The Effect of Inorganic Nitrate on Exercise Capacity in Heart Failure with Preserved Ejection Fraction

Running title: Zamani et al.; Inorganic nitrate in HFpEF

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Abstract

Background—Inorganic nitrate (NO₃⁻), abundant in certain vegetables, is converted to nitrite by bacteria in the oral cavity. Nitrite can be converted to nitric oxide (NO) in the setting of hypoxia. We tested the hypothesis that NO₃⁻ supplementation improves exercise capacity in HFrEF via specific adaptations to exercise.

Methods and Results—Seventeen subjects participated in this randomized, double-blind, cross-over study comparing a single-dose of NO₃-rich beetroot juice (NO₃⁻:12.9 mmoles) versus an identical nitrate-depleted placebo. Subjects performed supine-cycle maximal-effort cardiopulmonary exercise tests, with measurements of cardiac output (CO) and skeletal muscle oxygenation. We also assessed skeletal muscle oxidative function. Study endpoints included exercise efficiency (total work/total oxygen consumed), peak VO₂, total work performed, vasodilatory reserve, forearm mitochondrial oxidative function, and augmentation index (a marker of arterial wave reflections, measured via radial arterial tonometry). Supplementation increased plasma NO-metabolites (median 326 µM versus 10 µM; P=0.0003), peak VO₂ (12.6±3.7 vs. 11.6±3.1 mL O₂/min/kg; P=0.005), and total work performed (55.6±35.3 vs. 49.2±28.9 kJ; P=0.04). However, efficiency was unchanged. NO₃⁻ led to greater reductions in SVR (-42.4±16.6 vs. -31.8±20.3%; P=0.03) and increases in CO (121.2±59.9 vs. 88.7±53.3%; P=0.006) with exercise. NO₃ reduced aortic augmentation index (132.2±16.1 vs. 141.4±21.9%, P=0.03) and tended to improve mitochondrial oxidative function.

Conclusions—NO₃⁻ increased exercise capacity in HFrEF by targeting peripheral abnormalities. Efficiency did not change due to parallel increases in total work and VO₂. NO₃⁻ increased exercise vasodilatory and cardiac output reserves. NO₃ also reduced arterial wave reflections, which are linked to left ventricular diastolic dysfunction and remodeling.

Clinical Trial Registration Information—www.clinicaltrials.gov. Identifier: NCT01919177.

Key words: heart failure, exercise, nitric oxide, inorganic nitrate
Introduction

Heart failure with preserved ejection fraction (HFpEF) is associated with an approximate 30% heart failure readmission rate,\(^1\) significantly impaired quality of life, and 23% mortality over 3 years.\(^2\) Unfortunately, there are no guideline-recommended pharmacologic therapies that improve any of these frequencies.

Exercise intolerance is the hallmark of HFpEF, though the mechanism of this limitation is incompletely understood. Not only have abnormalities in diastolic and systolic function been identified,\(^3\) but evidence exists for peripheral derangements in the arteries and skeletal muscle. Subjects with HFpEF have impaired exercise vasodilatory reserve\(^4,\(^5\) and increased late systolic pressure augmentation from arterial wave reflections.\(^6\) Abnormalities of skeletal muscle have also been identified including greater fat deposition, a shift from slow-twitch oxidative fibers to more easily fatigable type-II glycolytic fibers, and reduced capillary-to-fiber ratios.\(^7,\(^8\) The reduction in blood flow to exercising muscle may lead to greater reliance on anaerobic glycolysis, predisposing to earlier exhaustion.

Historically, endogenous NO generation was thought to occur exclusively by nitric oxide synthases (NOS). More recently, however, the nitrate-nitrite-NO pathway has been recognized as an important alternative source of NO \textit{in vivo}. After ingestion, nearly 25% of the ingested dose is concentrated within the salivary glands before secretion into the oral cavity where anaerobic bacteria convert nitrate (NO\(_3^-\)) to nitrite (NO\(_2^-\)).\(^9,\(^10\) Subsequently, metalloproteins, such as deoxyhemoglobin and deoxymyoglobin, facilitate the reduction of systemically-absorbed NO\(_2^-\) to NO.\(^11,\(^12\) Importantly, whereas NO generation by the nitric oxide synthases requires molecular oxygen and may be limited by hypoxia,\(^10\) the conversion of NO\(_2^-\) to NO occurs \textit{preferentially} in the setting of hypoxia,\(^12,\(^13\) as would be found in exercising muscle. This would be especially true...
for fast-twitch muscles under blood-flow compromised conditions, such as in HFpEF.\textsuperscript{15}

Therefore inorganic nitrate may be a potent mediator of hypoxic vasodilation, a setting in which the classical NOS-mediated pathway is likely impaired.

Beyond vasodilation, inorganic nitrate has been shown to impact the $O_2$-cost of force generation, leading to less oxygen consumed per unit of work performed.\textsuperscript{16-19} The mechanism of this reduction remains incompletely understood, though a mitochondrial effect has been suggested. The putative impact of NO on the mitochondria include preservation of the proton gradient across the mitochondrial membrane, improved oxidative phosphorylation efficiency, a reduction in basal mitochondrial energy needs, reduced ATP cost for force generation, and a reduction in uncoupling proteins.\textsuperscript{18, 20, 21}

In this trial, we tested the hypothesis that inorganic nitrate administration improves exercise capacity in HFpEF. We also investigated the effect of inorganic nitrate on the vasculature and skeletal muscle to obtain insight into the mechanisms through which an effect on exercise tolerance may occur.

**Methods**

**Inclusion/Exclusion Criteria**

Inclusion criteria included symptomatic heart failure (orthopnea, paroxysmal nocturnal dyspnea, lower extremity edema, dyspnea on exertion) in the context of a preserved ejection fraction $>50\%$. Subjects were required to have a ratio of the early mitral inflow velocity (E) to septal tissue Doppler velocity ($e^\prime$)$>8$ and at least one other sign of chronically-elevated filling pressures including: (1) enlarged left atrium (left atrial volume index $>34 \text{ mL/m}^2$)\textsuperscript{22}; (2) an elevated NT-pro-BNP level within the past year; (3) chronic loop diuretic use for control of symptoms; or (4)
elevated filling pressures (mean pulmonary capillary wedge pressure > 12 mm Hg)\textsuperscript{23} on prior cardiac catheterization. Subjects had to be on stable medical therapy.

Exclusion criteria included non-cardiac conditions that limit exercise tolerance (orthopedic issues, peripheral arterial disease with claudication, neuromuscular disorders); gait instability; non-sinus rhythm; infiltrative/hypertrophic cardiomyopathy; pericardial disease; primary pulmonary arteriopathy; acute coronary syndrome or coronary revascularization within 60 days; clinically significant valvular disease (>mild aortic or mitral stenosis or > moderate aortic or mitral regurgitation); clinically significant lung disease felt to contribute to exercise intolerance; significant ischemia seen on stress testing within the past year which was not revascularized; or any condition which the investigators felt could compromise the subject’s ability to participate in the study or exercise safely.

**Study Design**

This was a randomized double-blind cross-over study of a single dose of inorganic nitrate given in the form of concentrated nitrate-rich beetroot juice (NO\textsubscript{3}\textsuperscript{-}, BEET IT Sport, James White Drinks Ltd., Ipswich, UK) containing 12.9 mmoles of NO\textsubscript{3} in 140 mL versus an otherwise identical nitrate-depleted placebo (PB, James White Drinks, LTD., Ipswich, UK) given 3 hours before maximal-effort cardiopulmonary exercise testing. After completion of the initial visit and all study procedures (Figure 1), subjects underwent a washout period of at least 5 days before crossing over to the other arm (mean 11.8 days, range 5-42 days).

We tested the hypothesis that inorganic nitrate supplementation would increase exercise capacity in HFpEF and assessed key peripheral mechanisms of this effect. Specifically, we assessed whether inorganic nitrate increases: (1) Our primary outcome of exercise efficiency (the ratio of total work performed to total oxygen consumed); and secondary outcomes which include
(2) peak VO₂; (3) total work performed; (4) vasodilatory reserve during exercise (change in peripheral vascular resistance from rest to peak exercise); (5) skeletal muscle mitochondrial oxidative function. Finally, we assessed the effect of inorganic nitrate on late systolic pressure augmentation from arterial wave reflections, which increase late systolic load on the LV and contribute to LV remodeling and abnormal myocardial relaxation, and on post-occlusive vasodilation in the forearm microvasculature. The protocol was approved by the Philadelphia Veterans Affairs Institutional Review Board. All subjects provided written informed consent before enrollment. This trial was registered on clinicaltrials.gov (NCT01919177).

**Study Procedures**

Subjects took all regularly prescribed medications on their schedule. Subjects were asked to refrain from using mouthwash on study days, as alterations in the oral flora impact nitrate metabolism. Subjects were also asked to avoid phosphodiesterase-5 inhibitors for at least two days prior to avoid any interaction with nitrate. Blood pressure was taken in the right arm with a validated oscillometric device (Omron HEM-705CP, Omron Corporation, Kyoto, Japan) after 5 minutes of rest. Subjects were then given 140 mL of nitrate-rich beetroot juice (NO₃⁻) or placebo (PB). Blood pressure was taken every 10 minutes for the next 2 hours using the same oscillometric device. After 2 hours, venipuncture was performed, and blood was centrifuged at 3000 RPM for 5 minutes before storage at -80°C. NT-pro-BNP levels were measured in a batch at the end of the study (Orthoclinical Diagnostic Vitros 3600; upper limit of normal: 124 pg/mL).

**Resting echocardiography and arterial hemodynamics at rest**

A standard transthoracic echocardiogram was performed using a Vivid 7 machine (General Electric, Fairfield, CT) in accordance with American Society for Echocardiography recommendations. Special attention was given to obtaining adequate left-ventricular outflow
tract (LVOT) Doppler velocity-time integrals (VTI) from the 5-chamber view to calculate stroke volume (SV, the product of LVOT VTI x LVOT cross-sectional area). Radial and carotid pressure waveforms were acquired using a high-fidelity STP-304 Millar tonometer (Millar Instruments, Houston, Texas). Doppler flow, tonometry, and ECG signals were recorded continuously in real-time using a PowerLab data acquisition module and Lab Chart Pro (AD Instruments, Version 7, Colorado Springs, CO) for Macintosh personal computers. Real-time streaming video from the echocardiography machine was recorded along with physiologic signals using a USB video interface (VGA2USB VGA Frame Grabber, Epiphan Systems Inc., Palo Alto, CA). After all subjects completed the study, the entire video for each study was reviewed by one investigator (PZ) in a blinded manner. LVOT Doppler envelopes were individually assessed and disregarded if the envelope was not felt to be representative of the stroke volume at the time of interrogation. Translational motion of the heart, arrhythmias, and optimal signal alignment were taken into account. Representative envelopes were selected, manually traced, and averaged. For each subject, the same LVOT diameter was used in the calculation of cardiac output for both studies.

**Maximal Effort Cardiopulmonary Exercise Test**

We used a supine cycle ergometer designed for stress echocardiography (Stress Echo Ergometer 1505, Medical Positioning, Inc., Kansas City, MO). Subjects underwent expired gas analysis using a ParvoMedics True One 2400 device (Parvomedics, Utah, USA). Subjects performed a maximal exertion-limited exercise test using a graded-exercise protocol. Resistance began at 12.5 Watts (W) for 3 minutes, increasing to 25W for 3 minutes, then increasing by 25W every 3 minutes thereafter. Breath-by-breath information was recorded. Oscillometric blood pressure, heart rate, and oxygen saturation were monitored during the test using a patient monitor.
(IntelliVue MP50, Philips Medical Systems, Andover, MA). Limited echocardiography was performed during each stage of exercise. Verbal encouragement was given to all subjects. Doppler LVOT VTI was acquired at peak exertion immediately at the cessation of exercise.

Custom-designed software was programmed in MATLAB (Version R2011b, MathWorks, Natick, MA) for processing and quantification of CPET data. All data quantification was blinded to treatment (NO$_3^-$ vs. PB). Breath-by-breath CPET data was visually assessed, and aberrant breaths were excluded. A Savitzky–Golay filter was then used to remove high-frequency breath-by-breath noise in an operator-independent manner. Peak oxygen uptake (VO$_2$) was determined as the average value during the final 30 seconds of exercise. The gas exchange/ventilatory threshold (VT) was determined using both the V-slope and ventilatory equivalent methods, with the results of the two measurements averaged.$^{31}$ VO$_2$/VCO$_2$ slope was calculated from the beginning of exercise to peak effort.$^{32}$ Respiratory exchange ratio (RER) was calculated as the ratio of VCO$_2$ to VO$_2$ at end-exercise. Arterial tonometry data was analyzed using SphygmoCor software (AtCor Medical, Australia).

Assessment of skeletal muscle oxygenation during exercise

We measured skeletal muscle oxygenation continuously during exercise using near-infrared spectroscopy (NIRS). In brief, the NIRS device emits two wavelengths (760 nm and 850 nm) of light corresponding to peaks in the absorption spectra of deoxyhemoglobin and oxyhemoglobin, respectively. The device measures the intensity of the transmitted and received light, with the absorbed fraction being a measure of the respective hemoglobin concentration. This allows for quantification of relative oxyhemoglobin and deoxyhemoglobin concentrations, with their sum being equal to the total hemoglobin concentration. Tissue Saturation Index (TSI), the ratio of oxyhemoglobin to total hemoglobin concentrations, was automatically calculated. The NIRS
device was placed on the largest circumference of the left gastrocnemius on its lateral aspect (Portamon, Artinis Medical System, The Netherlands) with an additional device (PortaLite, Artinis Medical System, The Netherlands) placed on the ipsilateral flexor digitorum superficialis (FDS) 3 cm below the elbow, thus interrogating non-exercising muscle. The maximum detector distance of 3 cm was chosen to allow for approximately 1.5 cm of tissue penetration. Subcutaneous fat thickness at the site of NIRS interrogation was measured with ultrasound to assure that skeletal muscle was being interrogated. Figure 2 provides a summary of the physiologic signals obtained during maximal-effort exercise testing.

**Constant-Intensity Protocol Cardiopulmonary Exercise Test**

Approximately 15 minutes after the maximal exertion test, subjects were again connected to the CPET circuit and underwent a 6-minute protocol at a constant 25W-resistance. Care was taken to ensure that the vital signs and RER returned to baseline prior to beginning the next exercise session. Steady-state VO2 was defined as the average VO2 during the final 60 seconds of exercise.

**Skeletal muscle mitochondrial oxidative function and post-occlusive hyperemia**

We performed skeletal muscle mitochondrial function testing using the technique developed by Ryan et al. which has been validated against 31P-MRI. Details of the procedure may be found in the supplement. In brief, with the subject sitting with his/her arms raised to the level of the heart and elbows placed in mild flexion, a cuff was placed around the dominant upper arm. A rapid inflator (E20 Rapid Cuff Inflator, D.E. Hokanson, Inc., Bellevue, WA), connected to a large-volume compressor (Hokanson AG101 Cuff Inflator Air Source, D.E. Hokanson, Inc., Bellevue, WA), was used to control cuff inflation and deflation. Baseline local O2 consumption (mVO2) was measured using a series of high-pressure inflations (200 mmHg), during which the...
decline in local muscle oxygen is due exclusively to consumption, as the arterial occlusion removes the confounding impact of arterial inflow. Thereafter, a brief standardized exercise protocol was used to increase mVO₂. Subsequent intermittent cuff inflations were used to track mVO₂ recovery by assessing the change in the slope of oxyhemoglobin signal decline (see Supplemental Figure). Such slopes plotted over time have been shown to follow a mono-exponential recovery described by a time constant (τ, tau), which corresponds to phosphocreatine recovery kinetics measured with MRI spectroscopy, thus providing an index of mitochondrial oxidative function.⁴ After 2 minutes, the exercise protocol was repeated, with the results of the 2 transients averaged.

Post-occlusive reactive hyperemia

A minimum of five minutes passed, after which baseline brachial artery diameter and flow velocities in the dominant arm were obtained using Doppler ultrasound with a dedicated vascular probe. The brachial cuff was then inflated for 5 minutes to suprasystolic pressures at 200 mm Hg. After cuff release, brachial artery diameter and velocities were obtained at 1-minute to compute volume flow (product of VTI x brachial artery cross-sectional area).

Plasma measurements

Plasma levels of nitric oxide-metabolites (NOₓ, primarily nitrate, nitrite, NO-metal complexes, and low-molecular-weight and protein cysteine-NO adducts) were measured in a batch at the end of the trial, using the method described by Lundberg and Govoni.⁹ In brief, samples were first deproteinized by passing through a 30kD cut off filter (AmiconUltra-0.5 Centrifugal Filter Unit, EMD Millipore). For quantification of NOₓ, samples were injected into a custom-made ice-water-cooled reaction chamber containing vanadium(III)/hydrochloric acid solution heated to 95°C. The NO generated from the reduction of NOₓ was quantified by its gas phase
chemiluminescence reaction with ozone (Nitric Oxide Analyzer; Sievers Instruments, Boulder, CO). Signal peaks (mV) were manually integrated, and the corresponding areas were used for the quantification of NOx concentration. To this end, authentic nitrate in the range of 0 to 50 μM was injected, and a ten-point standard curve was constructed by plotting area against nitrate content. The detection limit of the assay was 1.6 μM of nitrate.

Statistical analysis
Endpoints between the NO3− and PB measurements were compared using the paired t-test for normally-distributed data or the Wilcoxon signed-rank test for non-normally distributed data. A P-value < 0.05 was considered significant. Given the cross-over design, we pre-specified a modified intent-to-treat analysis, which included only subjects who completed both visits. Our study has 80% power to detect standardized differences ≥0.72 between the PB and NO3− groups, at a nominal alpha level of 0.05. All analyses were performed using STATA 13.1 (StataCorp, College Station, TX).

Results
A total of 162 subjects were screened, with 20 subjects entering the study (Figure 3). One subject was found to be in atrial fibrillation during the initial echocardiogram and therefore did not undergo any further procedures. Two subjects did not return for the second visit. Thus, seventeen subjects were included in the final modified intention-to-treat analysis.

Study Participants
The mean age of study participants was 65.5±8.9 years, with 15 (88%) males, and 14 (82%) African-Americans. Subjects were obese (BMI 35.4±5.4), had a high prevalence of hypertension (100%), and a 29% prevalence of an eGFR<60 mL/min/1.73m2. Median NT-pro-BNP was 144.0
(Q1-Q3: 60.3-192.0) pg/mL. Mean E/e’ ratio was 11.6±2.9, and the mean left atrial volume index was 35.7±10.9 mL/m² (Table 1).

Serum NO₃ was significantly greater after NO₃⁻ supplementation (median NO₃ 326.0 [Q1-Q3: 290.0-352.0] versus 10.0 [Q1-Q3: 9.0-13.0] μM, P=0.0003). Blood pressure was monitored for two hours after study drug administration and no change was found.

**Exercise efficiency and capacity (CPET)**

As shown in Table 2, peak VO₂ (12.6±3.7 versus 11.6±3.1 mL O₂/kg/min, mean difference 1.0±1.2; P=0.0051), total work performed (55.6±35.3 versus 49.2±28.9 kilo-Joules, mean difference 6.5±11.9; P=0.04) and exercise duration (15.3±4.9 versus 14.5±4.4 minutes, mean difference 0.8±1.3; P=0.02), were all significantly increased following NO₃⁻ supplementation. Because total work performed and oxygen consumption increased in tandem, exercise efficiency, the primary endpoint of the study, was no different after NO₃⁻ supplementation (4.5±0.8 versus 4.6±1.1 kilo-Joules per liter of O₂ consumed, mean difference -0.1±1.0; P=0.64). Ventilatory threshold was significantly greater following NO₃⁻ supplementation (7.6±1.8 versus 7.0±1.4 mL O₂/kg/min, mean difference 0.5±0.9; P=0.03).

**Arterial hemodynamics**

Inorganic nitrate supplementation significantly enhanced the reduction in systemic vascular resistance (SVR) at peak exercise (Table 3; Percent change in SVR NO₃⁻: -42.4±16.6 versus PB: -31.8±20.3%, mean difference -10.6±16.9; P=0.03). This was accompanied by a significant increase in the cardiac output (Percent change in cardiac output NO₃⁻: 121.2±59.9 versus PB: 88.7±53.3%, mean difference 32.5±41.0; P=0.006). The change in heart rate was significantly greater in the NO₃⁻ group (78.0±24.1 versus 65.6±21.0%, mean difference 12.4±13.2; P=0.001), with a tendency towards greater stroke volume (NO₃⁻ 22.6±22.4 versus 12.7±25.4%, mean
difference 9.8±24.9; \( P=0.13 \). Despite the increase in work, neither peak (\( P=0.14 \)) nor percent change (\( P=0.20 \)) in the A-V \( O_2 \) difference was significantly different between the NO\(_3^-\) and PB arms. Individual data for peak VO\(_2\), cardiac output reserve, systemic vascular resistance reserve, and the A-V \( O_2 \) difference reserve are presented in Figure 4.

**Skeletal muscle oxygenation during exercise**

There was no difference in the change in TSI during exercise between groups (\( P=0.55 \)). However, the percent change in oxyhemoglobin from baseline to its minimum during exercise tended to be less following NO\(_3^-\) supplementation (NO\(_3^-\) median -11.3 [Q1-Q3: -23.7-(-2.4)] versus PB median -15.8 [Q1-Q3: -49.5-(-9.5)]\%; median difference 7.4 (Q1-Q3: -0.02-15.8); \( P=0.07 \)).

**Constant-intensity exercise protocol**

Steady-state VO\(_2\) was no different after NO\(_3^-\) supplementation (Table 2, NO\(_3^-\) : 6.7±1.0 versus PB: 6.7±0.8 mL \( O_2 \)/kg/min, mean difference 0.06±0.60; \( P=0.70 \)). Only 10 of the 17 subjects had a VT during the maximal effort study above the 25W workload. In these subjects, there was no significant difference in oxygen consumption during 25W constant-load exercise following NO\(_3^-\) supplementation (\( P=0.77 \)).

**Dynamic exercise protocol and post-ischemia hyperemic flow**

Resting mVO\(_2\), measured using NIRS, was not different between the NO\(_3^-\) and PB arms (NO\(_3^-\) median 0.28 [Q1-Q3: 0.13-0.41] versus PB median 0.30 [Q1-Q3: 0.0-0.33]% of ischemic calibration/s, median difference 0.0 (Q1-Q3: -0.04-0.02); \( P=0.97 \)). After the standardized exercise protocol, time to mVO\(_2\)-recovery back to baseline tended to be shorter in the NO\(_3^-\) arm (49.5±17.2 versus 66.9±29.3 seconds, mean difference -17.5±33.3; \( P\)-value=0.08, \( n=13 \)).

The percent change in brachial artery flow, measured at 1-minute post cuff-release,
tended to be greater following NO$_3^-$ supplementation (NO$_3^-$ 362.3 [Q1-Q3: 206.6-663.9] versus PB median 209.3 [Q1-Q3: 81.9-307.6]%, median change 250.5 (Q1-Q3: -136.0-343.6); $P=0.11$).

**Augmentation index**

The aortic augmentation index (derived from radial tonometry) was significantly decreased by NO$_3^-$ supplementation (NO$_3^-$ 132.2±16.7 versus PB 141.4±21.9%, mean difference -9.1±15.4; $P=0.03$). At peak exercise, aortic AIx tended to decrease following NO$_3^-$ (109.6±16.4 versus 116.9±19.3%, mean difference -7.2±16.8; $P=0.13$).

**Discussion**

In this study, we tested the impact of inorganic nitrate on exercise. We did not find any change in efficiency, the primary end-point of the study. We demonstrate, however, that a single dose of inorganic nitrate (12.9 mmol) administered prior to exercise significantly improves peak VO$_2$ in subjects with HFpEF. This change was accompanied by a significant reduction in systemic vascular resistance and a significant increase in cardiac output at peak exercise, as well as an increase in the VO$_2$ at which VT occurred. Trends for improvements in skeletal muscle oxidative function and post-ischemic brachial artery flow were also found. Overall, our data suggest that NO$_3^-$ improves exercise capacity in HFpEF by improving the peripheral response to exercise and by providing greater O$_2$ delivery to exercising muscles. Inorganic nitrate also reduced late systolic aortic pressure augmentation, which suggests favorable effects on left ventricular pulsatile load.

In our trial, inorganic nitrate increased peak VO$_2$ in parallel with total work during a maximal exercise test. In contrast to what has been reported in healthy younger subjects, we did not observe an increase in efficiency, modeled as either the ratio of total work...
performed to total oxygen consumed or a reduction in the steady-state VO$_2$ during constant-intensity exercise. The reason behind this finding is unknown, although several possibilities exist. First, subjects with HFP EF may be sufficiently different from the young healthy individuals included in previous studies, such that inorganic nitrate may have differential effects on the mitochondria in this patient population. Indeed, in a recent study of healthy older individuals, NO$_3^-$ supplementation did not reduce the oxygen-cost of exercise, suggesting that perhaps age, and its consequent changes in mitochondria, may account for the difference.$^{39-41}$ Second, it is possible that subjects with HFP EF have an uncoupling between ATP generation and utilization. In accordance with other studies, we demonstrate a trend towards improvement in oxidative function using NIRS following NO$_3^-$ supplementation, suggesting improved ATP production.$^{20, 21}$ However, improved efficiency of oxygen consumption for a given workload depends on both the efficiency with which oxygen is converted into ATP as well as the mechanical efficiency of the system to generate force with the ATP generated.$^{21, 41}$ Previously, Smith et al. demonstrated abnormal creatine kinase shuttling in HFP EF using MRI and suggested that this finding may limit ATP availability to the myofibrils.$^{42}$ Restrictive ATP utilization may thus have limited any changes in efficiency.

We found a significant increase in peak VO$_2$ after a single dose of NO$_3^-$, which is highly relevant from the clinical standpoint. We demonstrate greater reduction in systemic vascular resistance following NO$_3^-$, likely contributing to the observed increase in cardiac output. This is consistent with the vasodilatory role of inorganic nitrate. As exercise capacity in heart failure is often limited by oxygen delivery, the improvement in cardiac output and associated improvement in muscle blood flow, was likely the main contributor to the improved peak VO$_2$ induced by NO$_3^-$ in this study.$^{15, 43, 44}$ The improvement in VT following NO$_3^-$ supplementation is
also consistent with an increased delivery of oxygen, leading to reduced stimulation of glycolytic pathways, and greater exercise times.\textsuperscript{15,45}

Unlike prior exercise intervention studies in HFpEF, where improvements were associated with increases in the systemic arteriovenous oxygen gradient,\textsuperscript{46} we did not find an increase in the A-V $O_2$ difference despite the greater workload. Instead, the increase in peak VO$_2$ in our study occurred in parallel to an increased cardiac output. Similarly, the absence of a lower local muscle oxyhemoglobin or tissue saturation levels with NO$_3^-$, despite greater workload and presumably local oxygen utilization, would be consistent with increased muscle blood flow. The greater post-occlusive flow within the brachial artery is similarly consistent with an enhancement of hypoxic vasodilation by inorganic nitrate.

Finally, we observed a reduction in central (aortic) augmentation index, a marker of wave reflections that has been shown to be increased in HFpEF.\textsuperscript{6} Late systolic load (from wave reflections) has been associated with increased left ventricular remodeling and diastolic dysfunction in animal experimental models\textsuperscript{25,47} and human studies\textsuperscript{27,48} and has been strongly associated with incident heart failure in humans.\textsuperscript{49} This change induced by NO$_3^-$, if sustained during chronic therapy, may have the potential for favorable disease-modifying effects on the left ventricle. This should be addressed in future studies.

Our study must be viewed in the context of its strengths and limitations. Strengths of this study include a comprehensive physiologic assessment of the adaptations to exercise, which quantified changes in the vasculature and skeletal muscle in addition to gas exchange. Our study was small, yet the cross-over design reduced measurement variability and enhanced detection of differences between treatments. Our study was composed primarily of males, limiting its generalizability. Our study showed a trend towards improved mitochondrial oxidative function
using NIRS. While this technique has been validated, these findings should be interpreted conservatively while more experience with NIRS accrues. Studies with more-established techniques, such as MRI-spectroscopy, would be desirable to confirm our findings. We studied subjects during supine exercise. It is possible that the values of peak VO₂ may have been different with upright exercise. Additionally, we used echocardiography to measure cardiac output at rest and at peak exercise. This technique is technically challenging and may have limited accuracy; however, our analyses were performed blinded to treatment assignment and demonstrated significant differences between groups. The optimal dose of nitrate supplementation is unknown, and perhaps a larger dose may have led to greater benefit. Finally, we made no adjustments for multiple comparisons in this pilot study, which introduces an increased chance of a type I error. However, the consistency of our findings with our pre-specified hypotheses makes it unlikely that our conclusions were reached by chance alone. Our results, demonstrating an improvement in exercise capacity with inorganic nitrate, should be confirmed in a larger study that also investigates the longer-term impact of NO₃⁻ in HFpEF.

Conclusions

A single dose of inorganic nitrate supplementation enhanced peak VO₂ and various peripheral adaptations to exercise in HFpEF including vasodilatory and cardiac output reserves. Inorganic nitrate also reduced aortic late systolic pressure augmentation, favorably impacting pulsatile load. Future longer-term trials are required to test inorganic nitrate as a therapy for HFpEF.

Conflict of Interest Disclosures: Dr. Zamani performed prior research in HFpEF that was funded by Gilead Life Sciences. Dr. Chirinos is named as Inventor in a patent application for the use of inorganic nitrate in HFpEF. Dr. Chirinos has received minor support (equipment loans)
from Atcor Medical, Cardiodynamics, and APC cardiovascular. Dr. Ischiropoulos is the Gisela and Dennis Alter Chair in Pediatric Neonatology at the Children's Hospital of Philadelphia and is supported by NIH grant HL54926. Dr. Margulies reports advisory committee membership for Novo Nordisk and Astra-Zeneca, and research grant support from Juventis Therapeutics, Celladon Corporation, Thoratec Corporation, Innolign Biomedical, LLC., and the U.S. National Institutes of Health (HL105993, HL110338, HL113777).

References:


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Table 1. Descriptive Variables of Subjects.

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<td>Race</td>
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<td>1.8 (0.08)</td>
</tr>
<tr>
<td>Weight (kg), mean (SD)</td>
<td>113.6 (23.5)</td>
</tr>
<tr>
<td>BMI (kg/m²), mean (SD)</td>
<td>35.4 (5.4)</td>
</tr>
<tr>
<td>Obese, n (%)</td>
<td>16 (94)</td>
</tr>
<tr>
<td>Current Smoker, n (%)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>17 (100)</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>12 (71)</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>12 (71)</td>
</tr>
<tr>
<td>Coronary Artery Disease, n (%)</td>
<td>3 (18)</td>
</tr>
<tr>
<td>Prior Stress Test, n (%)</td>
<td>16 (94)</td>
</tr>
<tr>
<td>Stress Test within 2 years, n (%)</td>
<td>14 (82)</td>
</tr>
<tr>
<td>NYHA Class, n (%)</td>
<td></td>
</tr>
<tr>
<td>Class I</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Class II</td>
<td>12 (71)</td>
</tr>
<tr>
<td>Class III</td>
<td>4 (24)</td>
</tr>
<tr>
<td>Class IV</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Drug Therapy, n (%)</td>
<td></td>
</tr>
<tr>
<td>Beta Blocker</td>
<td>11 (65)</td>
</tr>
<tr>
<td>ACE-Inhibitor/ARB</td>
<td>11 (65)</td>
</tr>
<tr>
<td>Calcium-Channel Blocker</td>
<td>7 (41)</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Statin</td>
<td>10 (59)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>15 (88)</td>
</tr>
<tr>
<td>Thiazide</td>
<td>4 (24)</td>
</tr>
<tr>
<td>Loop Diuretics</td>
<td>6 (35)</td>
</tr>
<tr>
<td>Laboratory Data</td>
<td></td>
</tr>
<tr>
<td>Serum Cr (mg/dL), mean (SD)</td>
<td>1.24 (0.37)</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²), mean (SD)†</td>
<td>73.0 (23.1)</td>
</tr>
<tr>
<td>eGFR &lt; 60 mL/min/1.73 m², n (%)</td>
<td>5 (29)</td>
</tr>
<tr>
<td>Hemoglobin (mg/dL), mean (SD)</td>
<td>13.0 (1.6)</td>
</tr>
<tr>
<td>NT-pro-BNP (picogram/mL), median (Q1-Q3)</td>
<td>144.0 (60.3-192.0)</td>
</tr>
<tr>
<td>NT-pro-BNP &gt; Upper Limit Normal‡, n (%)</td>
<td>9 (53)</td>
</tr>
<tr>
<td>Echocardiography</td>
<td></td>
</tr>
<tr>
<td>Left Ventricular Ejection Fraction (%), mean (SD)</td>
<td>63.5 (8.6)</td>
</tr>
<tr>
<td>Left Atrial Volume (mL), mean (SD)</td>
<td>83.9 (27.7)</td>
</tr>
<tr>
<td>Left Atrial Volume Index (mL/m²), mean (SD)</td>
<td>35.7 (10.9)</td>
</tr>
<tr>
<td>Mitral E-wave velocity (cm/s), mean (SD)</td>
<td>71.7 (16.4)</td>
</tr>
<tr>
<td>Mitral A-wave velocity (cm/s), mean (SD)</td>
<td>73.3 (24.2)</td>
</tr>
<tr>
<td>Mitral E/A Ratio, mean (SD)</td>
<td>1.05 (0.34)</td>
</tr>
<tr>
<td>Mitral Septal Tissue Doppler Velocity (e’, cm/s), mean (SD)</td>
<td>6.5 (1.7)</td>
</tr>
<tr>
<td>Mitral E/e’ Ratio, mean (SD)</td>
<td>11.6 (2.9)</td>
</tr>
<tr>
<td>Posterior Wall Thickness (cm), mean (SD)</td>
<td>1.37 (0.21)</td>
</tr>
<tr>
<td>Interventricular Septum Thickness (cm), mean (SD)</td>
<td>1.39 (0.29)</td>
</tr>
<tr>
<td>Relative Wall Thickness, mean (SD)</td>
<td>0.61 (0.12)</td>
</tr>
<tr>
<td>Meets European Society of Cardiology HFpEF Criteria, n (%)§</td>
<td>9 (53)</td>
</tr>
</tbody>
</table>

* Obesity defined as BMI >30 kg/m²; † eGFR was calculated using the Modification of Diet in Renal Disease (MDRD) Study equation; ‡ NT-pro-BNP upper limit of normal >124 pg/mL; § As defined by Paulus et al. 23
Table 2. Gas Exchange Data for Maximal Effort and Constant-Intensity (25W) Exercise Studies.

<table>
<thead>
<tr>
<th></th>
<th>Inorganic Nitrate Mean, SD</th>
<th>Placebo Mean, SD</th>
<th>Difference Between Inorganic Nitrate and Placebo Studies, Mean, SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maximal Effort Study</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exercise Duration, min</td>
<td>15.3 (4.9)</td>
<td>14.5 (4.4)</td>
<td>0.8 (1.3)</td>
<td>0.02</td>
</tr>
<tr>
<td>Total Work Done, kJ</td>
<td>55.6 (35.3)</td>
<td>49.2 (28.9)</td>
<td>6.5 (11.9)</td>
<td>0.04</td>
</tr>
<tr>
<td>Total Exercise Efficiency, kJ performed/L O₂ consumed</td>
<td>4.5 (0.8)</td>
<td>4.6 (1.1)</td>
<td>-0.1 (1.0)</td>
<td>0.64</td>
</tr>
<tr>
<td>Peak VO₂, mL O₂/min/kg</td>
<td>12.6 (3.7)</td>
<td>11.6 (3.1)</td>
<td>1.0 (1.2)</td>
<td>0.005</td>
</tr>
<tr>
<td>Peak Minute Ventilation (Vₑ), L/min</td>
<td>51.6 (17.6)</td>
<td>47.4 (16.1)</td>
<td>4.2 (5.6)</td>
<td>0.007</td>
</tr>
<tr>
<td>Peak Respiratory Exchange Ratio (RER)</td>
<td>1.02 (0.08)</td>
<td>0.99 (0.08)</td>
<td>0.03 (0.06)</td>
<td>0.06</td>
</tr>
<tr>
<td>Vₑ/VCO₂ Slope</td>
<td>32.7 (4.3)</td>
<td>32.3 (4.4)</td>
<td>0.5 (2.4)</td>
<td>0.45</td>
</tr>
<tr>
<td>Ventilatory Threshold, mL O₂/min/kg</td>
<td>7.6 (1.8)</td>
<td>7.0 (1.4)</td>
<td>0.5 (0.9)</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Constant-Intensity Protocol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steady-State VO₂, mL/min/kg</td>
<td>6.7 (1.0)</td>
<td>6.7 (0.8)</td>
<td>0.06 (0.6)</td>
<td>0.70</td>
</tr>
</tbody>
</table>
Table 3. Hemodynamic Reserve during Peak VO\textsubscript{2} Study.

<table>
<thead>
<tr>
<th></th>
<th>Inorganic Nitrate Mean (SD)</th>
<th>Placebo Mean (SD)</th>
<th>Difference between Inorganic Nitrate and Placebo Studies, Mean (SD)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline MAP\textsuperscript{*}, mm Hg</td>
<td>88.4 (12.1)</td>
<td>88.2 (12.8)</td>
<td>0.18 (13.7)</td>
<td></td>
</tr>
<tr>
<td>Peak MAP, mm Hg</td>
<td>104.0 (17.3)</td>
<td>104.3 (16.0)</td>
<td>-0.3 (11.8)</td>
<td></td>
</tr>
<tr>
<td>% change in MAP</td>
<td>18.0 (21.9)</td>
<td>18.2 (16.7)</td>
<td>0.2 (23.5)</td>
<td>0.98</td>
</tr>
<tr>
<td>Baseline HR\textsuperscript{†}, bpm</td>
<td>62.7 (8.8)</td>
<td>63.4 (9.1)</td>
<td>-0.7 (5.7)</td>
<td></td>
</tr>
<tr>
<td>Peak HR, bpm</td>
<td>115.2 (19.1)</td>
<td>113.8 (22.8)</td>
<td>1.5 (15.1)</td>
<td></td>
</tr>
<tr>
<td>% change in HR</td>
<td>78.0 (24.1)</td>
<td>65.6 (21.0)</td>
<td>12.4 (13.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>Baseline CO\textsuperscript{‡}, L/min</td>
<td>5.6 (1.5)</td>
<td>5.9 (1.8)</td>
<td>-0.3 (1.1)</td>
<td></td>
</tr>
<tr>
<td>Peak CO, L/min</td>
<td>12.1 (3.9)</td>
<td>10.8 (3.6)</td>
<td>1.2 (2.2)</td>
<td></td>
</tr>
<tr>
<td>% change in CO</td>
<td>121.2 (59.9)</td>
<td>88.7 (53.3)</td>
<td>32.5 (41.0)</td>
<td>0.006</td>
</tr>
<tr>
<td>Baseline SV\textsuperscript{§}, mL</td>
<td>81.9 (18.2)</td>
<td>83.4 (21.1)</td>
<td>-1.5 (13.8)</td>
<td></td>
</tr>
<tr>
<td>Peak SV, mL</td>
<td>99.7 (24.0)</td>
<td>92.5 (26.0)</td>
<td>7.2 (14.5)</td>
<td></td>
</tr>
<tr>
<td>% change in SV</td>
<td>22.6 (22.4)</td>
<td>12.7 (25.4)</td>
<td>9.8 (24.9)</td>
<td>0.13</td>
</tr>
<tr>
<td>Baseline SVR\textsuperscript{‖}, Wood Units</td>
<td>17.0 (5.2)</td>
<td>16.5 (5.3)</td>
<td>0.5 (4.5)</td>
<td></td>
</tr>
<tr>
<td>Peak SVR, Wood Units</td>
<td>9.7 (3.9)</td>
<td>11.2 (5.2)</td>
<td>-1.6 (2.2)</td>
<td></td>
</tr>
<tr>
<td>% change in SVR</td>
<td>-42.4 (16.6)</td>
<td>31.8 (20.3)</td>
<td>-10.6 (16.9)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\textsuperscript{*} MAP = mean arterial pressure; \textsuperscript{†} HR = heart rate; \textsuperscript{‡} CO = cardiac output; \textsuperscript{§} SV = stroke volume; \textsuperscript{‖} SVR = systemic vascular resistance
Figure Legends:

**Figure 1.** Protocol Flowchart.

**Figure 2.** Physiologic Signals Acquired During the Maximal-Effort Exercise Test.

**Figure 3.** Subject Consort Diagram.

**Figure 4.** Individual Data for Peak VO\(_2\), Cardiac Output Reserve, Systemic Vascular Resistance Reserve, and A-V \(O_2\) Difference Reserve.
Figure 1

Flowchart:

1. Consent and Randomization: Study Drug A or B
2. Serum NO$_x$ Determination
3. Baseline Echo
   - Maximal Effort VO$_2$
   - Constant Intensity (25 W) VO$_2$
   - Expired gases
   - Arterial tonometry
   - Echocardiography
   - Muscle oxygenation
4. Rest
5. Mitochondrial Oxidative Function using NIRS
   - Dynamic exercise protocol
   - Successive rapid cuff inflation
   - Protocol performed twice
   - Baseline brachial flow
   - 5-minute brachial occlusion
   - Brachial flow at 1 min.
6. Post-Occlusive Brachial Reactive Hyperemia
7. ~5 days
8. Study Drug B or A
9. Serum NO$_x$ Determination
10. Repeat Testing
Figure 2
Figure 3

162 Subjects screened for eligibility

142 - Met exclusion criteria
   50 - Unavailable to participate
   16 - No symptoms of HF
   19 - Inability to exercise
   12 - eGFR <30
   12 - Dyspnea from other reason
   7 - Significant valvular diseases
   6 - Current angina
   5 - Prior reduced LVEF
   5 - Current reduced LVEF
   4 - Any rhythm other than sinus
   2 - Severe liver disease
   2 - Current nitrates use
   1 - Significant anemia
   1 - Recent acute coronary syndrome

20 Subjects Entered Study

17 Subjects Completed Both Visits

3 Did not complete protocol:
   1 – Found to be in atrial fibrillation during baseline echocardiography
   2 – Did not return for Visit #2
+ Sign and Red Line connect mean/median values

Figure 4
The Effect of Inorganic Nitrate on Exercise Capacity in Heart Failure with Preserved Ejection Fraction
Payman Zamani, Deepa Rawat, Prithvi Shiva-Kumar, Salvatore Geraci, Rushik Bhuva, Prasad Konda, Paschalis-Thomas Doulias, Harry Ischiropoulos, Raymond R. Townsend, Kenneth B. Margulies, Thomas P. Cappola, David C. Poole and Julio A. Chirinos

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Supplement Material
Supplemental Methods

Skeletal muscle mitochondrial oxidative capacity and Post-Occlusive Hyperemia

We performed skeletal muscle mitochondrial function testing using the technique developed by Ryan et al. which has been validated against \( ^{31} \text{P-MRI} \).\(^1\) Fifteen minutes following the end of the square protocol, subjects were asked to sit comfortably with their arms raised on a table to the level of the heart. The elbows were placed in mild flexion at approximately 30 degrees to avoid venous pooling. NIRS devices were then placed over the FDS bilaterally. A blood pressure cuff was placed on the dominant arm and connected to a rapid inflator (E20 Rapid Cuff Inflator, D.E. Hokanson, Inc., Bellevue, WA), which was connected to a large-volume compressor (Hokanson AG101 Cuff Inflator Air Source, D.E. Hokanson, Inc., Bellevue, WA). Three initial high-pressure inflations (200 mm Hg) for 10s were performed to measure the rate of local O\(_2\) muscle oxygen consumption (MVO\(_2\)) at rest. During arterial occlusions, the inflow of oxygenated blood into muscle tissue is interrupted; thus, the rate of decrease in oxyhemoglobin concentration is solely proportional to MVO\(_2\).\(^2\) Subjects were then instructed to perform 8 maximal contractions at a rate of 0.5 Hz using a manual dynamometer in the dominant hand. Following the last contraction, a series of rapid cuff inflations (200 mm Hg) was performed in the following sequence: Cuff occlusions 1-5: 5 second on/5 seconds off, Cuff occlusions 6-10: 5 seconds on/10 seconds off; Cuff occlusions 11-15: 10 seconds on/20 seconds off (Supplemental Figure).\(^3\) A two-minute rest period was then given before the entire protocol was repeated. A custom-made video was used
to standardize the cadence and timing of contractions and occlusions between experiments.

The recovery of mVO$_2$ after brief exercise was assessed as previously described in detail.$^1,^4$ Oxy- and deoxyhemoglobin signals were corrected based on the principle that total blood-volume remains constant during arterial occlusions. This correction is performed under the assumption that decreases in oxyhemoglobin occur in a 1:1 ratio with increases in deoxyhemoglobin and that the rate of this change is proportional to local muscle oxygen consumption (mVO$_2$).$^4$

Second, to account for differences in the adipose layer overlying the muscle bed of interest, signals were normalized to the minimum values obtained during the 5-minutes of brachial artery occlusion and are thus presented as a percent of the ischemic calibration. These methods have been shown to remove the variability caused by differing degrees of adipose thickness, as well as differences due to the depth of interrogation.$^4$ Finally, the linear slope of the decrease in oxyhemoglobin over time was calculated for each arterial occlusion with linear regression, using a custom-made software interface programmed in Matlab (Supplemental Figure).

Such slopes plotted over time have been shown to follow a mono-exponential recovery described by a time constant (τ, tau). This recovery corresponds with PCr recovery kinetics measured with MRI spectroscopy and quantify mitochondrial oxidative capacity.$^1,^5,^6$ In some cases, slopes taken early after the cessation of exercise showed a rapid increase in mVO$_2$, followed by a monoexponential recovery. In these cases, only the monoexponential recovery portion was fitted to assess τ.
**Arterial tonometry**

A high-fidelity Millar radial tonometer was affixed to the right wrist for continuous measurement during exercise. Similarly, a high-fidelity Millar pen-like tonometer (Millar SPT 304; Millar Instruments, Houston, TX) was utilized to acquire carotid and brachial waveforms.

Tonometric signals were analyzed using the SphygmoCor software (AtCor Medical, Australia). A generalized transfer function was applied to the radial artery tonometry signals to generate an aortic pressure waveform.\(^7\) Augmentation Index (Alx) was calculated as the ratio of the amplitude of the second peak to the first peak \((P_2/P_1)\).
Supplemental Figure 1. Measurement of mitochondrial oxidative function
References:
Supplemental Figure 1.

Exercise  Transient Arterial Occlusions