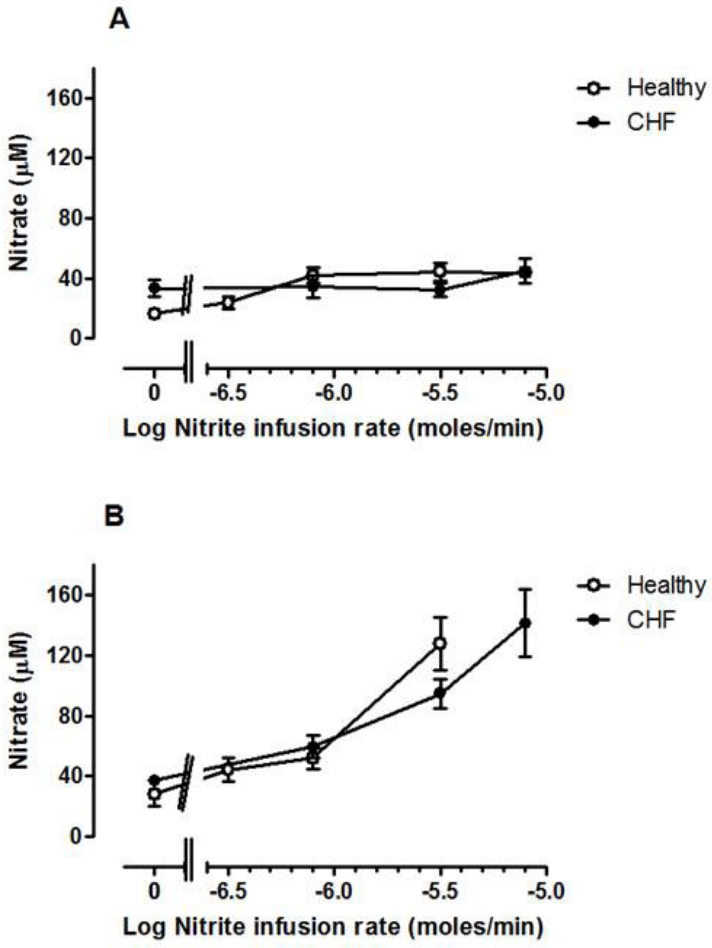

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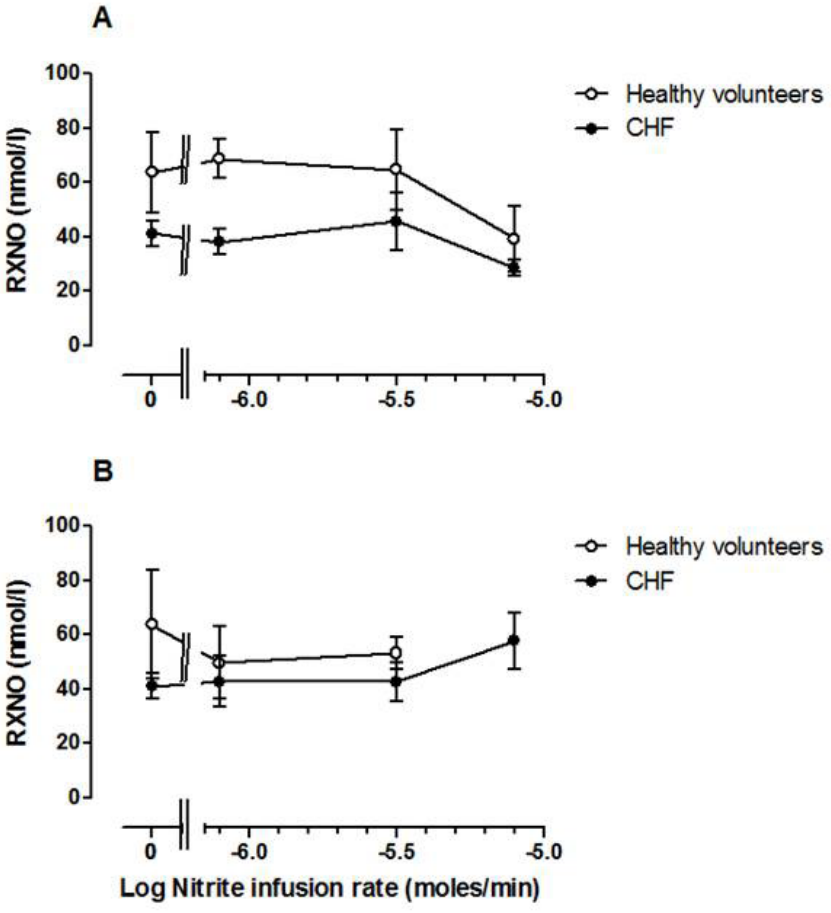

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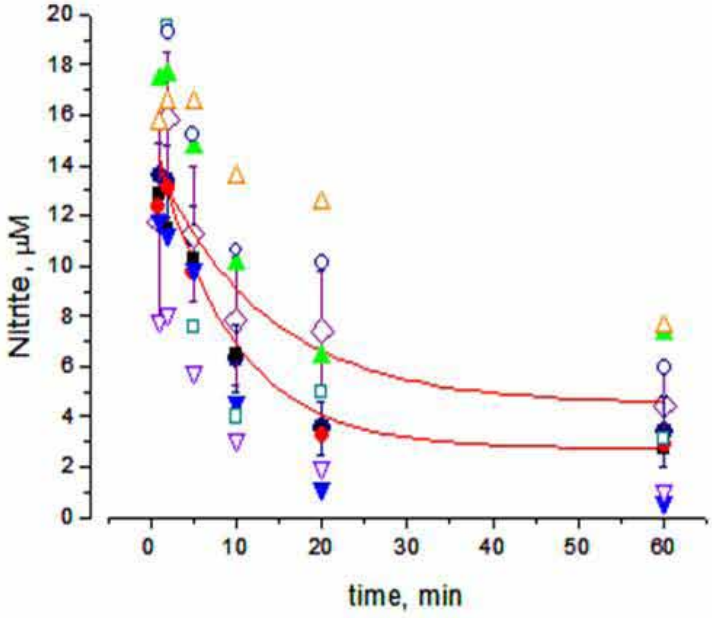

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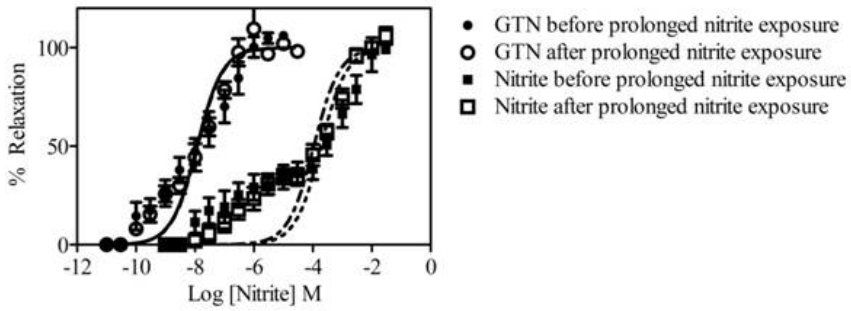

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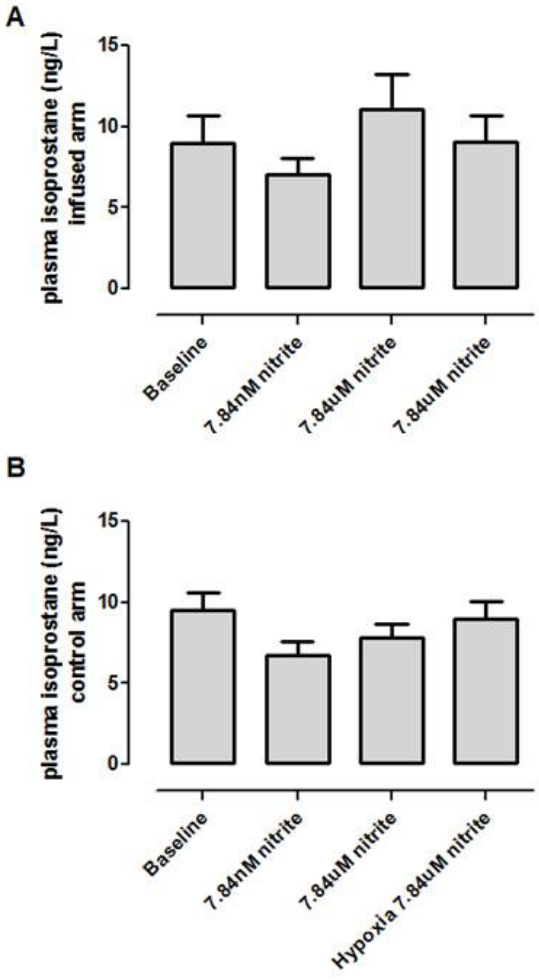

Figure 10.jpg