Beet the Best?

Dietary Inorganic Nitrate to Augment Exercise Training in Lower Extremity Peripheral Artery Disease With Intermittent Claudication

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Rationale: **A primary goal of therapy for patients with peripheral artery disease (PAD) and intermittent claudication is increased ambulatory function. Supervised exercise rehabilitation was recently shown to confer superior walking benefits to pharmacological or surgical interventions. Increases in plasma inorganic nitrite, via oral nitrate, have been shown to increase exercise performance in both human and animal models, especially in hypoxic conditions.**

Objective: **To determine whether a 36-session exercise rehabilitation program while consuming oral inorganic nitrate (4.2 mmol concentrated beetroot juice) would produce superior benefits over exercise plus placebo in pain-free walking and markers of increased skeletal muscle perfusion in patients with PAD and intermittent claudication.**

Methods and Results: **This was a randomized, double-blind, per-protocol study design. After the 12-week protocol, claudication onset time on a maximal treadmill test increased by 59.2±57.3 s for the exercise plus placebo group (n=13) and by 180.3±46.6 s for the exercise plus beetroot juice group (n=11; $P \leq 0.05$). This produced a between treatment medium to large standardized effect size (Cohen d) of 0.62 (95% CI, −0.23 to +1.44). The data for 6-minute walk distance showed a similar pattern with increases of 24.6±12.1 and 53.3±19.6 m ($P \leq 0.05$) in the exercise plus placebo and exercise plus beetroot juice groups, respectively. Measures of gastrocnemius perfusion, including ankle-brachial index, peak reactive hyperemic blood flow, and tissue deoxygenation characteristics, during exercise (assessed my near-infrared spectroscopy) all changed significantly for the exercise plus beetroot juice group with moderate-to-large effect sizes over exercise plus placebo changes.**

Conclusions: **Although it is premature to speculate on overall clinical utility of a nitrate-based therapy for PAD, this early pilot study evidence is encouraging. Specifically, our data suggests that increasing plasma nitrite before exercise may allow PAD subjects to train with less pain, at higher workloads for longer durations at each training session, thereby maximizing the beneficial peripheral vascular and skeletal muscle adaptations.**

Clinical Trial Registration: **URL: http://www.clinicaltrials.gov. Unique identifier: NCT01684930 and NCT01785524. (*Circ Res*. 2018;123:654-659. DOI: 10.1161/CIRCRESAHA.118.313131.)**

Key Words: exercise ■ humans ■ inorganic nitrate ■ nitric oxide ■ peripheral artery disease

Peripheral artery disease (PAD) is a highly prevalent[1] and costly[2] condition. Intermittent claudication (IC), defined as ischemic leg pain that occurs with walking and resolves with rest, affects 90% of symptomatic patients with PAD. PAD+IC results in functional impairment,[3] reduced daily physical activity,[4] a lower quality of life,[5] and an accelerated loss of mobility.[3] Accordingly, a primary goal of therapy for PAD+IC is increased ambulatory function.

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Although the pathophysiologic mechanisms contributing to the functional impairment and decline in PAD are not fully delineated, current evidence suggests that a range of tissue maladaptations in response to chronic underperfusion are involved. These include vascular endothelial dysfunction, reduced nitric oxide (NO) bioavailability,[6] capillary density rarefaction,[7,8] increased

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Novelty and Significance

What Is Known?

- Peripheral artery disease (PAD) with intermittent claudication is associated with a markedly impaired quality of life and a high perception of disability.
- Increased walking capacity is a primary goal of therapy for PAD+intermittent claudication.
- Supervised exercise training confers walking benefits superior to pharmacological or surgical interventions.

What New Information Does This Article Contribute?

- Regular oral inorganic nitrate supplementation before exercise training produced superior functional benefits in comparison with supervised exercise training plus placebo.
- Increasing plasma nitrite before exercise may allow PAD subjects to train with less pain, at higher workloads for longer durations at each training session, thereby maximizing the beneficial peripheral vascular and skeletal muscle adaptations.
- This relatively simple and inexpensive therapeutic approach could provide clinically meaningful changes in exercise capacity beyond current best practice.

PAD with intermittent claudication is defined as lower limb ischemic pain, which occurs with walking and is relieved by rest. It results in significant reductions in walking ability and quality of life. Here, we report that oral inorganic nitrate supplementation in conjunction with a 3-month supervised exercise rehabilitation program produces superior functional benefits than supervised exercise rehabilitation training plus placebo. This approach, if confirmed, could have the ability to provide clinically meaningful changes in exercise capacity beyond the current best practice option. This could significantly reduce the burden of exercise participation and would be a significant step forward in the available therapeutic options for patients with PAD+intermittent claudication.

Nonstandard Abbreviations and Acronyms

BR	beetroot juice
COT	claudication onset time
EX+BR	exercise plus beetroot juice
EX+PL	exercise plus placebo
IC	intermittent claudication
PAD	peripheral artery disease

reactive oxygen species,[9] mitochondrial dysfunction,[9] and a preferential loss of type I oxidative fibers.[10] Overall, this results in the uncoupling of skeletal muscle cellular metabolism from tissue perfusion[11] and the exhibition of a glycolytic phenotype, which promotes the early onset of fatigue and exercise intolerance.

NO is an important signaling molecule, which is essential for vascular function/health. In healthy human exercise studies, increased vascular NO bioavailability has been shown to provide ergogenic benefits, especially in low oxygen conditions or in high-intensity activities that rely predominantly on fast-twitch muscle fibres.[12] Interestingly, in vascular pathologies, including PAD, peripheral tissue maladaptations to chronic underperfusion and disuse (coupled with endothelial dysfunction and the inability to endogenously upregulate NO) may provide an ideal target for exogenous supplementation therapies. To date, the use of NO donor compounds has been limited in clinical applications, primarily because of systemic vascular effects often resulting in hypotension.

Plasma nitrite was once considered a biologically inert byproduct and marker of endothelial NO production.[13,14] However, several studies have demonstrated an endocrine-like role for NO equivalents,[15,16] including nitrite,[17,18] which function to conserve and transport NO and release it under hypoxic and acidic conditions.[17] This suggests increasing plasma nitrite may be an innovative way to create a delivery pool for NO, which would target the peripheral tissues that are underperfused because of occlusive vascular disease and maybe especially beneficial during exercise. It would also avoid systemic pressor effects.

In patients with PAD+IC, we have observed (1) a net consumption of plasma nitrite following maximal exercise,[6] (2) a change in plasma nitrite that predicted an increase in exercise performance,[19,20] and (3) a single oral dose of a inorganic nitrate (9 mmol) in the form of beetroot juice (BR) produced an 18% increase in pain-free walking (COT) on a graded treadmill test.[21] These findings suggest that elevated plasma nitrite allows patients with PAD+IC to exercise with less pain, at higher workloads for longer durations.

Currently, treatment options to increase function in PAD are limited. Supervised exercise rehabilitation has a class IA recommendation and confers superior ambulatory benefits than pharmacological or surgical interventions,[22,23] with lower costs, morbidity, and mortality.[24] Supervised exercise was recently approved for reimbursement coverage by the Centers for Medicare and Medicaid Services, so will likely be utilized on a much wider basis in the future. It is unknown whether the concurrent addition of an inorganic nitrate supplementation to exercise rehabilitation would beet the best available therapeutic approach for PAD+IC.

Accordingly, the primary hypothesis of this pilot trial was, in patients with PAD+IC, regular consumption of a high inorganic nitrate-containing supplement (BR), in conjunction with supervised exercise (exercise plus BR [EX+BR]) during a 12-week period would produce greater clinical benefit via increases in pain-free exercise tolerance (COT, 6-minute walk), relative to exercise plus placebo (EX+PL). A secondary hypothesis was that these differences would be mediated by increased NO bioavailability/signaling, skeletal muscle microvascular tissue perfusion, and tissue structural adaptations.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

The NO-PAD trial was a randomized, double-blind, per-protocol design, details of which have been published[25] and registered on http://www.clinicaltrials.gov (NCT01684930 and NCT01785524). Briefly, we recruited patients aged between 40 and 80 years with diagnosed PAD (ankle-brachial index, <0.90; Table). All subjects were required to have stable IC pain as the limiting factor in their exercise ability.

Table. Subject Demographics

	All (n=24)	EX+PL (n=13)	EX+BR (n=11)
Age, y	69.7±8.1	71.5±7.3	67.5±8.6
Men, n (%)	15 (62.5)	6 (46.2)	9 (82)
Height, cm	171.9±9.8	170.3±8.5	173.7±11.3
Mass, kg	80.4±15.8	79.7±12.6	81.3±19.6
BMI	27.1±4.4	27.5±4	26.6±5.1
Diabetes mellitus, n (%)	8 (33.3)	2 (15.4)	6 (54.5)
Blood glucose, mg/dL	128.3±29.7	139.5±39.2	118.9±14.2
Statin status, n (%)	18 (75)	10 (77)	8 (73)
Smoking status, n (%)			
Current	9 (37.5)	5 (38.5)	4 (36.4)
Former	9 (37.5)	5 (38.5)	4 (36.4)
Never	6 (25)	3 (23.0)	3 (27.2)
Myocardial infarct, n (%)	4 (16.7)	2 (15.4)	2 (18.2)
Stroke, n (%)	4 (16.7)	1 (7.6)	3 (27.3)
Periph intervention, n (%)	4 (16.7)	1 (7.6)	3 (27.3)
Race, n (%)			
White	15 (62.5)	7 (53.8)	8 (72.7)
Black	7 (29.2)	5 (38.5)	2 (18.2)
Asian	1 (4.2)	0 (0)	1 (9.1)
Hispanic	1 (4.2)	1 (7.7)	0 (0)

Mean±SD except where otherwise noted. There were no significant differences between any of the metrics. BMI indicates body mass index; BR, beetroot juice; EX, exercise; and PL, placebo.

The study was approved by the Duke University Medical Center Institutional Review Board, and all subjects signed an informed consent before participation.

After baseline exercise and vascular testing, subjects were randomly assigned to consume either 70 mL (4.2 mmol NO_3^-) BR (EX+BR) or an identical nitrate-depleted placebo (EX+PL), 3 hours before each exercise training visit (3× per week for 12 weeks). To check for beverage compliance and nitrate-to-nitrite conversion, several scheduled and unannounced blood draws were obtained. Based on previous studies and known variabilities in nitrate-to-nitrite conversion,[26] an a priori acceptable range for resting plasma NO_2^- concentrations (before exercise) was set at ≥400 nM nitrite for EX+BR and ≤400 nM for EX+PL (Figure 1).[25] Additionally, all subjects included in the per-protocol analysis completed at least 34 of the 36 supervised exercise training sessions. Each session included at least 30 minutes of actual walking, with the intensity tailored to each subjects' initial baseline maximal graded exercise test results. When claudication pain became moderately severe (3–4/5 on a 5-point claudication pain scale), they would step off the treadmill and rest until the pain subsided (rest periods were not included in the 30-minute exercise time). Typically, after a patient was able to walk 8 to 10 minutes at the initial workload, the grade was increased by 0.5%, or the speed increased by 0.1 mph as tolerated.

Statistics

This was a repeated-measures design, with the purpose of assessing change over time (12 weeks) for the 2 intervention groups. Because this was a pilot study, the overarching goal of the analyses is to derive effect sizes (ie, effectiveness) for this intervention for a future larger confirmatory study.

Comparisons were made using a general linear model ANOVA adjusted for baseline values. P <0.05 (2 tailed) was used as the criterion to declare statistical significance. Changes in individual and treatment group near-infrared spectroscopy spectra generated from the incident leg gastrocnemius muscle tissue monitoring during the pre- and post-treatment maximal graded exercise test were examined for statistical significance by χ^2 test between groups. Deoxyhemoglobin spectra were fit to a 3-parameter exponential decay curve ($f=Y_o+a\times e^{[b\times x]}$), whereas oxyhemoglobin spectra were fit to a single rectangular 2-parameter hyperbola ($f=a\times x/[b+x]$). Individual parameters were directly compared by Z score using the H_o of no significant difference between treatment groups.

Results

Thirty-five patients were enrolled in the study and after baseline testing were randomly allocated to EX+BR (n=17) or EX+PL (n=18). During the trial, 3 participants from each group withdrew participation. A further 3 subjects from EX+BR and 2 from EX+PL were excluded because of plasma nitrite concentrations outside the a priori-specified ranges.

There were no differences in plasma nitrate and nitrite concentrations at the initial baseline testing visit or at the end of the 12-week study testing (≈1 week after the final BR dosage; Figure 1).

Measures of clinical function (Figure 2) or tissue perfusion (Figure 3) were also not different at baseline (pre). After the 12-week protocol (post), COT increased by 59.2±57.3 s for the EX+PL group, and by 180.3±46.6 s for the EX+BR group (P≤0.05). This produced a between treatment medium to large standardized effect size (Cohen d) of 0.62 (95% CI, −0.23 to +1.44; Figure 2). The data for 6-minute walk showed a similar pattern with increases of 24.6±12.1 and 53.4±19.6 m (P≤0.05) in the EX+PL and EX+BR groups respectively with a standardized effect size of 0.43 (95% CI, −0.44 to +1.21; Figure 2). Peak walk time increased significantly for both groups 238.7±207.0 and 269.9±195.3 s, respectively (both P ≤0.01, data not shown).

Indices of gastrocnemius perfusion showed similar responses to treatments. Resting ankle-brachial index measures showed no changes after EX+PL but increased by 0.16±0.11 after EX+BR (P≤0.05) with a standardized effect size of 0.88 (95% CI, −0.01 to +1.70; Figure 3). Measures of limb blood flow at rest (using strain-gauge plethysmography) were not different between groups and did not change after either treatment. Responses after a 5-minute arterial occlusion of the thigh (peak reactive hyperemic blood flow) did not change (0.74+2.67 mL blood per 100-mL tissue per minute) for EX+PL but increased by 2.57±2.65 mL blood per 100-mL tissue per minute (P≤0.05) for EX+BR, with a between treatment effect size of 0.65 (95% CI, −0.21 to +1.46; Figure 3).

Similarly, measures of gastrocnemius tissue perfusion during the maximal graded exercise test revealed differences in the near-infrared spectroscopy spectra characteristics between treatments (Figure 3E and 3F; Online Figure I). There was no difference in the deoxygenation characteristics of the EX+PL group over time; however, the change in initial desaturation rate within the EX+BR group from pre to post was significantly reduced as reflected in an increased parameter a, (4.07±0.111; P≤0.05; Figure 3E). Conversely, the change in the oxyhemoglobin spectra over time indicated

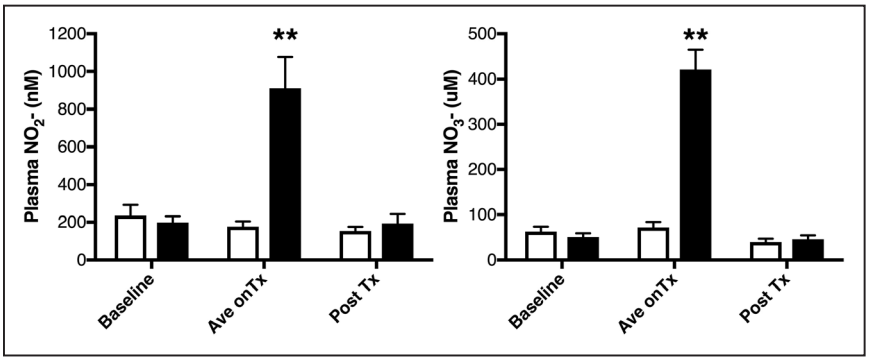

Figure 1. Venous plasma (left) NO_2^- and (right) NO_3^- concentrations at baseline, during the 12 wk of training (mean of several tests-Ave on Tx), and at the end of study testing (post Tx). Exercise (EX)+placebo (clear bars/circles) or EX+beetroot juice (black bars/circles). ***P*<0.01 vs other values.

a significant increase in the maintenance of oxygenation in both the EX+PL and EX+BR groups after the 12-week treatment period. This improvement was again significantly greater in the EX+BR treatment group, both in the initial rate (parameter a) and in plateau rate (parameter b; Figure 3F; Online Table I).

Discussion

This study shows that oral inorganic nitrate supplementation before exercise rehabilitation 3× per week for 12 weeks produced increases in pain-free exercise tolerance. However, the overall hypothesis that these changes would be superior to exercise rehabilitation alone could not be confirmed, although moderate to strong effect sizes were observed. COT was chosen as the major end point of this trial because it may best represent changes after treatment in gastrocnemius tissue perfusion and mitochondrial metabolism. Interestingly, the increase in COT for exercise alone was similar to our previous exercise-only PAD trial[19] confirming that the current pilot study was likely effective but underpowered.

In a practical context, the combination of exercise and BR improved COT by 121.1 s (95% CI, −23.7 to 265.9 s), which was a 200% increase over exercise alone. This is slightly >1 complete stage on the treadmill graded exercise test. For the 6-minute walk test, often regarded as more representative of activities of daily living,[27] a clinically meaningful change in patients with chronic heart failure, who have a similar functional level to PAD, is 30 m.[28] In the current study, the EX+BR group walked 28.8 m (95% CI, −16.3 to 73.9) further than the EX+PL group. These data are suggestive that the addition of BR to the intervention was clinically beneficial beyond the currently optimal treatment of exercise alone.

The secondary hypothesis that the differences were mediated by increased NO bioavailability/signaling is supported by the significantly greater plasma nitrite levels during the 12-week training intervention. Importantly, the ankle-brachial index and peak blood flow measurements were made after the training period but without acute nitrate/nitrite/NO supplementation. Therefore, the increases in ankle-brachial index and peak limb blood flow for the EX+BR group at the postintervention testing suggest greater tissue structural changes may have occurred. One possible mechanism for such a structural change is an increase in collateralization and downstream angiogenesis via NO-mediated mechanisms, which result in reduced tissue desaturation. In this regard, it is interesting that exercise alone produced no significant decrease in tissue deoxygenation within the gastrocnemius on exercise, as determined by near-infrared spectroscopy. However, the EX+BR

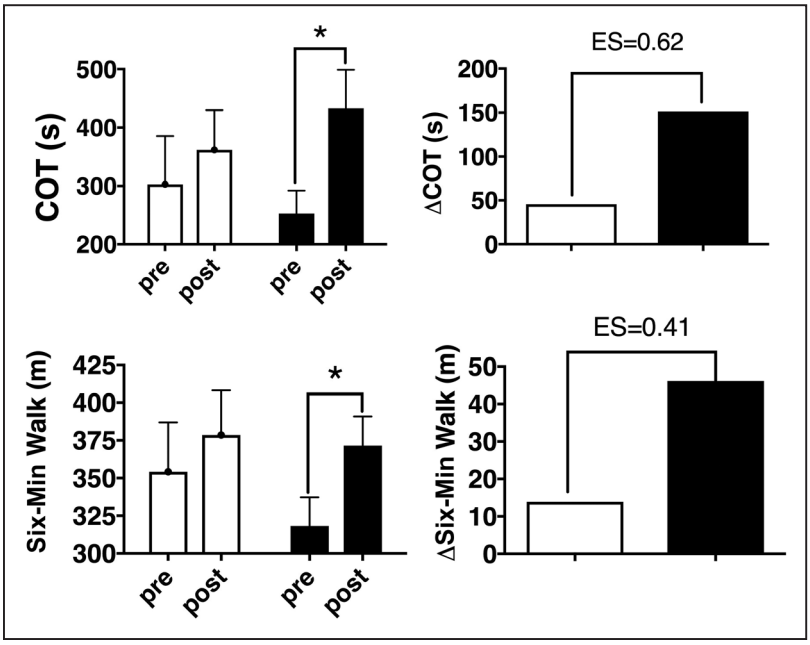

Figure 2. Left, Raw data and (right) group changes for (top) claudication onset time (COT) and (bottom) 6-min walk test after 12 wk of exercise (EX)+placebo (clear bars/circles) or EX+beetroot juice (black bars/circles). **P*<0.01 within treatment. ES indicates effect size.

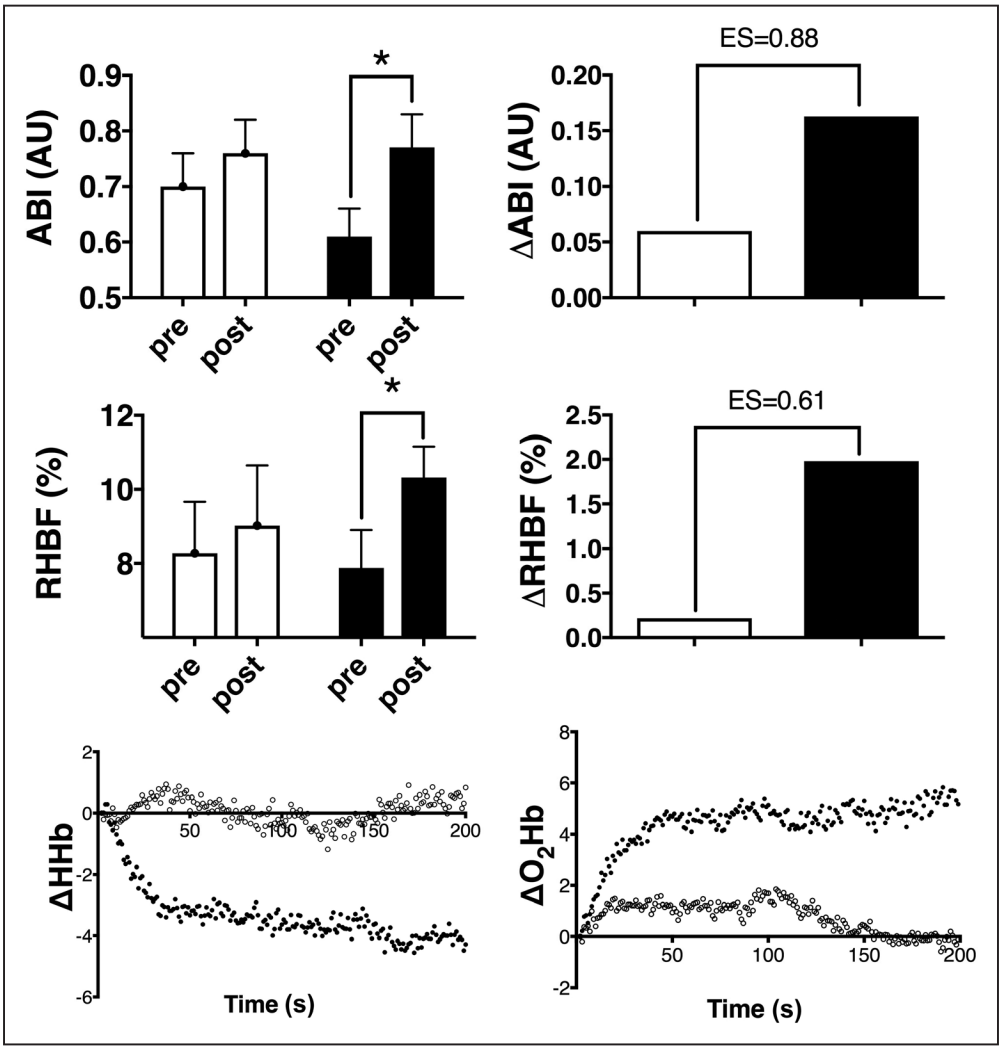

Figure 3. Left, Raw data and (right) group changes for (top) ankle-brachial index (ABI; arbitrary units) and (middle) reactive hyperemic blood flow (RHBF; mL/min per 100-mL tissue or %). Bottom, Group average changes in near-infrared spectroscopy spectra from pre to post-treatment (**left**) deoxyhemoglobin and (**right**) oxyhemoglobin. Exercise (EX)+placebo (clear bars/circles) or EX+beetroot juice (black bars/circles). *P<0.05 within treatment. ES indicates effect size.

combination resulted in reduced tissue deoxygenation on exercise initiation (Figure 3E). These data suggest that oxygen extraction within the working tissue is improved with BR supplementation. Although we have no direct evidence from tissue samples in this study, a murine hindlimb permanent femoral artery ligation model demonstrated a dose-dependant relationship between nitrite dose (via intraperitoneal injection BID for 7 days) and improved tissue perfusion via angiogenesis.[29] Coadministration of the NO scavenger carboxy-PTIO (2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide) with the nitrite completely abrogated the increase in perfusion suggesting the mechanism of effect is NO mediated.

Despite exercise training alone delivering substantial improvements in functional capacity for patients with PAD+IC, limb ischemia and claudication pain are still major limiting factors[30,31] in the intensity, duration, and progression that can be achieved in therapeutic exercise programs. These factors likely contribute to the poor adherence rates often experienced in PAD+IC rehabilitation programs. It is clear that new therapeutic approaches that can raise the ischemic threshold and reduce the burden of exercise in PAD+IC are needed.

The limitations of the current study include the small sample size, 17% drop out rate (not unusual in this population) and lack of an intent to treat design make it premature to speculate on overall clinical utility of a nitrate-based therapy for PAD. However, the early evidence is encouraging. Specifically, our data suggest that increasing plasma nitrite before exercise may allow PAD subjects to train with less pain, at higher workloads for longer durations at each training session,[21] thereby maximizing the beneficial peripheral vascular and skeletal muscle adaptations.

The data from this study suggest that a sample size of 58 subjects per group will have 80% power for detecting a ≥90 s difference in mean COT improvement, at the 2-sided significance level of 0.05. To plan for a 20% loss to follow-up, 73 individuals (58/[1−0.20]≈73) would need to be recruited in each treatment arm.

If our hypotheses are confirmed by such additional clinical trials and basic science research, this novel, relatively simple, and inexpensive-to-implement therapeutic approach would provide clinically meaningful changes in exercise capacity beyond the current best practice option. This outcome could

significantly reduce the burden of exercise participation and may lead a paradigm shift in the treatment and rehabilitation recommendations for PAD+IC.

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Disclosures

None.

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