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The inorganic Nitrate and eXercise performance in Heart Failure (iNIX-HF) phase II clinical trial: Rationale and study design

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ABSTRACT
Background: Heart failure (HF) is a debilitating and often fatal disease that affects millions of people worldwide. Diminished nitric oxide synthesis, signaling, and bioavailability are believed to contribute to poor skeletal muscle function and aerobic capacity. The aim of this clinical trial (iNIX-HF) is to determine the acute and longer-term effectiveness of inorganic nitrate supplementation on exercise performance in patients with HF with reduced ejection fraction (HFrEF).

Methods: This clinical trial is a double-blind, placebo-controlled, randomized, parallel-arm design study in which patients with HFrEF (n = 75) are randomized to receive 10 mmol potassium nitrate (KNO3) or a placebo capsule daily for 6 wk. Primary outcome measures are muscle power determined by isokinetic dynamometry and peak aerobic capacity (VO2peak) determined during an incremental treadmill exercise test. Endpoints include the acute effects of a single dose of KNO3 and longer-term effects of 6 wk of KNO3. The study is adequately powered to detect expected increases in these outcomes at P < 0.05 with 1-β > 0.80.

Discussion: The iNIX-HF phase II clinical trial will evaluate the effectiveness of inorganic nitrate supplements as a new treatment to ameliorate poor exercise capacity in HFrEF. This study also will provide critical preliminary data for a future ‘pivotal’, phase III, multi-center trial of the effectiveness of nitrate supplements not only for improving exercise performance, but also for improving symptoms and decreasing other major cardiovascular endpoints. The potential public health impact of identifying a new, relatively inexpensive, safe, and effective treatment that improves overall exercise performance in patients with HFrEF is significant.

1. Background
There are over six million patients with heart failure (HF) in the United States and millions more worldwide [1]. The hallmark symptom of this debilitating and often ultimately fatal disease is exercise intolerance. This is due in part to a diminished peak O2 consumption (VO2peak) [2] and especially an exaggerated ventilatory response to exercise, resulting from elevated afferent signaling from the exercising limbs [3]. The muscles of patients with HF are also weaker, slower, and less powerful than those of healthy individuals [4–6], even when participants are carefully matched for physical activity level, muscle mass, and statin use [6]. Together, these maladaptations lead to functional deficits, loss of independence, and reduced quality of life, and are powerful predictors of mortality in patients with HF [7–10]. Unfortunately, despite treatment advances HF remains not only a mortal disease but also a leading cause of disability. New treatments that operate via novel pathways to improve exercise capacity are therefore needed and have the potential to enhance clinical care dramatically.

Numerous factors underlie the impairments in exercise performance described above. These include a reduction in cardiac output due to ventricular dysfunction [2], alterations in blood flow regulation/distribution [11], abnormalities in muscle energetics [12], and molecular
In addition, a reduction in nitric oxide (NO) signaling may also play a role. NO regulates many physiological processes vital to exercise performance, including cardiac function [14] and skeletal muscle blood flow [15,16], energy metabolism [17], and contractile function [18]. Importantly, NO bioavailability is reduced in patients with HF, due to a decreased rate of NO synthesis [19] and/or an increased rate of NO destruction resulting from increased oxidative stress [20]. Thus, a relative deficiency in NO likely contributes to the diminished exercise capacity in patients with HF.

Based on the above mechanisms, we [21,22] and others [23–26] have investigated the effects of nitrate (NO\textsuperscript{−}) on exercise performance in patients with HFrEF. Most [21–25], albeit not all [26–28], of these studies have found that BRJ supplementation can ameliorate the functional deficits described above. In particular, we have demonstrated that ingestion of a single dose of NO\textsuperscript{−}-rich BRJ can improve muscle power and peak VO\textsubscript{2} in patients with HFrEF [21] and VO\textsubscript{2}peak in patients with non-ischemic HF with reduced ejection fraction (HFrEF) [22] (Fig. 1). The magnitudes of these changes are likely to be clinically relevant. For example, NO\textsubscript{−} ingestion increased maximal muscle power by 13%, which would be sufficient to erase 1/3rd of the deficit normally observed in HFrEF [6]. Similarly, VO\textsubscript{2}peak increased by 8% following NO\textsubscript{−} intake, which theoretically would reduce the annual risk of cardiac transplantation or death by almost 10% [31]. Notably, these improvements were observed in patients already receiving stable, optimal pharmacotherapy, including use of beta blockers, angiotensin converting enzyme inhibitors/receptor blockers, and/or aldosterone antagonists. Indeed, quantitatively the effect of acute dietary NO\textsubscript{−} intake on VO\textsubscript{2}peak compares favorably to chronic treatment with the first two medications [32–38] (aldosterone antagonists do not alter VO\textsubscript{2}peak in patients with HFrEF [39]), and unlike these conventional therapies, dietary NO\textsubscript{−} also improves muscle power. Finally, this approach provides potential advantages over other methods for increasing NO production and/or signaling in HFrEF, as shown in Table 1.

Inorganic NO\textsubscript{−} supplementation is therefore a promising new treatment for improving muscle and cardiovascular dysfunction in patients with HFrEF. Before this promise can be realized, however, the above-described findings need to be corroborated in a larger study of such individuals. It also needs to be determined whether the beneficial effects of dietary NO\textsubscript{−} on VO\textsubscript{2}peak and muscle power are maintained, or perhaps even enhanced, with repeated ingestion, or if tolerance or endothelial dysfunction develops as with inorganic nitrates, e.g., glycercyl trinitrate [40]. These negative consequences are thought to be due to increased production of highly-reactive oxygen species during drug activation, which does not occur with inorganic nitrate supplementation [41]. Finally, in addition to efficacy the longer-term safety and tolerability of NO\textsubscript{−} supplementation needs to be established. Supported by a grant from the National Heart, Lung, and Blood Institute (R61 HL155858), the inorganic Nitrate and eXercise performance in Heart Failure (iNIX-HF) phase II registered clinical trial (NCT05562167) has therefore been designed to achieve the following specific aims:

Specific Aim 1 (R61 phase). To complete all the regulatory, procedural, and safety steps required to launch the iNIX-HF clinical trial and enroll the first participants;

Specific Aim 2 (R33 phase). To determine the short-term (acute) effectiveness of dietary NO\textsubscript{−} on exercise performance in patients with HFrEF; and,

Specific Aim 3 (R33 phase). To determine the longer-term effectiveness of dietary NO\textsubscript{−} on exercise performance in patients with HFrEF.

These aims will be addressed using a double-blind, placebo-controlled, randomized parallel-arm study design to determine the effects of short- (i.e., an acute dose) and longer-term (i.e., once daily for 6 wk) supplementation with 10 mmol KNO\textsubscript{3} on muscle power and VO\textsubscript{2}peak (co-primary outcomes) in patients with HFrEF. We hypothesize that an acute dose of KNO\textsubscript{3} will increase both muscle power and VO\textsubscript{2}peak, as we observed in our preliminary studies [21,22], and that this positive effect will be maintained, but not enhanced, after 6 wk of treatment.

2. Methods

2.1. Study participants

Patients with known HFrEF will be recruited from various sources,
including the Washington University School of Medicine (WUSM) HF clinic, the WUSM Cardiology clinic, the WUSM Cardiac Diagnostic Laboratory’s database of patients with outpatient transthoracic echo-cardiograms, the WUSM Volunteer for Health Research Participant Registry, WUSM electronic medical records, and from three other hospitals in the St. Louis metropolitan area. Inclusion and exclusion criteria for the trial are shown in Table 2. All potential participants will provide written, informed consent before participating in the study, which is approved by the WUSM Institutional Review Board and the Indiana University Human Subjects Office.

2.2. Experimental protocol

As shown in Fig. 2, participants will complete three study visits, each involving identical assessments of exercise capacity. Visit 1 will be conducted without ingestion of any study drug. Participants will then be randomized to KNO or placebo therapy, stratifying by sex and ischemic/nonischemic status. The effect of an acute dose of 10 mmol KNO3 or placebo on VO2peak and muscle power will then be assessed during Visit 2. Afterwards, participants will continue on the same daily dose of 10 mmol KNO3 or placebo for 6 wk before returning for Visit 3, during which the longer-term effects of KNO3 (or placebo) will be determined.

2.2.1. Screening/phenotyping/baseline assessment (visit 1)

Participants will report to the WUSM Clinical Translational Research Unit (CTRU) in the morning after a 12 h fast and complete a medical history form, the Kansas City Cardiomyopathy Questionnaire (KCCQ), and the Minnesota Living with HF Questionnaire (MLHFQ). They will then undergo a physical examination, including measurement of heart rate (HR) and blood pressure (BP), and phlebotomy for screening/phenotyping laboratories: NT-proBNP, chemistries (including K+), glucose, and creatinine (for estimation of glomerular filtration rate). Women of child-bearing age will be administered a urine pregnancy test. Participants will also undergo a standard 2D Doppler, tissue Doppler, and strain imaging echocardiographic study to quantify left ventricular structure and function in accordance with ASE guidelines. If there are clinically significant abnormalities that are new relative to the patient’s medical history (e.g., low renal function), they will be withdrawn from the study and the patient’s cardiologist will be informed of the results. Eligible participants will proceed with measurement of body composition via DXA, pulse wave velocity (PWV) at time 0 and 1 h, and HR, BP, plasma NO2 and NO3, and breath NO at time 0, 1, 2, 3, and 4 h. During this period, participants will be familiarized with all procedures and will also perform practice exercise tests as described below.

2.2.2. Randomization

Participants will be randomized to the KNO3 or placebo arm using the REDCap randomization module, with stratification for sex and non-ischemic/ischemic cardiomyopathy status. The assignment order will be generated using SAS and then fed into REDCap.

2.2.3. Acute dose visit (visit 2)

As with Visit 1, participants will report to the CTRU fasted (and not having used mouthwash) and have blood drawn for measurement of K+ and creatinine. If hyperkalemia is present or if eGFR is <45 mL min⁻¹·1.73 m²⁻², the patient will be withdrawn from the study and their cardiologist contacted; otherwise, the study visit will proceed as follows. HR, BP, plasma NO2 and NO3, and breath NO will be measured at time 0 and hourly for 3 h and PWV will be measured at time 0 and 1 h after the participant ingests a single gelatin capsule containing either 10 mmol KNO3 or placebo (microcrystalline cellulose). This dose was chosen after preliminary experiments demonstrated that a higher dose (i.e., 20 mmol) did not result in greater improvements in muscle power or VO2peak, but was accompanied by a 3-fold higher frequency of nausea (i.e., 27% vs. 9%) [42]. Based on our novel pharmacokinetic model of the NO3/NO2 system [43], a dose of 10 mmol is expected to increase average 24 h plasma NO3 and NO2 concentrations by ~1200% and ~50%, respectively (Table 3). The capsules will be produced under subcontract by the University of Iowa Pharmacy using USP-grade KNO3 (Spectrum Chemical, New Brunswick, NJ) and tested for appearance, disintegration, weight variation, capsule closure, microbial enumeration (USP 61), and absence of E. Coli (USP 62) before use. After allowing 2 h for attainment of peak plasma NO3, plasma NO2, and breath NO levels, the power of the knee extensor muscles will be determined using isokinetic dynamometry. After 10 min of recovery, participants will perform an incremental treadmill exercise test to determine their VO2peak and ventilatory responses to exercise. Details of the exercise protocols are provided in sections 2.4 and 2.5.

2.2.4. Intervention

After completion of Visit 2, participants will be given a 6 wk supply of capsules (i.e., n = 42) containing either 10 mmol KNO3 or placebo, based on the randomization scheme. Participants will be instructed not to use mouthwash before ingesting the study capsules and to not change their diet or level of physical activity during the study (in particular, to not begin or cease an exercise program while enrolled in the study).

2.2.4.1. 6 wk dose visit (visit 3). The procedures for this visit will be identical to those described for Visit 2.

2.3. Measurement of plasma NO3, plasma NO2 and breath NO

Blood samples will be rapidly centrifuged to obtain plasma, which will be frozen at −80 °C until being shipped on dry ice to Indiana University Purdue University Indianapolis (IUPUI) for analysis. There,
plasma NO$_3$ and NO$_2$ concentrations will be determined using a dedicated high-performance liquid chromatography system (ENO-30, Eicom USA, San Diego, CA) as previously described [22]. To reduce variability, all samples from a single participant will be analyzed together.

The level of NO in each participant’s breath, a biomarker of whole-body NO production, will be measured using a portable electrochemical analyzer (NIOX VERO, Aerocrine Inc., Morrisville, NC) following American Thoracic Society/European Respiratory Society guidelines [44].

2.4. Measurement of muscle contractile function

A Biodex System4 isokinetic dynamometer (Biodex Medical Systems, Shirley, NY) will be used to determine the maximal power (P$_{\text{max}}$) and velocity (V$_{\text{max}}$) of the quadriceps muscles of the participant’s dominant leg as previously described [21]. Briefly, peak torque and hence peak power ($=\text{torque} \times \text{angular velocity}$) will be measured during 3–4 knee extensions performed at angular velocities of 0, 1.57, 3.14, 4.71, and 6.28 rad/s (0, 90, 180, 270, and 360°/s). The peak power-velocity data will then be fit with a parabolic function to determine P$_{\text{max}}$ and V$_{\text{max}}$. Strong verbal encouragement will be provided throughout the test.

2.5. Measurement of VO$_{\text{peak}}$ and ventilatory responses

These measures will be determined during an incremental treadmill exercise test (modified Naughton protocol [45]) performed to volitional fatigue. Respiratory gas exchange will be measured at 15 s intervals using a ParvoMedics TrueOne 2400 metabolic cart. VO$_{\text{peak}}$ (mL min$^{-1}$·kg$^{-1}$) will be defined as the highest average of four consecutive VO$_2$ values. The ventilatory threshold (VT; mL min$^{-1}$·kg$^{-1}$) will be

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Table 3
Pharmacokinetic model-predicted changes in steady-state plasma NO$_3$ and NO$_2$ in response to various dietary NO$_3$ dosing regimens.

<table>
<thead>
<tr>
<th>10 mmol NO$_3$ QD</th>
<th>5 mmol NO$_3$/kg BD</th>
<th>20 mmol NO$_3$/kg QD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma NO$_3$</td>
<td>Plasma NO$_3$</td>
<td>Plasma NO$_3$</td>
</tr>
<tr>
<td>$\Delta$C$_{\text{min}}$ (μmol/L)</td>
<td>209</td>
<td>0.090</td>
</tr>
<tr>
<td>$\Delta$C$_{\text{max}}$ (μmol/L)</td>
<td>568</td>
<td>0.245</td>
</tr>
<tr>
<td>$\Delta$C$_{\text{ave}}$ 0–24 h (μmol/L)</td>
<td>380</td>
<td>0.164</td>
</tr>
<tr>
<td>$\Delta$C$_{\text{ave}}$ 0–12 h (μmol/L)</td>
<td>476</td>
<td>0.205</td>
</tr>
</tbody>
</table>

QD, once per day. BD, twice per day. $\Delta$C$_{\text{min}}$, minimum increase in concentration above baseline. $\Delta$C$_{\text{max}}$, maximum increase in concentration above baseline. $\Delta$C$_{\text{ave}}$, 0–24 h, average increase in concentration above baseline over 24 h. $\Delta$C$_{\text{ave}}$, 0–12 h, average increase in concentration during first 12 h after ingestion. Calculations are based on data from Refs. 21,22,42, and unpublished observations. For context, in our hands average baseline (fasting) plasma NO$_3$ and NO$_2$ concentrations in patients with HFpEF are $32 \pm 17$ and $0.366 \pm 0.415$ μmol/L, respectively.
Sample size estimates.

The ratios of ventilation ($V_e$) to VO$_2$ (i.e., $V_e$/VO$_2$) and VCO$_2$ (i.e., $V_e$/VCO$_2$) will be determined by regressing $V_e$ on VO$_2$ and VCO$_2$, respectively, using all data obtained during sub-VT exercise. The oxygen uptake efficiency slope (OUES; L O$_2$/L $V_e$) will be calculated in a similar fashion by regressing VO$_2$ (in L/min) on the log of $V_e$ (also in L/min) throughout exercise [47]. Total exercise duration will also be recorded.

2.6. Other measures

Aortic stiffness will be determined by measuring the carotid-to-femoral PWV in duplicate with the cuff-based Sphygmocor XCEL (AtCor Medical, Itasca, IL) [48]. This device enables simultaneous acquisition of carotid (via tonometer) and femoral (via cuff) pulse waves. Transit time between carotid and femoral pressure waves will be calculated with the “foot-to-foot method”. Wave “feet” will be identified with intersecting tangent algorithms. PWV (m/s) will be calculated as distance traveled by the pulse wave divided by pulse transit time. In addition, these data will be subjected to pulse wave analysis to derive novel measures of arterial hemodynamics and wave reflections that have been recently shown to be associated with clinical outcomes in patients with HFrEF [49].

Before and immediately after the treadmill test, 2D and Doppler echocardiography recordings will be obtained via the suprasternal notch while the participant is seated upright in a chair next to the treadmill. These data will be used to determine pulsatile arterial hemodynamics with HFrEF [49].

Bioavailability of NO is decreased in HF, particularly HFrEF. Taken together, findings from these studies illustrate that NO is a key mediator of muscle function at the molecular level. NO increases muscle glucose metabolism, muscle protein synthesis, muscle force, and muscle endurance. NO has been shown to increase muscle mass and muscle strength, and NO improves muscle blood flow, muscle function, and exercise capacity. NO has been shown to increase muscle mass and muscle strength, and NO improves muscle function, exercise capacity, and exercise performance, but also for improving quality of life and reducing the morbidity of HF. NO can be increased using pharmacological agents such as L-arginine, which increases NO bioavailability, and small studies have shown that dietary NO increases NO production and improves exercise performance in patients with HFpEF. The inorganic Nitrate and Exercise performance In Heart Failure (iNIX-HF) phase II clinical trial will further evaluate the effectiveness of dietary NO as a new treatment to ameliorate poor exercise capacity in HFpEF. This study will also provide critical preliminary data for a future ‘pivotal’, phase III, multicenter trial of the effectiveness of dietary NO, not only for improving exercise performance, but also for improving symptoms and decreasing other major cardiovascular endpoints. The potential public health impact of identifying a new, relatively inexpensive, safe, and effective treatment that improves overall exercise performance in patients with HFpEF is significant.

3. Summary

HF is a mortal and morbid disease, impairing the ability of millions of people to exercise and even to perform routine activities of daily living. Improving aerobic exercise capacity, decreasing ventilatory effort, and bolstering muscle speed and power should decrease the morbidity of HF. NO is a key mediator of muscle function at the molecular level. Oral availability of NO is decreased in HF, particularly HFpEF. Taken orally, inorganic NO increases NO bioavailability, and small studies have shown that dietary NO increases NO production and improves exercise performance in patients with HFpEF. The inorganic Nitrate and Exercise performance In Heart Failure (iNIX-HF) phase II clinical trial will further evaluate the effectiveness of dietary NO as a new treatment to ameliorate poor exercise capacity in HFpEF. This study will also provide critical preliminary data for a future ‘pivotal’, phase III, multicenter trial of the effectiveness of dietary NO, not only for improving exercise performance, but also for improving symptoms and decreasing other major cardiovascular endpoints. The potential public health impact of identifying a new, relatively inexpensive, safe, and effective treatment that improves overall exercise performance in patients with HFpEF is significant.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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derived from the relation between oxygen uptake and minute ventilation during incremental exercise, J. Am. Coll. Cardiol. 28 (1996), 1567–1572.


