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RESEARCH PAPER

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

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BACKGROUND AND PURPOSE

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

EXPERIMENTAL APPROACH

The acute haemodynamic effects of brachial artery infusion of NO_2^- (0.31 to 7.8 μmol·min^{-1}) 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 NO_2^- 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.

Abbreviations

ALDH, aldehyde dehydrogenases; FBF, forearm blood flow; FVV, forearm venous volume; GTN, glyceryl trinitrate; RXNO, protein-bound NO; UVV, unstressed venous volume

Introduction

Nitrite (NO_2^-), present in plasma at submicromolar concentrations, has been regarded in the past as a relatively inert product of NO catabolism and dietary nitrate/ NO_2^- ingestion. NO_2^- 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 NOS (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 (CHF).

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 (Dupuis *et al.*, 1990; Atherton *et al.*, 1997; 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 (Muir and Nolan, 1991; Manyari *et al.*, 1993) 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 (Chirkov *et al.*, 1999; 2001; Anderson *et al.*, 2004). 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 theorized 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 NO_2^- would not vary between patients with CHF and normal subjects. The secondary hypothesis was that the pharmacokinetics of NO_2^- would not differ significantly between the two groups. Additional experiments were performed to determine whether prolonged NO_2^- 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 New York Heart Association 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 h. Pre-study dietary nitrate/NO_2^- intake was not modified.

Experimental protocol

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

Haemodynamic assessment. The principal haemodynamic 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 utilizing 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.

NO_2^- *infusion.* Figure 1 is a schematic of the overall experimental design. After determination of baseline data, NO_2^- was infused into the non-dominant brachial artery. Infusion rates were 0.31 $\mu mol \cdot min^{-1}$ for 30 min, thereafter increasing to 0.78 $\mu mol \cdot min^{-1}$, 3.1 $\mu mol \cdot min^{-1}$ and 7.8 $\mu mol \cdot min^{-1}$, each for further 30-min infusion periods. Changes in haemodynamic parameters were measured 5, 12 and 20 min after initiation of each infusion rate.

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,

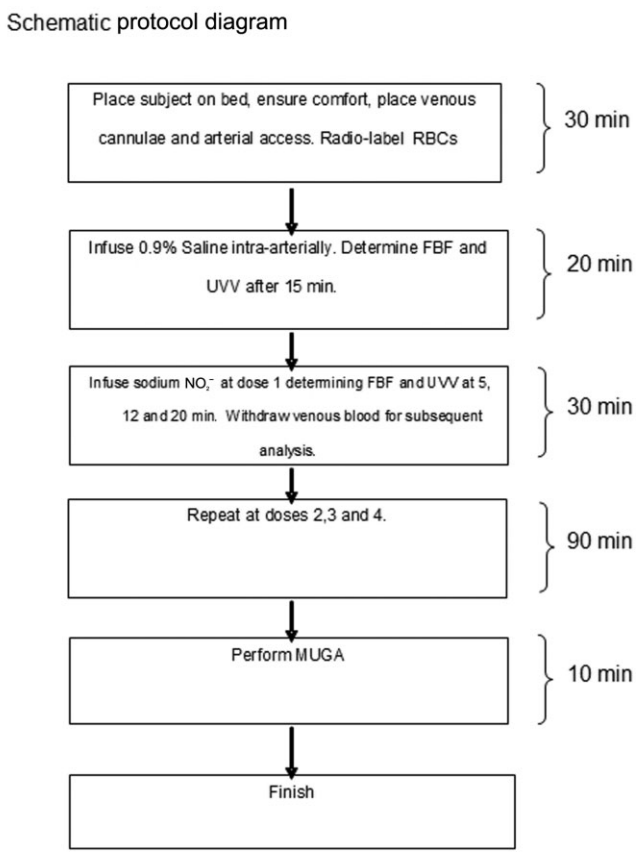

Figure 1

Schematic diagram of study protocol.

oxygen and methaemoglobin concentrations (Bayer Rapidlab 865, Siemens, Erlangen, Germany). Blood for determination of venous plasma NO_2^-, nitrate and protein-bound NO (RXNO) concentrations was taken into EDTA tubes and immediately centrifuged (200 rpm for 10 min at 4°C). Samples were stored at –80°C prior to assay. Plasma NO_2^-, nitrate and RXNO content were determined via ozone-based chemiluminescence (Pinder *et al.*, 2009) or HPLC (Rassaf *et al.*, 2002) as previously described.

Assessment of NO_2^- *clearance in human plasma* in vitro. To ensure that NO_2^- 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 NO_2^- *in vitro* to final concentrations of 2 and 20 μM; after spiking samples were incubated under gentle agitation at 37°C with aliquots being removed after 1, 2, 5, 10, 20 and 60 min prior to addition of N-ethylmaleimide (10 mM), centrifugation and assay.

Reagents. Sodium NO_2^- was purchased from Martindale Pharmaceuticals, UK. HPLC-grade NO_2^--free water (Fisher Scientific, Loughborough, UK) was utilized for extractions and dilutions.

In vitro *tolerance induction experiments*. *In vitro* studies were performed to address the possibility that prolonged infusion of NO_2^- 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 h, 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 1 g, as previously described (Sage *et al.*, 2000) and segments were equilibrated for 60 min before being constricted with 120 mmol·L^{-1} KCl; vessel segments in which constriction was less than 1 g were discarded. After a further 30 min 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 $NaNO_2$ (4×10^{-9} to 1.2×10^{-2} M) and GTN (4×10^{-9} to 1.2×10^{-5} M) in order to obtain cumulative vasodilator concentration-response curves. The order of administration of $NaNO_2$ and GTN was randomized. A washout period of 30 min 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 min. After a further washout period of 30 min, $NaNO_2$ and GTN concentration-response curves were repeated, again in random order. In each experiment, control vessels were utilized 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.

Assessment of NO_2^-*infusion upon levels of oxidative stress*. Heart failure patients ($n = 15$) were exposed to saline infusion (20 min), followed by two incremental doses of NO_2^- (7.84 nM and 7.84 μM; 20 min i.v. infusion for each dose) under normoxic conditions. The patients were then exposed to 12% hypoxia for 20 min and infused with 7.84 μM NO_2^-. At the end of each infusion, blood samples were taken for plasma 8-isoprostane analysis.

Assessment of oxidative stress. Total plasma 8-iso prostaglandin F2α (8-isoprostane) was measured using a commercial 8-isoprostane EIA assay (Cayman Chemical Company, Ann Arbor, MI, USA). 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 hydrolysing 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.

Table 1
Demographic data for both groups of subjects

Demographic and clinical features	CHF patients (mean ± SEM)	Healthy controls (mean ± SEM)	*P*-value
Age (year)	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 ($kg \cdot m^{-2}$)	30.0 ± 1.2	26.8 ± 0.6	0.05
Serum cholesterol ($mmol \cdot L^{-1}$)	5.3 ± 0.4	5.4 ± 0.2	0.69
Plasma glucose ($mmol \cdot L^{-1}$)	5.8 ± 0.4	4.7 ± 0.1	0.05
Serum creatinine ($\mu mol \cdot L^{-1}$)	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	

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 utilizing non-paired *t*-tests (two-sided) for normally distributed parameters, and Wilcoxon tests for skewed data. Categorical data were compared using a Fisher's exact test. Serial changes in FBF and UVV, pH and venous O_2 saturation, and concentrations of NO_2^- and RX 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.

Table 2
Summary of the medication taken by the CHF patients

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%

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 $mL \cdot min^{-1}$, mean plasma creatinine levels were marginally abnormal in the CHF group, typical of populations with heart failure.

Haemodynamic effects of NO_2^- infusion

Sodium NO_2^- 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 NO_2^- infusion respectively. For CHF patients, the mean arterial pressure values were 73 ± 4 and 70 ± 8 mmHg at baseline and peak NO_2^- infusion, respectively, while the corresponding values for heart rate were 62 ± 5 and 65 ± 4 $beats \cdot min^{-1}$ in normal subjects and 66 ± 4 and 69 ± 4 $beats \cdot min^{-1}$ in CHF patients. Maximal venous methaemoglobin concentrations in the infused arm were less than 2% of total haemoglobin at all NO_2^- 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 NO_2^- 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 $\mu mol \cdot min^{-1}$ and higher. In CHF patients, vasodilator responses were induced already at the lowest infusion rates of NO_2^-, but with similar

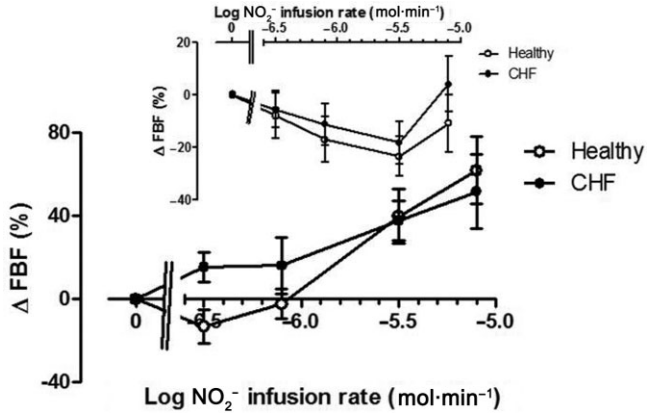

Figure 2

Changes in 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 versus normal subjects.

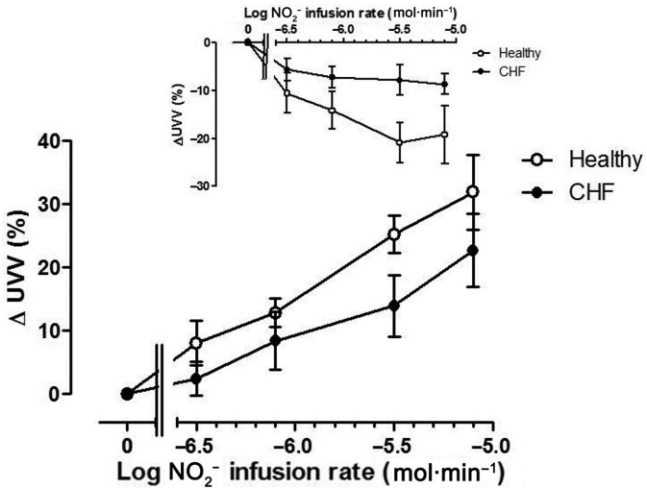

Figure 3

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 versus normal subjects.

responses at doses >3.14 μmol·min^{-1}. Logit transformation of concentration-response data confirmed that the gradients of the linearized 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 3.1 μmol·min^{-1}.

UVV changes are summarized 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 NO_2^- 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 NO_2^- 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 NO_2^- 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 NO_2^- infusion are summarized in Figure 5A and B. Resting plasma NO_2^- concentrations did not differ statistically between groups (0.60 ± 0.0.07 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 NO_2^- infusion (F = 32.7; P < 0.0001). In normal subjects, venous NO_2^- 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 NO_2^- infusion rate was disproportionately small: approximately sevenfold 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 B) suggesting that the degree of oxidation of NO_2^- to nitrate did not differ between groups.

RXNO concentrations

In the non-infused arm (Figure 7A), RXNO concentrations were significantly greater in normal subjects than in patients

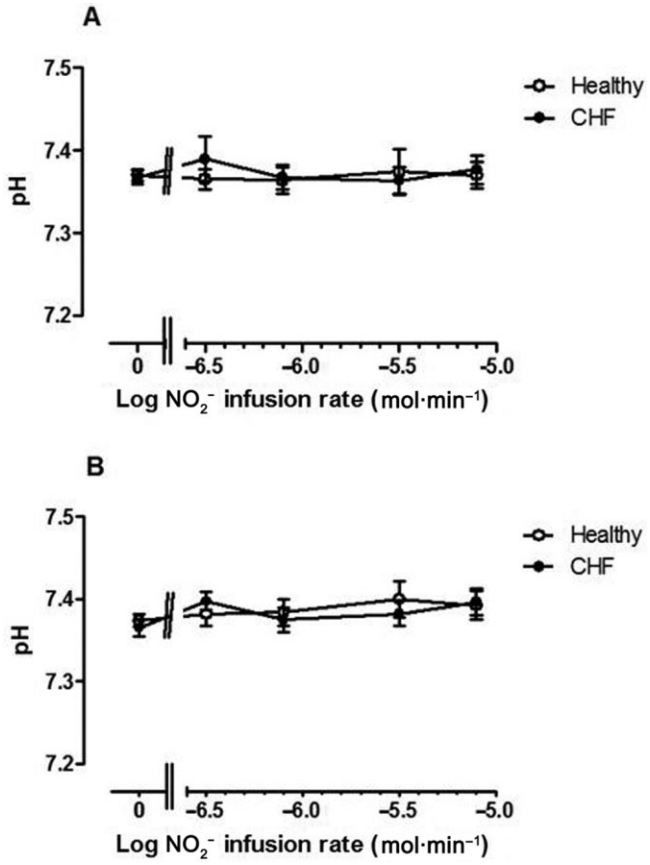

Figure 4

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.

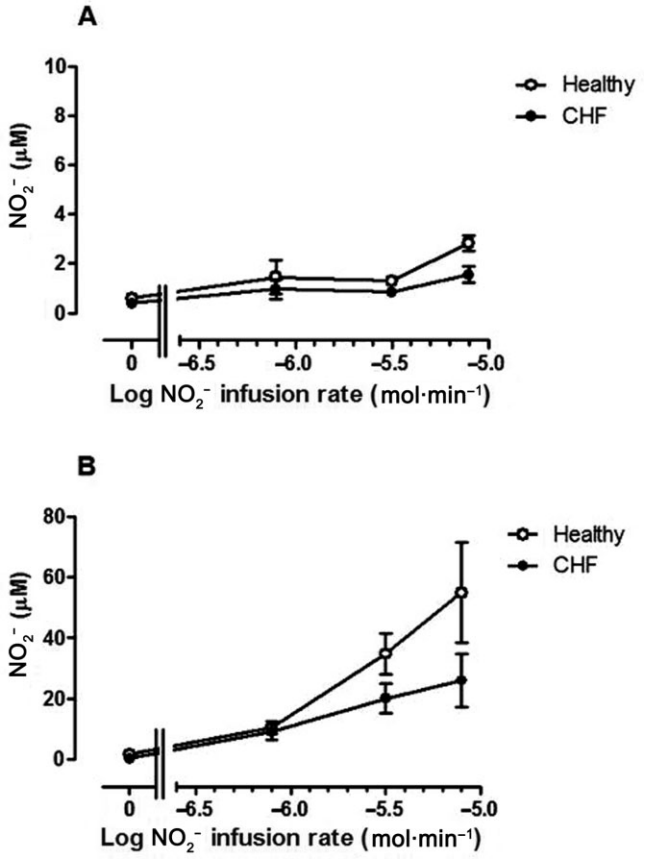

Figure 5

Venous plasma NO_2^- concentration (μM) in (A) non-infused arm and (B) infused arm in normal subjects (open symbols) and CHF patients (closed symbols). Baseline NO_2^- concentrations were marginally lower in CHF patients than in normal subjects (P = 0.08). NO_2^- 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 versus normal subjects.

with CHF (F = 8.6; P = 0.04). In the infused arm, this difference was attenuated during NO_2^- infusion, becoming non-significant. Furthermore, RXNO tended to increase with the highest NO_2^- infusion rate.

Assessment of in vitro NO_2^- *clearance in human plasma*

Following spiking of whole blood with NO_2^- *in vitro*, plasma NO_2^- decayed similarly (P = 0.66) in the two groups, with an apparent half-life of 5–6 min, irrespective of the initial concentration of NO_2^- achieved, as summarized 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*, NO_2^- induced vasorelaxation with a bi-sigmoidal concentration-response characteristic (Figure 9). After exposure to very high concentrations of NO_2^- (10^{-2} M) for 45 min followed by 30 min washout, there was no significant shift in the NO_2^- 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 NO_2^- tolerance induction respectively).

Assessment of NO_2^- *infusion on oxidative stress*

Plasma total isoprostane levels (Figure 10) did not increase significantly with NO_2^- 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 NO_2^-', 'higher dose NO_2^-' and during hypoxia respectively (P = 0.44)].

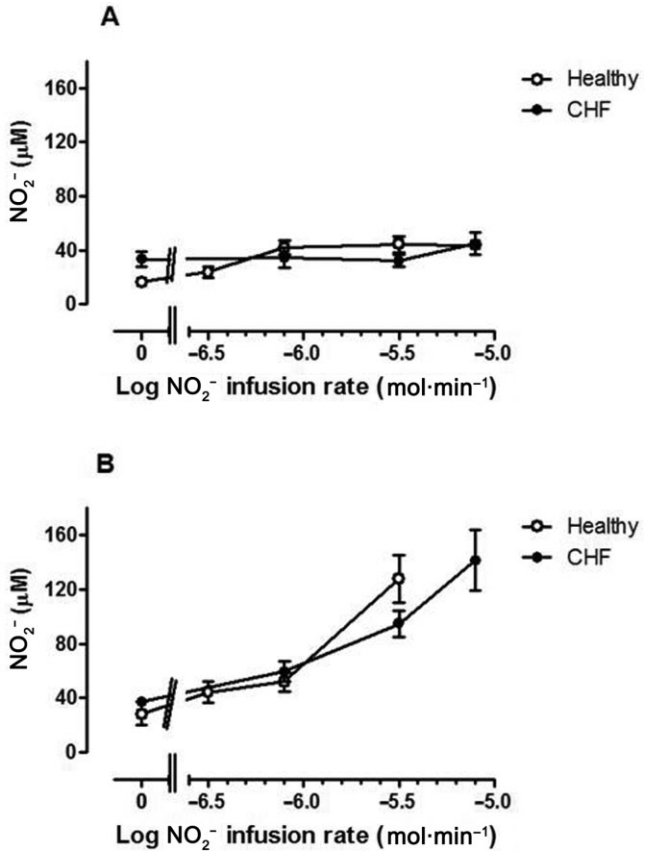

Figure 6

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 NO_2^- infusion, nitrate concentrations increased (F = 16.2 P < 0.0001) in the infused arm, but without significant difference between normal subjects and CHF patients.

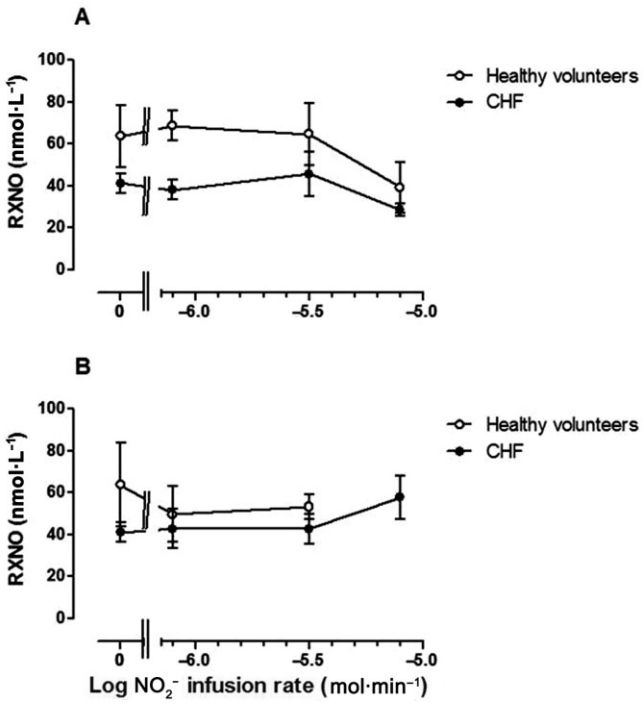

Figure 7

Venous plasma RXNO (nmol·L^{-1}) 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 NO_2^- 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.

Discussion

Previous studies have suggested that infused NO_2^- 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 NO_2^-, 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 (Shiva *et al.*, 2007; Ingram *et al.*, 2010; Milsom *et al.*, 2010). The precise mechanism(s) underlying this accentuated effect remain incompletely understood.

In the present study, effects of NO_2^- 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 were again

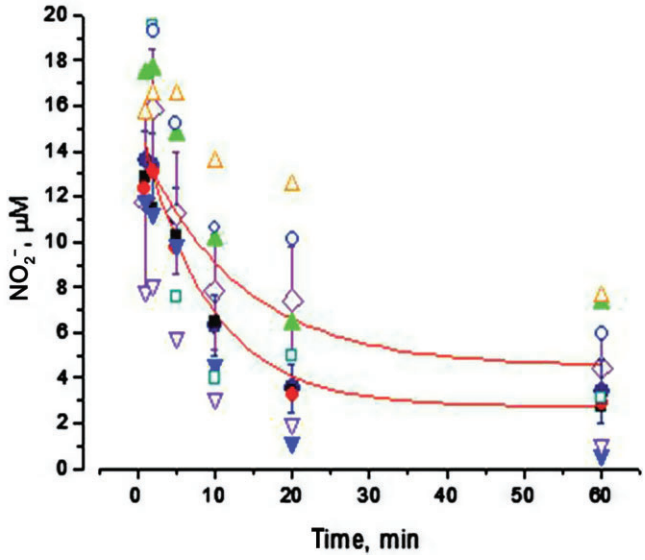

Figure 8

Clearance of NO_2^- *in vitro* from human plasma. Closed symbols represent data from normal subjects; open symbols represent data from CHF patients.

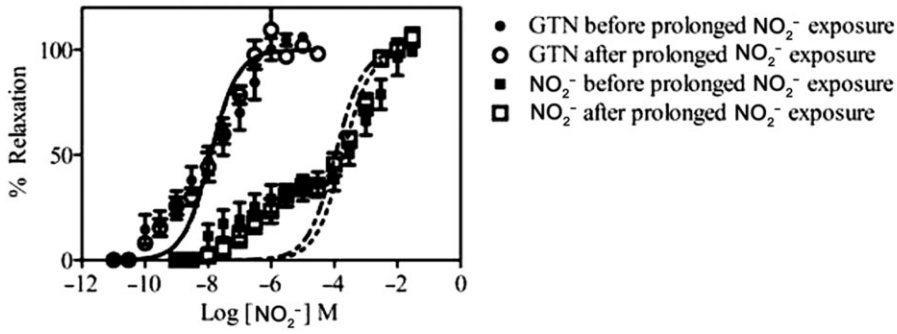

Figure 9

In vitro tolerance/cross-tolerance induction study. Concentration-response curves are shown for NO_2^- and GTN, indicating percentage relaxation of saphenous vein rings, before and after prolonged incubation with NO_2^-.

documented in normal subjects: the lowest two infusion rates of NO_2^- 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 NO_2^- is altered, with enhancement of arteriolar dilatation, but attenuation of venodilator responses.

The finding that CHF patients had lower RXNO and marginally lower NO_2^- concentrations than normal subjects is consistent with previous findings in subjects with endothelial dysfunction (Lauer *et al.*, 2001; Kleinbongard *et al.*, 2003; Heiss *et al.*, 2006). Steady-state NO_2^- concentrations (in the absence of dietary or i.v. 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 RXNO concentrations reflect the generation of S-nitrosoproteins from either NO or NO_2^- (Bryan *et al.*, 2005), as well as a measure of prevalent redox stress (Ng *et al.*, 2004). With sodium NO_2^- 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 NO_2^- infusion rate. Furthermore, NO_2^- levels increased far less markedly with increasing sodium NO_2^- 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 (bioactivation), 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 NO_2^- to nitrate, there was nevertheless a similar increase in plasma nitrate levels in the two groups upon infusion of NO_2^-, 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 NO_2^- disappearance in the two groups, thus excluding sampling artefacts as a possible explanation for the differences in NO_2^- concentrations between normal subjects and CHF patients.

As regards the additional potential formation of S-nitrosoproteins via NO_2^- metabolism, venous RXNO concentrations tended to increase in the infused arm in the CHF patients (coupled with a relative decrease in RXNO concentrations in the controls), attenuating the difference between baseline levels in the two groups. These findings are also consistent with selective bioactivation 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 bioactivation 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 NO_2^-, 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 NO_2^- 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 NO_2^- on venous capacitance and, to a lesser extent, to the effects on FBF at high infusion rates. These findings may

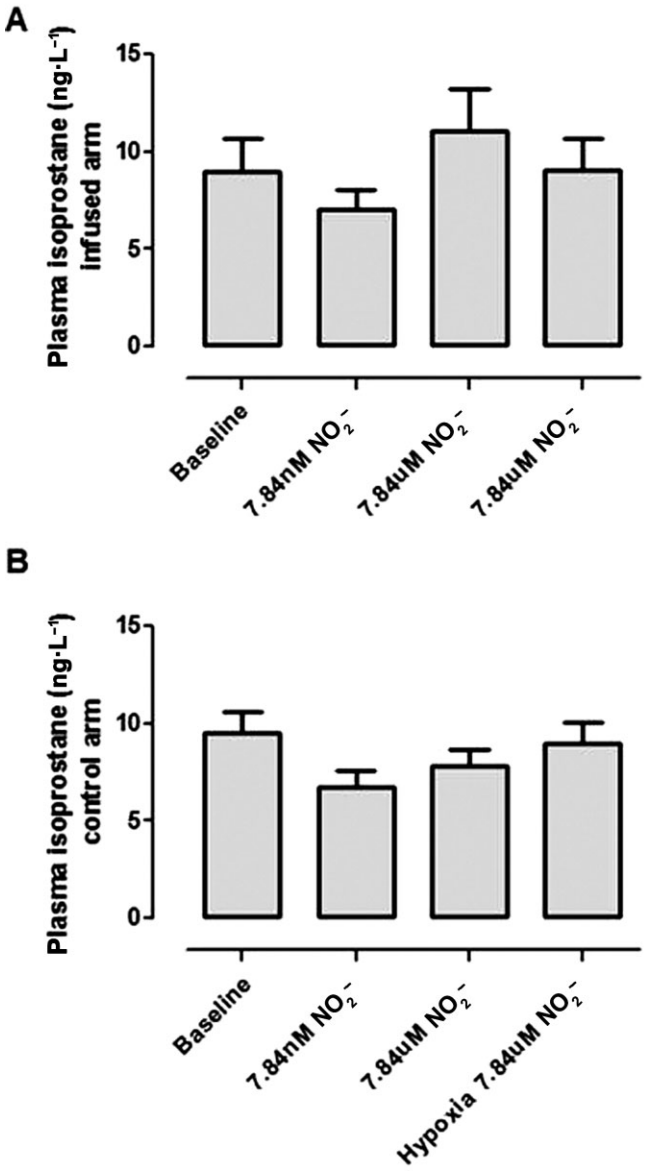

Figure 10

Plasma 8-isoprostane concentrations following NO_2^- infusion in heart failure patients. Values did not vary significantly between infused (A) and non-infused (B) arms.

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 NO_2^- 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; Towell *et al.*, 1986; Chen *et al.*, 2002; D'Souza *et al.*, 2011). 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 NO_2^- is catalysed by a large number of NO_2^- reductases, which may include ALDH (Feelisch *et al.*, 2008), there is a theoretical potential both for the induction of tolerance after prolonged administration of NO_2^- and for the associated induction of redox stress. We therefore evaluated the potential for NO_2^- tolerance induction utilizing 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, NO_2^- did not induce tolerance to itself (consistent with the findings of Dejam *et al.*, 2007) or to GTN. Furthermore, prolonged systemic administration of NO_2^- 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 utilized 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 NO_2^- 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 NO_2^- may exhibit vasoactivity independent of bioconversion and that NO_2^- 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 NO_2^- concentrations. Furthermore, the precise proportion of NO_2^- 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 oxidized-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 by-products 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 NO_2^-, 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 methaemoglobin (a possible concern), we did not observe a significant increase in venous methaemoglobin 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 ACE inhibitors and angiotensin receptor blockers improve NO production/endothelial function, we could not find any evidence to suggest any impact upon NO_2^- 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, NO_2^- 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 NO_2^- effect in chronic heart failure (e.g. via administration of dietary sources of nitrate) and from the i.v. infusion of NO_2^- 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^- bioactivation, perhaps counterbalanced 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 i.v. NO_2^- 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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Conflict 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.

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