Heart Failure

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

Payman Zamani, MD; Deepa Rawat, MD; Prithvi Shiva-Kumar, MD, MS; Salvatore Geraci, RDCS; Rushik Bhuva, MD; Prasad Konda, MD; Paschalis-Thomas Doulias, PhD; Harry Ischiropoulos, PhD; Raymond R. Townsend, MD; Kenneth B. Margulies, MD; Thomas P. Cappola, MD, ScM; David C. Poole, PhD, DSc; Julio A. Chirinos, MD, PhD

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 in the setting of hypoxia. We tested the hypothesis that NO3− supplementation improves exercise capacity in heart failure with preserved ejection fraction via specific adaptations to exercise.

Methods and Results—Seventeen subjects participated in this randomized, double-blind, crossover study comparing a single dose of NO3−-rich beetroot juice (NO3−, 12.9 mmol) with an identical nitrate-depleted placebo. Subjects performed supine-cycle maximal-effort cardiopulmonary exercise tests, with measurements of cardiac output and skeletal muscle oxygenation. We also assessed skeletal muscle oxidative function. Study end points 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 nitric oxide metabolites (median, 326 versus 10 μmol/L; P=0.0003), peak VO2 (12.6±3.7 versus 11.6±3.1 mL O2·min−1·kg−1; P=0.005), and total work performed (55.6±35.3 versus 49.2±28.9 kJ; P=0.04). However, efficiency was unchanged. NO3− led to greater reductions in systemic vascular resistance (−42.4±16.6% versus −31.8±20.3%; P=0.005) and increases in cardiac output (121.2±59.9% versus 88.7±53.3%; P=0.006) with exercise, NO3− reduced aortic augmentation index (132.2±16.7% versus 141.4±21.9%; P=0.03) and tended to improve mitochondrial oxidative function.

Conclusions—NO3− increased exercise capacity in heart failure with preserved ejection fraction by targeting peripheral abnormalities. Efficiency did not change as a result of 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—URL: http://www.clinicaltrials.gov. Unique identifier: NCT01919177. (Circulation. 2015;131:371-380. DOI: 10.1161/CIRCULATIONAHA.114.012957.)

Key Words: exercise ■ heart failure ■ nitrates ■ nitric oxide

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

Exercise intolerance is the hallmark of HFpEF, although 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 reserve4,5 and increased late systolic pressure augmentation from arterial wave reflections.6 Abnormalities of skeletal muscle, 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, have also been identified.7 The reduction in blood flow to exercising muscle may lead to greater reliance on anaerobic glycolysis, predisposing to earlier exhaustion.

Historically, endogenous nitric oxide (NO) generation was thought to occur exclusively by NO synthases. More recently, however, the nitrate-nitrite-NO pathway has been recognized as an important alternative source of NO in vivo. After ingestion, nearly 25% of the ingested dose is concentrated within

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.114.012957/-/DC1.

Correspondence to Julio A. Chirinos, MD, PhD, 3800 Woodland Ave, Room 8B111, Philadelphia, PA 19104. E-mail Julio.Chirinos@uphs.upenn.edu

© 2014 American Heart Association, Inc.

Circulation is available at http://circ.ahajournals.org

DOI: 10.1161/CIRCULATIONAHA.114.012957

371
the salivary glands before secretion into the oral cavity, where anaerobic bacteria convert nitrate (NO$_3^-$) to nitrite (NO$_2^-$).

Subsequently, metalloproteins such as deoxyhemoglobin and deoxymyoglobin facilitate the reduction of systemically absorbed NO$_2^-$ to NO. Importantly, whereas NO generation by the NO synthases requires molecular oxygen and may be limited by hypoxia, the conversion of NO$_2^-$ to NO occurs preferentially in the setting of hypoxia, 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. Therefore, inorganic nitrate may be a potent mediator of hypoxic vasodilation, a setting in which the classic NO synthase–mediated pathway is likely impaired.

Beyond vasodilation, inorganic nitrate has been shown to affect the O$_2$ cost of force generation, leading to less oxygen consumed per unit of work performed. The mechanism of this reduction remains incompletely understood, although a mitochondrial effect has been suggested. The putative impact of NO on the mitochondria includes 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.

In this trial, we tested the hypothesis that inorganic nitrate supplementation would increase 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') >8 and at least 1 other sign of chronically elevated filling pressures, including an enlarged left atrium (left atrial volume index >34 mL/m$^2$), an elevated N-terminal pro-brain natriuretic peptide (NT-pro-BNP) level within the past year, long-term loop diuretic use for control of symptoms, or elevated filling pressures (mean pulmonary capillary wedge pressure >12 mmHg) on prior cardiac catheterization. Subjects had to be on stable medical therapy.

Exclusion criteria included noncardiac conditions that limit exercise tolerance (orthopedic issues, peripheral arterial disease with claudication, neuromuscular disorders); gait instability; nonsinus rhythm; infiltrative/hypertrophic cardiomyopathy; pericardial disease; primary pulmonary arteriopathy; acute coronary syndrome or coronary revascularization within 60 days; clinically significant valvular disease (more than mild aortic or mitral stenosis or more than 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 that was not revascularized; or any condition that the investigators felt could compromise the subject’s ability to participate in the study or to exercise safely.

**Study Design**

This was a randomized, double-blind, crossover study of a single dose of inorganic nitrate given in the form of concentrated nitrate-rich beetroot juice (NO$_3^-$, BEET IT Sport, James White Drinks Ltd, Ipswich, UK) containing 12.9 mmol NO$_3^-$ in 140 mL versus an otherwise identical nitrate-depleted placebo (James White Drinks, Ltd) 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 our primary outcome of exercise efficiency (the ratio of total work performed to total oxygen consumed) and secondary outcomes that include peak VO$_2$, total work performed, vasodilatory reserve during exercise (change in peripheral vascular resistance from rest to peak exercise), and skeletal muscle mitochondrial oxidative function. Finally, we assessed the effect of inorganic nitrate on late systolic pressure augmentation from arterial wave...
cardiopulmonary exercise testing data were 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 (VO₂) was determined as the average value during the final 30 seconds of exercise. The gas exchange/ventilatory threshold was determined with both the V-slope and ventilatory equivalent methods, with the results of the 2 measurements averaged. The Vd/V̇E slope was calculated from the beginning of exercise to peak effort. Respiratory exchange ratio was calculated as the ratio of VO₂ to V̇E at the end of exercise. Arterial tonometry data were analyzed by use of SphygmCor software (AtCor Medical, West Ryde, NSW, 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 2 wavelengths (760 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 quantification of relative oxyhemoglobin and deoxyhemoglobin concentrations, with their sum being equal to the total hemoglobin concentration. Tissue saturation index, 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 (Portamonitor, Artinis Medical System, the Netherlands) with an additional device (PortaLite, Artinis Medical System) placed on the ipsilateral flexor digitorum superficialis 3 cm below the elbow, thus interrogating nonexercising muscle. The maximum detector distance of 3 cm was chosen to allow ±1.5 cm of tissue penetration. Subcutaneous fat thickness at the site of NIRS interrogation was measured with ultrasound to ensure that skeletal muscle began interrogated. Figure 2 provides a summary of the physiological 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 cardiopulmonary exercise testing circuit and underwent a 6-minute protocol at a constant 25-W resistance. Care was taken to ensure that the vital signs and respiratory exchange ratio had returned to baseline before the next exercise session began. Steady-state VO₂ was defined as the average VO₂ during the final 60 seconds of exercise.

Skeletal Muscle Mitochondrial Oxidative Function and Postocclusive Hyperemia

We performed skeletal muscle mitochondrial function testing using the technique developed by Ryan et al., which has been validated against P-31 magnetic resonance imaging. Details of the procedure may be found in the online-only Data 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) was used to control cuff inflation and deflation. Baseline local O₂ consumption (mVO₂) was measured with a series of high-pressure inflations (200 mmHg), during which the decline in local muscle oxygen is due exclusively to consumption because 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 Figure I in the online-only Data Supplement). Such slopes plotted over time have been shown to follow a monoexponential

Resting Echocardiography and Arterial Hemodynamics at Rest

A standard transthoracic echocardiogram was performed with a Vivid 7 machine (General Electric, Fairfield, CT) in accordance with American Society for Echocardiography recommendations. Special attention was given to obtaining adequate LV outflow tract (LVOT) Doppler velocity-time integrals from the 5-chamber view to calculate stroke volume (the product of LVOT velocity-time integral and LVOT cross-sectional area). Radial and carotid pressure waveforms were acquired with a high-fidelity STP-304 Millar tonometer (Millar Instruments, Houston, TX). Doppler flow, tonometry, and ECG signals were recorded continuously in real time with a PowerLab data acquisition module and LabChart Pro (AD Instruments, version 7, Colorado Springs, CO) for Macintosh personal computers. Real-time streaming video from the echocardiography machine was recorded, along with physiological signals, with a USB video interface (VGA2USB VGA Frame Grabber, Epiphyn Systems Inc, Palo Alto, CA). After all subjects completed the study, the entire video for each study was reviewed by an investigator (P.Z.) in a blinded manner. LVOT Doppler envelopes were individually assessed and disregarded if the envelope was not thought 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 with a Parvo Medics True One 2400 device (Parvo Medics, Sandy, UT). Subjects performed a maximal exertion-limited exercise test using a graded-exercise protocol. Resistance began at 12.5 W for 3 minutes, increasing to 25 W for 3 minutes, and then increasing by 25 W every 3 minutes thereafter. Breath-by-breath information was recorded. Oscillometric blood pressure, heart rate, and oxygen saturation were monitored during the test with the use of a patient monitor (IntelliVue MP50, Philips Medical Systems, Andover, MA). Limited echocardiography was performed during each stage of exercise. Oral verba...
recovery described by a time constant ($\tau$), which corresponds to phosphocreatine recovery kinetics measured with magnetic resonance imaging spectroscopy, thus providing an index of mitochondrial oxidative function.$^{14}$ After 2 minutes, the exercise protocol was repeated, with the results of the 2 transients averaged.

### Postocclusive Reactive Hyperemia

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

### Plasma Measurements

Plasma levels of NO metabolites (NO$_x$, 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 30-kD cutoff filter (AmiconUltra-0.5 Centrifugal Filter Unit, EMD Millipore). For quantification of NO$_x$, 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 NO$_x$ 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 NO$_x$ concentration. To this end, authentic nitrate in the range of 0 to 50 μmol/L was injected, and a 10-point standard curve was constructed by plotting area against nitrate content. The detection limit of the assay was 1.6 μmol/L nitrate.

### Statistical Analysis

End points between the NO$_x$ and placebo measurements were compared by use of the paired $t$ test for normally distributed data or the Wilcoxon signed-rank test for nonnormally distributed data. A value of $P<0.05$ was considered significant. Given the crossover design, we prespecified 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 placebo and NO$_3$ groups at a nominal $\alpha$ level of 0.05. All analyses were performed with 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, 17 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%) male and 14 (82%) black. Subjects were obese (body mass index, 35.4±5.4 kg/m$^2$), had a high prevalence of hypertension (100%), and had a 29% prevalence of an estimated glomerular filtration rate <60 mL-min$^{-1}$·1.73 m$^2$. Median NT-pro-BNP was 144.0 pg/mL (quartiles 1–3, 60.3–192.0 pg/mL). The mean E/e’ ratio was 11.6±2.9, and the mean left atrial volume index was 35.7±10.9 mL/m$^2$ (Table 1).

Serum NO$_x$ was significantly greater after NO$_3$ supplementation (median NO$_x$, 326.0 μmol/L [quartiles 1–3, 290.0–352.0 μmol/L] versus 10.0 μmol/L [quartiles 1–3, 9.0–13.0 μmol/L]; $P=0.0003$). Blood pressure was monitored for 2 hours after study drug administration, and no change was found.

### Exercise Efficiency and Capacity (Cardiopulmonary Exercise Test)

As shown in Table 2, peak $V_{O_2}$ (12.6±3.7 versus 11.6±3.1 mL·kg$^{-1}$·min$^{-1}$; mean difference, 1.0±1.2 mL·kg$^{-1}$·min$^{-1}$; $P=0.0051$), total work performed (55.6±35.3 versus 49.2±28.9 kJ; mean difference, 6.5±11.9 kJ; $P=0.04$), and exercise duration (15.3±4.9 versus 14.5±4.4 minutes; mean difference 0.8±1.3 minutes; $P=0.02$) were significantly increased after NO$_3$ supplementation. Because total work performed and oxygen consumption increased in tandem, exercise efficiency, the primary end point of the study, was no different after NO$_3$ supplementation (4.5±0.8 versus 4.6±1.1 kJ/L·O$_2$ consumed; mean difference −0.1±1.0 kJ/L·O$_2$ consumed; $P=0.64$). Ventilatory threshold was significantly greater after NO$_3$ supplementation.
(7.6±1.8 versus 7.0±1.4 mL O₂·kg⁻¹·min⁻¹; mean difference, 0.5±0.9 mL O₂·kg⁻¹·min⁻¹; P=0.03).

**Arterial Hemodynamics**

Inorganic nitrate supplementation significantly enhanced the reduction in systemic vascular resistance at peak exercise (Table 3; percent change in systemic vascular resistance: NO₃⁻, −42.4±16.6% versus placebo, −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 placebo, 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 toward greater stroke volume (NO₃⁻, 22.6±22.4% versus placebo, 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 arteriovenous O₂ difference was significantly different between the NO₃⁻ and placebo arms. Individual data for peak VO₂, cardiac output reserve, systemic vascular resistance reserve, and the arteriovenous O₂ difference reserve are presented in Figure 4.

**Skeletal Muscle Oxygenation During Exercise**

There was no difference in the change in the tissue saturation index during exercise between groups (P=0.55). However, the percent change in oxyhemoglobin from baseline to its minimum during exercise tended to be less after NO₃⁻ supplementation (NO₃⁻ median, −11.3% [quartiles 1–3, −23.7% to −2.4%] versus placebo median, −15.8% [quartiles 1–3, −49.5% to −9.5%]; median difference, 7.4% [quartiles 1–3, −0.02% to 15.8%; P=0.07]).

**Constant-Intensity Exercise Protocol**

Steady-state VO₂ was no different after NO₃⁻ supplementation (NO₃⁻, 6.7±1.0 mL O₂·kg⁻¹·min⁻¹ versus placebo, 6.7±0.8 mL O₂·kg⁻¹·min⁻¹; mean difference, 0.06±0.60 mL O₂·kg⁻¹·min⁻¹; P=0.70; Table 2). Only 10 of the 17 subjects had a ventilatory threshold during the maximal effort study above the 25-W workload. In these subjects, there was no significant difference in oxygen consumption during 25-W constant-load exercise after NO₃⁻ supplementation (P=0.77).

**Dynamic Exercise Protocol and Postischemia Hyperemic Flow**

Resting mVO₂, measured with NIRS, was not different between the NO₃⁻ and placebo arms (NO₃⁻ median, 0.28% [quartiles 1–3, 0.13%–0.41%] versus placebo median, 0.30% [quartiles 1–3, 0.0%–0.33%] of ischemic calibration per second; median difference, 0.0% [quartiles 1–3, −0.04% to 0.02%]; P=0.97). After the standardized exercise protocol, time to mVO₂ recovery back to baseline tended to be shorter in the NO₃⁻ arm (49.5±17.2 versus 66.9±29.3 seconds; mean difference, −17.5±33.3 seconds; P=0.08; n=13). The percent change in brachial artery flow, measured at 1 minute after cuff release, tended to be greater after NO₃⁻ supplementation (NO₃⁻, 362.3% [quartiles 1–3, 206.6%–663.9%] versus placebo median, 209.3% [quartiles 1–3,
Table 1. Descriptive Variables of Subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD), y</td>
<td>65.5 (8.9)</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>15 (88)</td>
</tr>
<tr>
<td>Race, n (%)</td>
<td>Black 14 (82) White 3 (18)</td>
</tr>
<tr>
<td>Height, mean (SD), m</td>
<td>1.8 (0.08)</td>
</tr>
<tr>
<td>Weight, mean (SD), kg</td>
<td>113.6 (23.5)</td>
</tr>
<tr>
<td>Body mass index, mean (SD), kg/m²</td>
<td>35.4 (5.4)</td>
</tr>
<tr>
<td>Obesity, 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 mellitus, n (%)</td>
<td>12 (71)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>17 (100)</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 y, n (%)</td>
<td>14 (82)</td>
</tr>
<tr>
<td>NYHA class, n (%)</td>
<td>I 1 (6) II 12 (71) III 4 (24) IV 0 (0)</td>
</tr>
<tr>
<td>Drug therapy, n (%)</td>
<td>β-Blocker 11 (65) ACE inhibitor/ARB 11 (65) Calcium channel blocker 7 (41) Spironolactone 1 (6) Statin 10 (59) Aspirin 15 (88) Thiazide 4 (24) Loop diuretics 6 (35)</td>
</tr>
<tr>
<td>Laboratory data</td>
<td>Serum creatine, mean (SD), mg/dL 1.24 (0.37) eGFR, mean (SD), mL/min·1.73 m² 73.0 (23.1) eGFR &lt;60 mL/min·1.73 m², n (%) 5 (29) Hemoglobin, mean (SD), mg/dL 13.0 (1.6) NT-pro-BNP, median (Q1–Q3), pg/mL 144.0 (60.3–192.0) NT-pro-BNP above the upper limit of normal, n (%)‡ 9 (53)</td>
</tr>
<tr>
<td>Echocardiography</td>
<td>LV ejection fraction, mean (SD), % 63.5 (8.6) Left atrial volume, mean (SD), mL 83.9 (27.7) Left atrial volume index, mean (SD), mL/m² 35.7 (10.9) Mitral E-wave velocity, mean (SD), cm/s 71.7 (16.4) Mitral A-wave velocity, mean (SD), cm/s 73.3 (24.2) Mitral E/A ratio, mean (SD) 1.05 (0.34) Mitral septal tissue Doppler velocity (e'), mean (SD), cm/s 6.5 (1.7) Mitral E/e' ratio, mean (SD) 11.6 (2.9) Posterior wall thickness, mean (SD), cm 1.37 (0.21) Interventricular septum thickness, mean (SD), cm 1.39 (0.29) Relative wall thickness, mean (SD) 0.61 (0.12)</td>
</tr>
<tr>
<td>Meets European Society of Cardiology HFpEF criteria, n (%)§§</td>
<td>9 (53)</td>
</tr>
</tbody>
</table>

ACE indicates angiotensin-converting enzyme; ARB, angiotensin receptor blocker; eGFR, estimated glomerular filtration rate; HFpEF, heart failure with preserved ejection fraction; LV, left ventricular; NT-pro-BNP, N-terminal brain natriuretic peptide; NYHA, New York Heart Association; and Q1–Q3, quartiles 1 through 3. n=17.

*Obesity defined as body mass index >30 kg/m².

†eGFR was calculated from 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.25

81.9%–307.6%], median change, 250.5% [quartiles 1–3, −136.0% to 343.6%]; P=0.11).

Augmentation Index

The aortic augmentation index (derived from radial tonometry) was significantly decreased by NO₃⁻ supplementation (NO₃⁻, 132.2±16.7% versus placebo, 141.4±21.9%; mean difference, −9.1±15.4%; P=0.03). At peak exercise, aortic augmentation index tended to decrease after NO₃⁻ (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 before exercise significantly improves peak VO₂ 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 VO₂ at which ventilatory threshold occurred. Trends for improvements in skeletal muscle oxidative function and post-ischemic brachial artery flow were also found. Overall, our data suggest that NO₃⁻ improves exercise capacity in HFpEF by improving the peripheral response to exercise and by providing greater O₂ delivery to exercising muscles. Inorganic nitrate also reduced late systolic aortic pressure augmentation, which suggests favorable effects on LV pulsatile load.

In our trial, inorganic nitrate increased peak VO₂ in parallel with total work during a maximal exercise test. In contrast to what has been reported in healthy younger subjects,16,17,19,36–38 we did not observe an increase in efficiency, modeled as either the ratio of total work performed to total oxygen consumed during a maximal effort test or a reduction in the steady-state VO₂ during constant-intensity exercise. The reason behind this finding is unknown, although several possibilities exist. First, subjects with HFpEF may be sufficiently different from the young healthy individuals included in previous studies that inorganic nitrate may have differential effects on the mitochondria in this patient population. Indeed, in a recent study of healthy older individuals, NO₃⁻ supplementation did not reduce the oxygen cost of exercise, suggesting that perhaps age, with its consequent changes in mitochondria, may account for the difference.39–41 Second, it is possible that subjects with HFpEF have an uncoupling between ATP generation and utilization. In accordance with other studies, we demonstrate a trend toward improvement in oxidative function using NIRS after NO₃⁻ supplementation, suggesting improved ATP production.30,21 However, improved efficiency of oxygen consumption for a given workload depends on both the efficiency with which oxygen is converted into ATP and the mechanical efficiency of the system to generate force with the ATP generated.31,41 Previously, Smith et al42 demonstrated abnormal creatine kinase shuttling in HFpEF using magnetic resonance imaging and suggested that this finding may limit ATP availability to the myofilibrils. Restrictive ATP utilization may thus have limited any changes in efficiency.

We found a significant increase in peak VO₂ after a single dose of NO₃⁻, which is highly relevant from the clinical standpoint. We demonstrate a greater reduction in systemic vascular resistance...
after NO$_3^-$, likely contributing to the observed increase in cardiac output. This is consistent with the vasodilatory role of inorganic nitrate. Because exercise capacity in heart failure is often limited by oxygen delivery, the improvement in cardiac output and associated improvement in muscle blood flow were likely the main contributors to the improved peak VO$_2$ induced by NO$_3^-$ in this study.\textsuperscript{15,43,44} The improvement in ventilatory threshold after 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 in which improvements were associated with increases in the systemic arteriovenous oxygen gradient,\textsuperscript{46} we did not find an increase in the arteriovenous O$_2$ difference despite the greater workload. Instead, the increase in peak VO$_2$ in our study occurred in parallel with 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 postocclusive 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

<table>
<thead>
<tr>
<th>Table 2. Gas Exchange Data for Maximal-Effort and Constant-Intensity (25 W) Exercise Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal-effort study</td>
</tr>
<tr>
<td>Exercise duration, min</td>
</tr>
<tr>
<td>Total work done, kJ</td>
</tr>
<tr>
<td>Total exercise efficiency, kJ consumed</td>
</tr>
<tr>
<td>Peak VO$_2$, mL O$_2$·min$^{-1}$·kg$^{-1}$</td>
</tr>
<tr>
<td>Peak VO$_2$, L/min</td>
</tr>
<tr>
<td>Peak respiratory exchange ratio</td>
</tr>
<tr>
<td>Ventilatory threshold, mL O$_2$·min$^{-1}$·kg$^{-1}$</td>
</tr>
<tr>
<td>Constant-intensity protocol</td>
</tr>
<tr>
<td>Steady-state VO$_2$, mL·min$^{-1}$·kg$^{-1}$</td>
</tr>
</tbody>
</table>

Values are mean (SD). CO indicates cardiac output; HR, heart rate; MAP, mean arterial pressure; SV, stroke volume; and SVR, systemic vascular resistance.

<table>
<thead>
<tr>
<th>Table 3. Hemodynamic Reserve During Peak VO$_2$ Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline MAP, mm Hg</td>
</tr>
<tr>
<td>Peak MAP, mm Hg</td>
</tr>
<tr>
<td>Change in MAP, %</td>
</tr>
<tr>
<td>Baseline HR, bpm</td>
</tr>
<tr>
<td>Peak HR, bpm</td>
</tr>
<tr>
<td>Change in HR, %</td>
</tr>
<tr>
<td>Baseline CO, L/min</td>
</tr>
<tr>
<td>Peak CO, L/min</td>
</tr>
<tr>
<td>Change in CO, %</td>
</tr>
<tr>
<td>Baseline SV, mL</td>
</tr>
<tr>
<td>Peak SV, mL</td>
</tr>
<tr>
<td>Change in SV, %</td>
</tr>
<tr>
<td>Baseline SVR, Wood units</td>
</tr>
<tr>
<td>Peak SVR, Wood units</td>
</tr>
<tr>
<td>Change in SVR, %</td>
</tr>
</tbody>
</table>

Values are mean (SD). CO indicates cardiac output; HR, heart rate; MAP, mean arterial pressure; SV, stroke volume; and SVR, systemic vascular resistance.
to be increased in HFpEF. Late systolic load (from wave reflections) has been associated with increased LV remodeling and diastolic dysfunction in animal experimental models, and human studies, and has been strongly associated with incident heart failure in humans. This change induced by NO₃⁻, if sustained during long-term therapy, may have the potential for favorable disease-modifying effects on the LV. 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 physiological 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 crossover design reduced measurement variability and enhanced detection of differences between treatments. Our study was composed primarily of men, limiting its generalizability. Our study showed a trend toward improved mitochondrial oxidative function using NIRS. Although this technique has been validated, these findings should be interpreted conservatively while more experience with NIRS accrues. Studies with more established techniques such as magnetic resonance imaging 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 by investigators 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 prespecified 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 affecting pulsatile load. Future longer-term trials are required to test inorganic nitrate as a therapy for HFpEF.

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 National Institutes of Health grant HL54926. Dr Margulies reports advisory committee membership for Novo Nordisk and AstraZeneca, as well as research grant support from Juventis Therapeutics, Celladon Corp, Thoratec Corp, Innolign Biomedical, LLC, and the National Institutes of Health (HL105993, HL110338, HL113777). The other authors report no conflicts.

References


17. Lang RM, Bierig M, Devereux RB, Flachskampf FA, Foster E, Pellikka PA, Picard MH, Roman MJ, Seward J, Shanevish JS, Solomon SD, Spencer KT, Sutton MS, Stewart WJ; Chamber Quantification Writing Group, American Society of Echocardiography’s Guidelines and Standards Committee, European Association of Echocardiography. Recommendations for chamber quantification: a report from the American Society of Echocardiography’s Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology. J Am Soc Echocardiogr. 2005;18:1440–1463.


---

**CLINICAL PERSPECTIVE**

Heart failure with preserved ejection fraction is a debilitating condition without any pharmacological therapies in contemporary use that improve either the morbidity or the mortality associated with this disease. Many studies have demonstrated abnormalities in the arterial system, microvasculature, and skeletal muscles of subjects with heart failure with preserved ejection fraction, suggesting that peripheral abnormalities may play an important role in the functional limitation seen in these subjects. Specifically, subjects with heart failure with preserved ejection fraction have an impaired ability to decrease systemic vascular resistance with exercise and to redistribute blood flow to exercising muscles. We demonstrate that inorganic nitrate supplementation significantly improves exercise capacity and systemic vasodilation during exercise, consistent with the known role of inorganic nitrate in mediating hypoxia-induced vasodilation. Inorganic nitrate also reduced late systolic left ventricular load from arterial wave reflections, which are known to exert deleterious effects on the left ventricle. Our pilot study introduces a new potential pathway in the treatment of heart failure with preserved ejection fraction, focusing on the peripheral abnormalities of the disease. Future study is now warranted on the potential role for long-term supplementation with inorganic nitrate in this disease population.
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

_Circulation._ 2015;131:371-380; originally published online December 22, 2014; doi: 10.1161/CIRCULATIONAHA.114.012957
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/131/4/371

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2014/12/22/CIRCULATIONAHA.114.012957.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/
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 $^{31}$P-MRI.\textsuperscript{1, 2} 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$_2$.\textsuperscript{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).\textsuperscript{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 ($\tau$, 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 $\tau$. 
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. Augmentation Index (Alx) was calculated as the ratio of the amplitude of the second peak to the first peak (P₂/P₁).
Supplemental Figure 1. Measurement of mitochondrial oxidative function
References:
Supplemental Figure 1.

Exercise  Transient Arterial Occlusions