

Effects of dietary nitrate supplementation on the oxygen cost of exercise and walking performance in individuals with type 2 diabetes: a randomised, double blind, placebo-controlled cross-over trial.

Running title: Dietary nitrate supplementation in T2DM

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ABSTRACT

Background: Dietary nitrate supplementation has been shown to reduce the oxygen (O₂) cost of exercise and enhance exercise tolerance in healthy individuals. This study assessed whether similar effects could be observed in individuals with type 2 diabetes (T2DM).

Methods: In a randomised, double blind, placebo-controlled cross-over study, 48 participants with T2DM supplemented their diet for four days with either nitrate rich beetroot juice (70 ml/day, 6.43 mmol nitrate/day) or nitrate depleted beetroot juice as placebo (70 ml/day, 0.07 mmol nitrate/day). After each intervention period, resting plasma nitrate and nitrite concentrations were measured subsequent to participants completing moderate-paced walking. Pulmonary gas exchange was measured to assess the O₂ cost of walking. Following a rest period, participants performed the six-minute walk test (6MWT).

Results: Relative to placebo, beetroot juice resulted in a significant increase in plasma nitrate (placebo: 57 ± 66 vs. beetroot: 319 ± 110 μ M; $P < 0.001$) and plasma nitrite concentration (placebo: 680 ± 256 vs. beetroot: 1065 ± 607 nM; $P < 0.001$). There were no differences between placebo juice vs. beetroot juice for the O₂ cost of walking (946 ± 221 vs. 939 ± 223 ml·min⁻¹, respectively; $P = 0.59$), and distance covered in the 6MWT (550 ± 83 vs. 554 ± 90 m, respectively; $P = 0.17$).

Conclusion: Nitrate supplementation did not affect the O₂ cost of moderate-paced walking or improve performance in the 6MWT. These findings indicate that dietary nitrate supplementation does not modulate the response to exercise in individuals with T2DM.

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Abbreviations:

ACEi – Ace inhibitor

ARB – Angiotensin receptor blocker

Asymmetric dimethylarginine - ADMA

Carbon dioxide – CO₂

Heart rate – HR

MRT – Mean response time

Nitric oxide – NO

Nitric oxide synthase – NOS

Oxygen – O₂

Oxygen uptake - $\dot{V}O_2$

Reactive oxygen species - ROS

Six-minute walk test – 6MWT

Type 2 diabetes – T2DM

INTRODUCTION

Individuals with type 2 diabetes mellitus (T2DM) have profound reductions in their tolerance to exercise compared to healthy individuals ¹. The discomfort experienced by individuals with T2DM during exercise may impact upon their ability or willingness to attain their recommended level of exercise, which is a key aspect of disease management ². Exercise intolerance in this population has been attributed to abnormalities at multiple points during the transport of oxygen (O₂) from the lungs to its site of utilisation in the muscles³⁻⁵. There is evidence that individuals with T2DM have reductions in the biosynthesis and bioavailability of the biological messenger nitric oxide (NO) ^{6,7}. NO is known to play an important role in muscle contractility, skeletal muscle glucose uptake, calcium handling ⁸, vascular function, blood flow regulation ⁹, and mitochondrial respiration and biogenesis ¹⁰.

There are two known pathways by which NO is synthesised, the L-arginine pathway and the entero-salivary pathway. The L-arginine pathway involves the synthesis of NO from L-arginine by the nitric oxide synthase (NOS) family of enzymes. The entero-salivary pathway involves a stepwise conversion of nitrate to nitrite, and subsequently, nitrite to NO. Briefly, nitrate from the diet (or from NO/nitrite oxidation) is absorbed into the circulation where it is concentrated in the salivary glands. Nitrate is then reduced to nitrite via facultative anaerobic bacteria on the surface of the tongue ¹¹ and subsequently swallowed. Some of the swallowed nitrite is reduced to NO in the acidic environment of the stomach, with important local effects on gastric function and protection against enteric pathogens ¹², and the remainder enters the circulation. Circulating nitrite acts as a reservoir for NO, with its reduction to NO potentiated in acidic or hypoxic areas, such as contracting skeletal muscle ¹³. It has been suggested that the entero-salivary pathway is a complementary system for NO synthesis ¹⁴.

There is extensive evidence in healthy individuals that nitrate supplementation, via either sodium nitrate or nitrate rich beetroot juice, increases plasma nitrite concentration ^{15,16}, lowers blood pressure (BP) ^{17,18}, and reduces the O₂ cost of exercise ^{15,19,20}. The reduction in the O₂ cost of exercise has been proposed to be related to a reduction in the ATP cost of muscle force production ¹⁷, an improvement in mitochondrial efficiency ²¹, or a combination of both ²².

It has been reported that the bioavailability of NO is reduced in individuals with T2DM due to increased scavenging and/or reduced synthesis. Reactive oxygen species (ROS) ²³ and hyperglycaemia ²⁴ may result in lower L-arginine derived NO. Further, there is evidence that NO production is suppressed by NOS inhibitors such as asymmetric dimethylarginine (ADMA) ²⁵ and caveolin-1 ²⁶, which are elevated in T2DM. Conversely, others have reported increased plasma nitrate and nitrite concentrations in individuals with T2DM ^{27,28}, which may indicate a quenching of NO activity or upregulation of NO synthesis to counteract resistance. It is possible that dietary nitrate supplementation in this population could ameliorate the impact of T2DM on NOS dependent NO synthesis by increasing the amount of NO produced via the entero-salivary pathway ¹⁴. We recently reported no effect of dietary nitrate supplementation on the 24h ambulatory BP in individuals with T2DM ¹², but the impact on the O₂ cost of exercise, and exercise tolerance in individuals with T2DM remains unknown.

The aim of this study was, therefore, to examine the effects of dietary nitrate supplementation (nitrate rich beetroot juice) on the O₂ cost of exercise and walking performance, and to confirm our previous finding regarding resting BP, in individuals with T2DM. It was hypothesised that, compared to nitrate depleted beetroot juice, nitrate rich beetroot juice would reduce the amount of O₂ required per unit of time to perform moderate-paced walking, and increase the distance covered in the 6-minute walk test.

METHODS

Patients

Forty eight patients (35 males) with T2DM (see Table 1 for characteristics) volunteered to participate in this randomised, double blind, placebo-controlled cross-over study. Participants were recruited from the NIHR Exeter Clinical Research Facility, Exeter 10,000 cohort, a database of individuals who have consented to be contacted for research. Ethical approval was granted by the Cornwall and Plymouth NRES Committee 12/SW/0118 and the study was registered as a clinical trial on the ClinicalTrials.gov website, ID # NCT02206074. All participants provided written informed consent. Patients were recruited if they had been diagnosed with T2DM (as defined by the WHO) at least five years prior to enrolment in the study and were 35–75 years of age. Patients were excluded from the study if they had significant renal impairment (eGFR <30), uncontrolled hypertension, a BMI <25 or >35 (kg/m²), a history of myocardial infarction or cerebro-vascular event, were taking regular organic nitrates or nicorandil, or were smokers (or who had stopped smoking within the previous three months). Patients taking phosphodiesterase inhibitors were asked to refrain from doing so for the duration of the study. On experimental days, participants were asked to arrive at the laboratory in a rested and fully hydrated state, at least 3 hours postprandial, and having avoided caffeine and alcohol for 6 and 24 hours, respectively. Participants were also asked to avoid exercise for 24 hours prior to each testing session. Participants were asked to record their diet for 24 hours prior to each experimental visit. Following the crossover the participant was asked to replicate their previous diet and this was verbally confirmed at the second visit.

Table 1. Characteristics of patients included in the final analysis. Data are mean \pm SD, or as a % of the cohort on a medication.

Age (years)	63.35 \pm 7.27
Diabetes Duration (years)	10.31 \pm 5.26
HbA1c (mmol/mol)	60.83 \pm 13.37
Body Mass Index (kg/m ²)	30.17 \pm 2.93
Baseline systolic BP (mmHg)	142.13 \pm 12.84
Baseline diastolic BP (mmHg)	80.85 \pm 7.36
No. of 30+ minutes of exercise per week	3.83 \pm 3.16
Portions of fruit and vegetables per day	3.83 \pm 2.00
Metformin %	83.33
Insulin %	25.00
ARB & ACEi %	47.92
Statins %	83.33

Pre-experimental tests

Participants attended the NIHR Exeter Clinical Research Facility where we completed informed consent, a medical history, anthropometric measures, and a resting ECG. A familiarisation session was performed for the treadmill exercise, allowing the determination of the walking speed for each participant that would be used in the experimental visits. The target speed was a pace equivalent to the participants' usual walking speed that could comfortably be maintained for 6 minutes.

Experimental Overview

Following the pre-experimental visit, participants were assigned in a double-blind, randomised, crossover design to consume 70 ml/day of nitrate rich beetroot juice (beetroot juice; containing 6.43 mmol of nitrate; Beet it, James White Drinks Ltd., Ipswich) or nitrate depleted beetroot juice (placebo; nitrate depleted beetroot juice containing 0.07 mmol of nitrate; Beet it, James White Drinks Ltd., Ipswich) for four days. The dose of 6.43 mmol of nitrate equated to 0.072 mmol.kg⁻¹ per day for 3.5 days. Similar dosing regimens (0.068¹⁷; 0.064²⁹; 0.086³⁰mmol.kg⁻¹) to ours have been shown to be effective at this relative dose. Production of the placebo juice has been detailed previously³¹, with the final product being indistinguishable in taste, colour, texture, appearance and odour to the nitrate rich beetroot juice³². The final 70 ml of juice was consumed ~3 hours before the commencement of exercise on the morning of testing. Participants were required to abstain from using antibacterial mouthwash and chewing gum throughout the study, as this has been shown to reduce the concentration of oral bacteria responsible for the reduction of nitrate to nitrite³³.

During the two experimental visits, resting BP was measured and venous blood samples were drawn into lithium-heparin tubes (Sarstedt S-Monovette, Nümbrecht, Germany) and prepared for nitrate and nitrite analysis as previously described³⁴. Subsequently, participants performed 3 bouts of walking on a motorised treadmill (NordicTrack T14.0, Chaska, MN, USA) at the target speed that was determined in the pre-experimental visit. Each bout was preceded by a resting baseline period of three minutes, with the three bouts separated by 15 minutes of passive recovery. Following a further 15 minutes of seated rest, participants performed a 6 minute walk test (6MWT) to assess functional capacity. This test was conducted indoors on a straight, flat course between 2 cones which were 32.2 meters apart, with patients instructed to

cover as much distance as possible in the allotted time. Standardised verbal encouragement was given throughout. Following their first experimental visit, participants began a washout period (10-14 days) before entering the opposing arm of the study.

Measurements

Prior to any exercise testing during the experimental visits, resting BP of the brachial artery was measured using an automated sphygmomanometer (Omron M6, Kyoto, Japan). Five measurements were taken in total, with the mean of the final three measurements being recorded.

For the determination of plasma nitrite concentration, venous blood samples (~4 ml) were drawn into lithium-heparin tubes (Sarstedt S-Monovette, Nümbrecht, Germany), centrifuged, aliquoted and immediately frozen in liquid nitrogen and stored at -80°C. Prior to analysis all samples were deproteinized using a variant of the protocol used by Higuchi, Motomizu³⁵. Fresh reagents were created each day and checked for contamination. No contamination was detectable for water, sodium hydroxide, zinc sulphate or sodium iodide. Determination of plasma nitrate and nitrite concentrations was performed on a nitric oxide analyser via ozone chemiluminescence (Sievers NOA 280; Analytix Ltd, Durham, UK) using the protocol described by Bateman, Ellis, Freeman³⁶.

Pulmonary gas exchange was measured breath by breath during all treadmill walking exercise (MedGraphics Cardio₂ Cardiopulmonary Diagnostic Systems, St. Paul, MN, USA). The volume transducer was calibrated before each test with a 3-liter calibration syringe (Hans Rudolph, Kansas City, MO, USA) and the O₂ and CO₂ analysers were calibrated using gases of known concentration. During treadmill walking exercise, heart rate (HR) was measured every 5 seconds via telemetry (Polar R5400sd, Kempele, Finland).

Data Analysis

The breath by breath oxygen uptake ($\dot{V}O_2$) data were initially inspected for errant breaths (e.g. associated with coughing and swallowing), with values lying more than four SDs from the local mean being removed. The breath-by-breath data were linearly interpolated to provide second-by-second values and, for each individual, the three bouts were time-aligned to the start of exercise and ensemble-averaged. A nonlinear least-square algorithm was used to fit the data, as described in the following equation:

$$\dot{V}O_2(t) = \dot{V}O_{2\text{ baseline}} + A_p(1 - e^{-(t - TD_p)/\tau_p})$$

where $\dot{V}O_2(t)$ represents the absolute $\dot{V}O_2$ at a given time t ; $\dot{V}O_{2\text{ baseline}}$ represents the mean $\dot{V}O_2$ in the baseline period (when participants were stood still on the treadmill); A_p , TD_p , and τ_p represent the amplitude, time delay, and time constant, respectively, describing the increase in $\dot{V}O_2$ above baseline when exercise had commenced. The end-exercise $\dot{V}O_2$ was defined as the mean $\dot{V}O_2$ measured over the final 30 seconds of exercise. The mean response time (MRT) was calculated by fitting a single exponential curve to the data with no time delay from the onset to the end of exercise. The O_2 deficit was calculated as the product of the $\dot{V}O_2$ response amplitude (i.e. the baseline to the point that a steady state was attained) and the MRT. For a schematic representation of the $\dot{V}O_2$ kinetics parameters, see Figure 1.

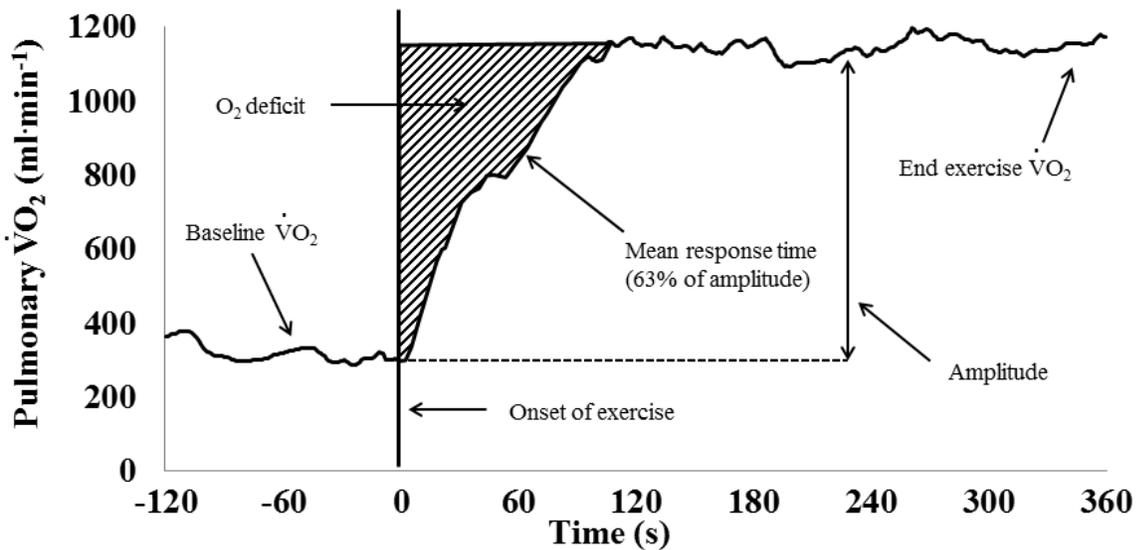


Figure 1. Schematic showing the parameters of the oxygen uptake kinetics on a representative participant's data.

Juice analysis

It has previously been suggested that the antioxidant content of beetroot juice may have beneficial effects³⁷. In order to establish whether the concentrations of known antioxidants in beetroot juice (polyphenols and betacyanins) were different in the active and placebo juice, both were analysed using the high-performance liquid chromatography technique according to our previously described methods³⁸.

Sample size and randomisation

An *a priori* sample size calculation was performed by a statistician from the University of Exeter Medical School. A pilot study (n=6) in individuals who have T2DM revealed a mean difference between beetroot juice and placebo juice of 45 ml·min⁻¹ for end exercise $\dot{V}O_2$ (1SD) during cycling exercise. For 90% power and an α -level set at $P = 0.05$ (two tailed), to detect a 0.5

SD difference 44 patients were required. In order to account for dropout, we anticipated that 48 patients would begin the study. Each participant was randomised by a research nurse, who also supplied them with the appropriate juice.

Data and statistical analysis

All data were tested for normality and are presented as means \pm standard deviation (*SD*) unless otherwise stated. Differences in plasma nitrate and nitrite concentrations, BP, $\dot{V}O_2$, and distance covered in the 6-minute walk test between the conditions were analysed using two-tailed, paired-samples *t*-tests. Where normality of data was not met, differences between the aforementioned data were tested using a non-parametric test (Wilcoxon rank-sum test). When the sample was split for patients taking different classes of medications, independent samples *t*-tests were performed. Pearson product-moment correlation coefficients were used to assess the relationships between variables. Statistical analyses were performed on SPSS software version 21.0 (Chicago, IL, USA), with statistical difference accepted when $P < 0.05$.

Results

48 subjects completed the study. For detailed information regarding participant recruitment and withdrawal, see Figure 2. Participants' self-reported adherence to beetroot juice and avoidance of mouth wash was 100% for both arms of the study. All participants reported similar physical activity and dietary patterns for both supplementation periods. The ingestion of both juices was well tolerated with no adverse side effects. As in previous studies using beetroot juice supplementation, participants reported beeturia (red urine) and red stools ¹⁵.

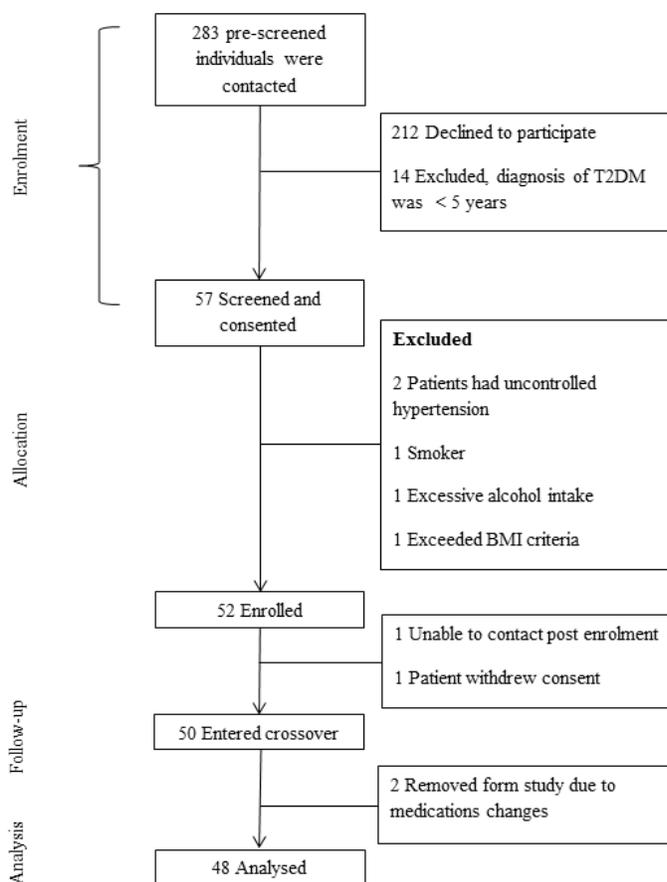


Figure 2. Flow diagram of trial

Plasma nitrate and nitrite concentrations: Relative to placebo, beetroot juice resulted in a significant increase in plasma nitrate concentration (placebo: 57 ± 66 vs. beetroot juice $319 \pm 90 \mu\text{M}$, $P < 0.001$, 95% CI -220, 302; Figure 3A). Plasma nitrite concentration also increased for beetroot juice in comparison to placebo (placebo: 680 ± 256 vs. beetroot juice 1065 ± 607 nM, $P < 0.001$, 95% CI -220, 548; Figure 3B). There were no differences in baseline (placebo) plasma nitrite when subjects were split into groups based on drug classes; ACEi and ARB ($n = 25$; difference vs. those not on these drugs -68 ± 75 nM, $P = 0.37$, 95% CI -218, 83), metformin ($n = 40$; difference -18 ± 105 nM, $P = 0.87$, 95% CI -231, 196), insulin ($n = 12$; difference 3 ± 86 nM,

$P = 0.97$, 95% CI -171, 177) statins ($n = 40$; difference 164 ± 97 nM, $P = 0.09$, 95% CI -31, 360) and sulphonylurea ($n = 15$; difference 35 ± 134 nM, $P = 0.79$, 95% CI -237, 306). Other classifications of drugs were prescribed, however the sample size was too small to make inferences; gliptins ($n = 2$), exenatide ($n = 2$), sulfazalazine ($n = 1$), alpha blocker ($n = 3$), beta blocker ($n = 1$), calcium channel blocker ($n = 6$), loop diuretic ($n = 1$), thiazide ($n = 2$) $P > 0.05$).

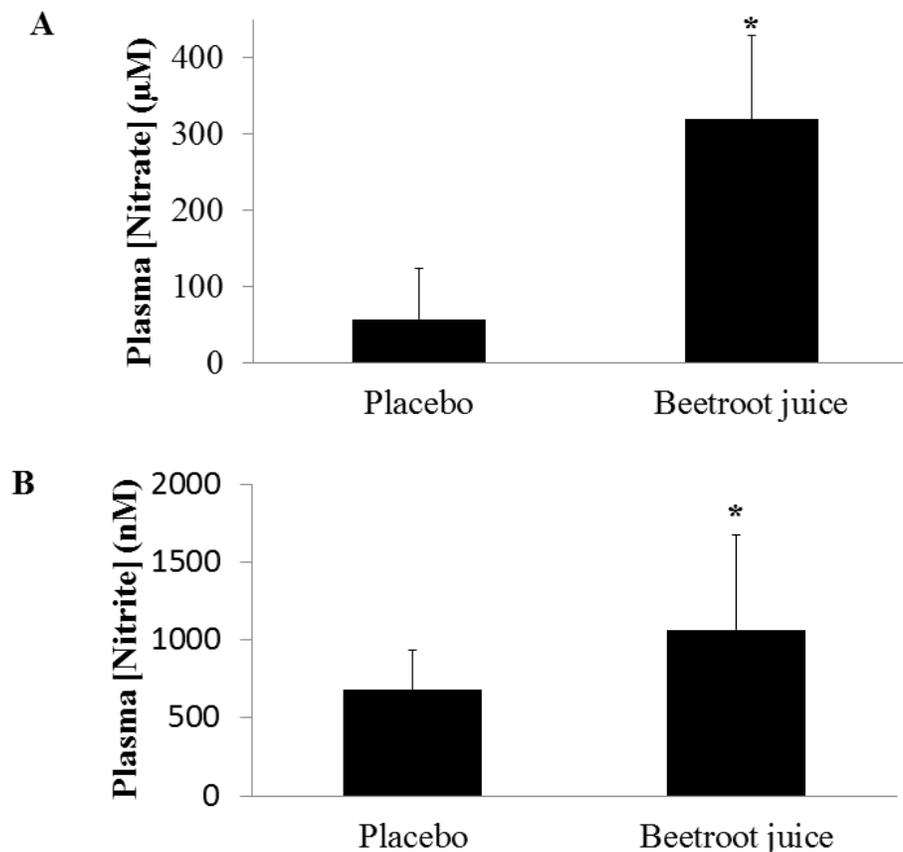


Figure 3. A depicts plasma nitrate and B shows plasma nitrite group mean concentrations analysed via chemiluminescence. Values are means \pm SD. *significantly different from the placebo $P < 0.001$.

Treadmill walking, $\dot{V}O_2$ kinetics, and heart rate: Relative to placebo, beetroot juice supplementation had no effect on baseline (placebo: 282 ± 50 vs. beetroot juice: 281 ± 46 ml.min⁻¹; $P = 0.82$, 95% CI -12.9, 10.21) or end-exercise $\dot{V}O_2$ (placebo: 939 ± 223 vs. beetroot

juice: $946 \pm 221 \text{ ml}\cdot\text{min}^{-1}$; $P = 0.60$, 95% CI -20, 34.3; Figure 4). The time constant and MRT of $\dot{V}O_2$ response, and the O_2 deficit were also not different between conditions (see Table 2). End-exercise heart rate was not different between conditions (placebo: 96 ± 12 vs. beetroot juice: 94 ± 11 BPM, $P = 0.36$, 95% CI -4.7, 1. 7). No significant correlation was observed between the change in plasma nitrite concentration and end-exercise $\dot{V}O_2$ ($r = 0.04$, $P = 0.77$).

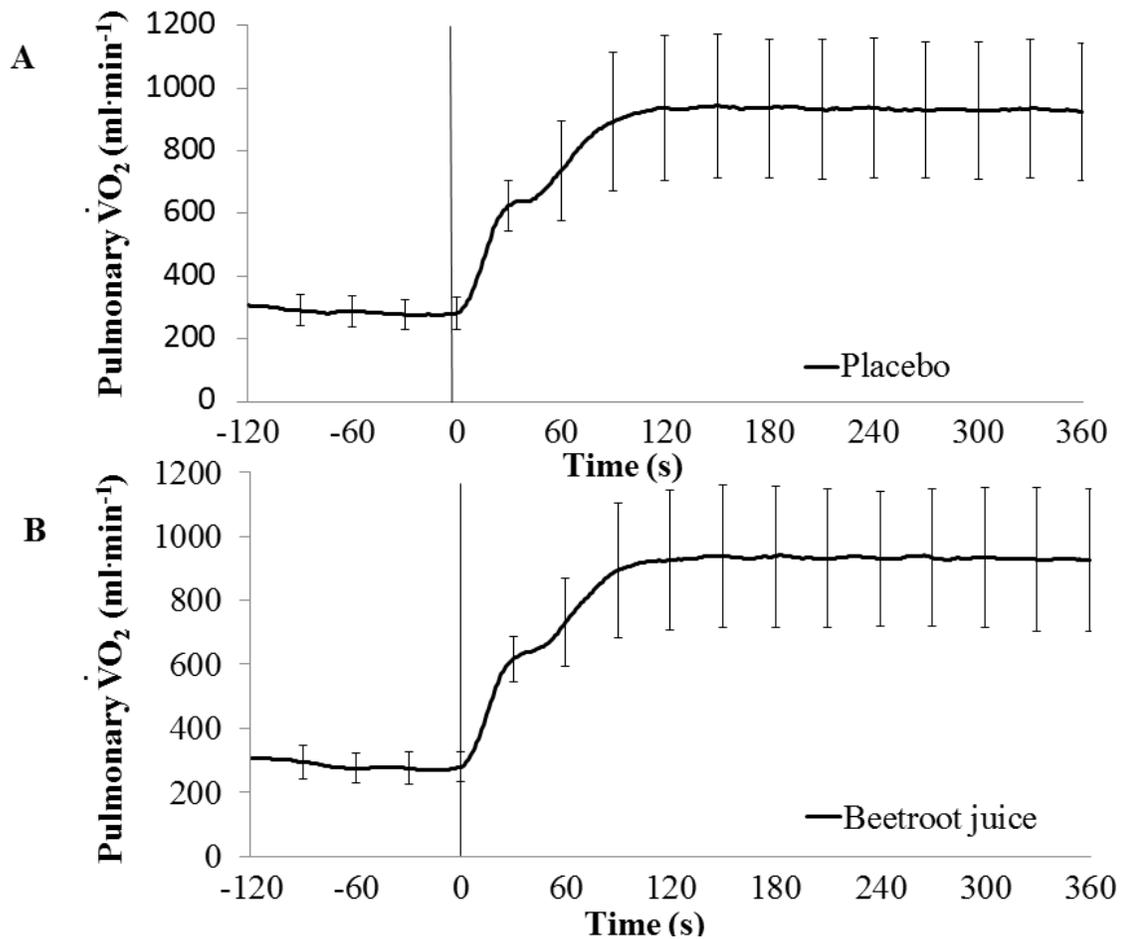


Figure 4. The group mean pulmonary oxygen uptake response for placebo (A) and nitrate-rich beetroot (B). The vertical line represents the initiation of walking exercise from the standing baseline. Group mean responses are shown with error bars every 30s.

Table 2. Oxygen uptake kinetics during walking exercise with placebo and nitrate-rich beetroot supplementation. Data are mean \pm SD.

	Placebo	Beetroot juice
Baseline (ml·min ⁻¹)	282 \pm 50	281 \pm 46
Primary amplitude (ml·min ⁻¹)	656 \pm 205	665 \pm 199
End-exercise (ml·min ⁻¹)	938 \pm 223	946 \pm 221
O ₂ deficit (L·min ⁻¹)	0.48 \pm 0.17	0.50 \pm 0.19
Mean response time (s)	45 \pm 10	45 \pm 10

Six minute walk test: No difference was found between placebo and beetroot juice for distance covered during the six minute walk test (placebo: 554 \pm 90 vs. beetroot juice: 550 \pm 83 m, $P = 0.17$, 95% CI -11, 2.04). No difference was observed for distance covered during the six minute walk test when the group was split for ACEi and ARB compared with individuals not on this classification of drug (change -7.6 \pm 44.6 m, $P = 0.25$, 95% CI -20.6, 5.4).

Blood pressure: Comparisons between placebo and beetroot juice revealed no statistically significant effect on systolic BP (placebo: 134 \pm 10 vs. beetroot juice: 132 \pm 12 mmHg, $P = 0.17$, 95% CI -4.09, 1.17) or diastolic BP (placebo: 77 \pm 7 vs. beetroot juice: 76 \pm 11 mmHg, $P = 0.27$, 95% CI -4.04, 0.74; Figure 5 A and B).

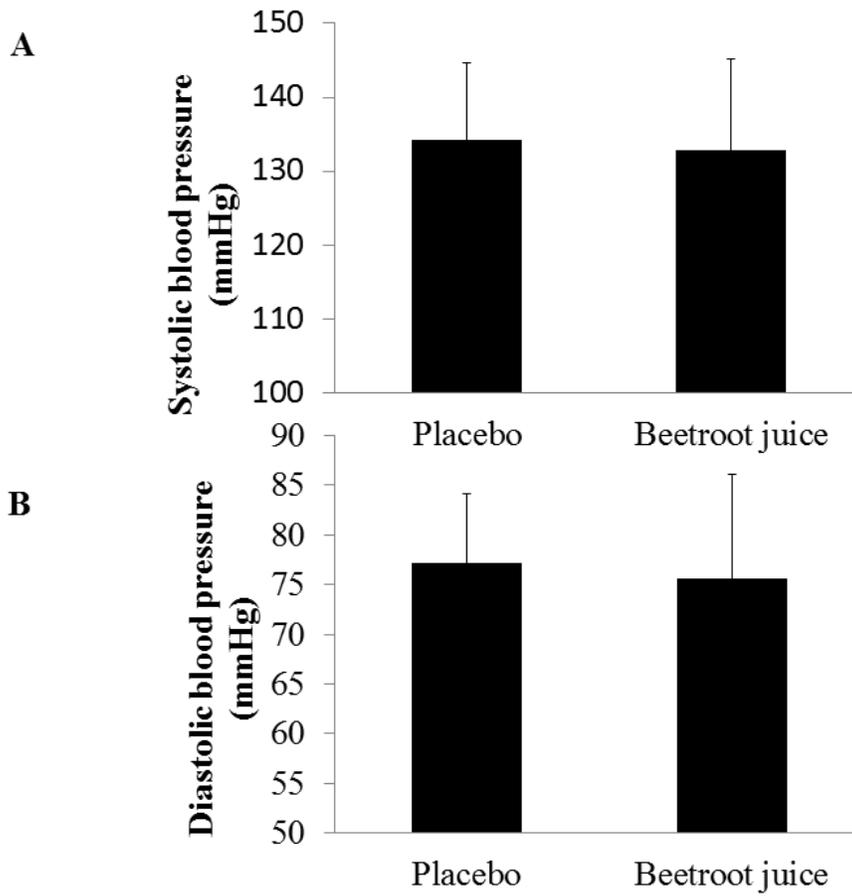


Figure 5. Effects of nitrate on blood pressure, A: Groups means for systolic blood pressure and B depicts the group mean for diastolic blood pressure. Values are means \pm SD.

Antioxidants in beetroot juice: Antioxidant concentrations in placebo and active beetroot juice are displayed in Table 3.

Table. 3. The content of total betacyanins and polyphenol compounds in two different beetroot juices. Table A, depicts all identified compounds and figure B shows the compound groups. Mean value \pm standard deviation (in mg/100 ml of fresh juice).

A

Identified compound	Beetroot juice	Placebo
Betanin-3- <i>O</i> -glucoside	452.71 \pm 19.56	519.31 \pm 11.43
Betanidine	6.03 \pm 0.40	3.61 \pm 0.19
Gallic acid	1.80 \pm 0.12	1.43 \pm 0.14
Chlorogenic acid	3.03 \pm 0.55	2.10 \pm 0.22
Caffeic acid	0.90 \pm 0.20	0.58 \pm 0.11
Ferulic acid	0.56 \pm 0.00	0.53 \pm 0.01
Rutinoside-3- <i>O</i> -quercetin	1.97 \pm 0.08	1.81 \pm 0.04
Glucoside-3- <i>O</i> -quercetin	0.89 \pm 0.04	0.83 \pm 0.02
Myricetin	0.44 \pm 0.01	0.47 \pm 0.02
Luteolin	0.23 \pm 0.01	0.16 \pm 0.01
Quercetin	0.25 \pm 0.01	0.19 \pm 0.00
Kaempferol	0.22 \pm 0.01	0.17 \pm 0.00

B

Group of compounds	Beetroot juice	Placebo
Total betacyanins	458.73 \pm 19.73	522.92 \pm 11.43
Total polyphenols	10.29 \pm 0.72	8.30 \pm 0.34
Total phenolic acids	6.30 \pm 0.67	4.65 \pm 0.34
Total flavonoids	3.99 \pm 0.10	3.65 \pm 0.05

Discussion

This is the first study to investigate the effects of dietary nitrate supplementation on the exercise responses of individuals with T2DM. The principal findings of this investigation were that short-term dietary nitrate supplementation did not reduce the O₂ cost of walking, or increase the distance covered in the six minute walk test in this population. We also confirmed our previous finding that nitrate supplementation had no effect on resting blood pressure in individuals with T2DM. These findings may be considered surprising given the compelling effects of dietary nitrate supplementation reported in other populations. Possible explanations for the lack of effects in the present study relate to an elevated baseline plasma nitrite concentration in the control condition, and/or reductions in the bioavailability of NO in individuals with T2DM.

Nitrate supplementation and plasma nitrate and plasma nitrite concentration.

Plasma nitrate and nitrite concentrations were significantly elevated following nitrate supplementation, which is consistent with previous studies examining young^{15,16} and older healthy participants³⁹, and individuals with peripheral arterial disease⁴⁰. The post placebo plasma nitrite concentration was approximately two to six-times higher in the present study than those reported in the aforementioned studies. The elevated plasma nitrite concentration in individuals with T2DM in the placebo condition may be associated with the habitual up-regulation of iNOS which is endemic in this population⁴¹. It is possible that the elevated baseline (placebo) plasma nitrite concentration is indicative of disease pathology and mitigates the attainment of the benefits of dietary nitrate supplementation that have been reported in other populations.

The plasma nitrite concentration was 680 ± 256 nM in the placebo arm in the present study. In the only other study which could be directly comparable, the plasma nitrite concentration was 232 nmol/L (200, 265), median (IQR) in the placebo arm ³⁴. The control plasma from Gilchrist, Winyard, Aizawa, Anning, Shore, Benjamin ³⁴ was re-measured during the current study to establish agreement between the nitrite values measured in the two studies. It is therefore unlikely that analytical error is the reason for our elevated 'baseline' plasma nitrite concentrations. There are notable differences between the two studies with respect to the timing of beetroot juice doses and plasma sampling. In the present work, studies were conducted at midday, with subjects having had breakfast including the beetroot juice and their usual morning hypoglycaemic and antihypertensive medications 3 hours previously. In our previous study in subjects with T2DM ³⁰, plasma sampling occurred after an overnight fast, with subjects having omitted their usual morning hypoglycaemic and antihypertensive medications, 16 hours after their last beetroot juice.

Multiple agents within these broad classes of medication have been shown to up-regulate eNOS activity ⁴²⁻⁴⁴. When the current cohort was split for drug classifications, no differences in baseline plasma nitrite were detectable. It is therefore unlikely that drug classification affected plasma nitrite concentrations, though the study was not powered to detect any such difference. Furthermore, there are data to suggest circadian variation in eNOS activity, with the lowest levels in the morning, rising through the day before falling again at night ⁴⁵. This could further influence the difference between plasma nitrite concentrations in the two groups. With a growing number of studies in patient groups, understanding the effect of concomitant medication on plasma nitrite and nitrate concentrations is becoming increasingly important. Accordingly, sufficiently powered research is required to examine the pharmacokinetics and dynamics of

medications and NO_x concentrations in order to elucidate the possibility of nitrate related therapeutic effects on exercise performance.

Nitrate supplementation and pulmonary oxygen uptake

There was no difference between active and placebo juice in the $\dot{V}O_2$ responses to low intensity walking exercise in the present study. A reduction in the baseline³¹ and steady state^{15,31,46} O₂ cost of low intensity exercise has previously been reported in young healthy individuals following similar nitrate dosage regimens to that which was implemented in the present study. No change in the O₂ cost of exercise was reported in healthy older individuals following nitrate supplementation³⁹. However, these authors reported a significant speeding of the $\dot{V}O_2$ kinetics, something that was not observed in the present study. It is therefore unlikely that aging *per se* explains the lack of effect on the $\dot{V}O_2$ kinetics in the present study. Other studies have reported no significant difference in the $\dot{V}O_2$ response to exercise subsequent to nitrate supplementation in healthy, well-trained athletes^{47,48}. This lack of effect has also been reported in individuals with COPD^{30,49,50}. These studies all had relatively small sample sizes (n = 8⁴⁷, 11^{48,51}, 13⁴⁹, and 15³⁰).

One possible explanation for the lack of effect on the oxygen cost of exercise may relate to the elevated plasma nitrite concentration in the placebo condition (baseline) of this study which might reduce the scope for the further increases in plasma nitrite concentration that have been realised via nitrate supplementation in other populations. Thus the lack of effect of dietary nitrate supplementation on the O₂ cost of exercise noted in the present study may indicate the existence of an upper limit for the baseline plasma nitrite concentration, beyond which the scope for positive effects from further increasing plasma nitrite concentration is reduced. In support of this suggestion, a significant negative correlation has been reported between the changes in

plasma nitrite concentration with beetroot juice vs. placebo juice and the change in performance during cycling exercise (i.e. participants whose plasma nitrite increased more following nitrate supplementation experienced a greater improvement in exercise performance, and *vice versa*)⁴⁷. However, in the current study there was no correlation between either placebo plasma nitrite concentration, or change in plasma nitrite concentration with nitrate rich vs. placebo juice, and the O₂ cost of exercise.

An alternative suggestion for the lack of effect of nitrate supplementation on the O₂ cost of exercise may be related to the reduction in the bioavailability of NO which has been reported in individuals with T2DM⁵²⁻⁵⁴. This reduced bioavailability has been linked with the concentration of plasma ADMA (an analogue of L-arginine), which is elevated in individuals with T2DM⁵⁵. ADMA is known to inhibit all three NOS isoforms, particularly eNOS⁵⁶, leading to eNOS uncoupling and the generation of superoxide radicals⁵⁷, ultimately resulting in increased oxidative stress. When NO and superoxide react, peroxynitrite is generated. This is known to result in a quenching of NO activity and thus a reduction in its bioavailability, which may serve to diminish any positive impact of nitrate supplementation^{58,59}. Furthermore, sustained exposure to oxidative and nitrative stress could result in damage to mitochondrial membranes⁶⁰ and thus reduce the P/O ratio (i.e more O₂ would be required to produce a given amount of ATP). It is possible that damaged mitochondria associated with T2DM⁶⁰ means that this population are less likely to benefit from the improved mitochondrial function subsequent to nitrate supplementation, which may underpin, in part, the reduced O₂ cost of exercise in healthy individuals¹⁰.

A further possible explanation for the lack of effect of nitrate supplementation on the exercise response in individuals with T2DM may relate to pathological consumption of nitrite

during exercise. Post-exercise plasma nitrite concentration has been shown to fall markedly in individuals with cardiovascular risk factors ⁶¹ compared with the response reported in healthy individuals ⁶². This may suggest that either the production of NO cannot keep up with metabolic demand, or the NO produced is scavenged more rapidly in individuals with cardiovascular risk factors compared with healthy individuals. It is plausible that a similar effect is seen in individuals with T2DM, with a marked net consumption of plasma nitrite during exercise. It should be noted, however, that this would not explain the lack of effect of nitrate supplementation on BP in our study as these measurements were conducted at rest.

It should be noted that although we have postulated a pro-oxidant state as an explanation for the lack of effect of nitrate supplementation in individuals with T2DM, beneficial effects of nitrate have been seen in other patient groups who are likely to be in a pro-oxidant state. Zamani et al., ⁶³ reported an improvement in exercise capacity (but no reduction in the oxygen cost of exercise) following beetroot juice supplementation in individuals with heart failure. Similar findings have been reported in individuals with peripheral artery disease ⁴⁰ and chronic obstructive pulmonary disease ^{30,49,51}. It should be noted, however, that to date no study has shown a reduction in the oxygen cost of exercise in a patient group, despite reporting improvements in exercise tolerance. It is feasible that the improvement in exercise tolerance in the aforementioned studies is associated with elevated muscle blood flow consequent to an elevated plasma nitrite concentration ⁶⁴, although this requires further investigation to confirm.

Nitrate supplementation and functional Capacity.

There was no difference between the active and placebo juice conditions for the distance covered in the 6MWT. This is consistent with the only other study to have investigated the impact of nitrate supplementation on exercise performance using the 6MWT (in healthy older

individuals)³⁹. Since any improvement in walking performance could reasonably be assumed to be underpinned by alterations in the $\dot{V}O_2$ response to exercise, and considering that this was not modulated by nitrate supplementation, it is perhaps not surprising that functional capacity was also not different between conditions. It is likely that the explanation for the lack of effect of nitrate supplementation on walking performance is synonymous with the potential explanations for the lack of effect on the O_2 cost of exercise (see previous section).

Walking performance has been shown to be enhanced in individuals who are taking ACEi⁶⁵. Our cohort included 48% of individuals on ACEi and/or ARBs, thus any potential improvements in walking performance subsequent to nitrate supplementation may have been masked. When we separated the group into those prescribed ACEi or ARBs compared to the remaining individuals, no difference was seen between groups for walking performance. The groups were split 48% (n = 23) with prescribed ACEi or ARBs and 52% (n = 25) not taking ACEi/ARB. One possible explanation for the finding is that the 6MWT was the last of the experimental procedures to be conducted and followed 18 minutes of treadmill walking. The prior exercise (and time since last nitrate supplementation) may have meant that some of the additional nitrite which was available following nitrate supplementation had been utilised, especially given our cohort's elevated cardiovascular risk⁶¹.

Nitrate supplementation and resting blood pressure.

In agreement with our previous study examining the effect of dietary nitrate supplementation on blood pressure in individuals with T2DM³⁴, we found no difference in BP between conditions. Nitrate doses similar to that which was administered in the present study (6.43 mmol per day) have elicited reductions in diastolic BP in healthy young individuals¹⁵ and

those with peripheral arterial disease ⁴⁰, and reduced systolic BP in healthy young ^{15,17,31} and old ³⁹ individuals.

There are a number of possible explanations for the lack of effect of nitrate supplementation on the resting BP of individuals with T2DM. A reduced NO responsiveness has been linked to vascular stiffening in older individuals ⁶⁶. However, aging *per se* is unlikely to explain the lack of effect in the present study as BP was significantly reduced subsequent to nitrate supplementation in healthy older adults (on no medication) ³⁹. Secondly, the elevated oxidative stress prevalent in individuals with T2DM would be expected to result in an increase in the scavenging of NO, potentially diminishing any hypotensive effects from nitrate supplementation.

Antioxidants in beetroot juice

It has been demonstrated that the beneficial effects of beetroot juice are largely explained by its nitrate content ³¹. However, it has also been suggested that an antioxidant effect may also be occurring, such as is observed under in vitro experiments with polyphenols ⁶⁷. Though there are small differences in antioxidants between the two juices used (placebo and active), the magnitude of this difference is unlikely to be physiologically relevant. Typical total daily polyphenol intake across multiple populations has been estimated to be in the region of 1g daily ⁶⁸; the effective dose from either juice in the current study therefore represents less than 1% of average total intake. Betacyanins were present in much higher quantities. The bioavailability of these compounds is however uncertain, but it appears to be very low with typical estimates from <1% to 4% of an oral dose, with some individuals having far greater absorption ^{69,70}. Furthermore, the removal of betanin from plasma is rapid, with a $t_{1/2}$ of 0.94 ± 0.07 hours. These

factors suggest the betacyanin content of beetroot juice is unlikely to have a clinically meaningful effect.

Strengths and limitations.

To date this is the largest trial, implementing robust methods, to examine the effect of inorganic nitrate or pharmacological nitrate supplementation on the O₂ cost of exercise. The *a priori* sample size calculation was designed to enable the study to detect an 8% reduction in the O₂ cost of low intensity exercise with a 0.05 alpha level and an 80% power. From the 95% CI it is likely that the actual difference for the O₂ cost of low intensity exercise following nitrate supplementation in individuals with T2DM was between -2.1% and 3.6%. Therefore the minimum detectable reduction in the O₂ cost of exercise in our study is 2.1%. This is very similar to the day to day variability of $\dot{V}O_2$ measurement, thus it is unlikely that a larger sample size would elucidate a clinically significant difference in the O₂ cost of walking exercise in this patient group. A potential limitation of the present study is that the 6MWT was completed up to 4 hours subsequent to the consumption of the final beverage, and after 18 minutes of walking when the increments in nitrite from supplementation may have been utilised already. As plasma nitrite concentration was not determined prior to the 6MWT, we cannot be certain that this remained elevated following nitrate supplementation compared to the placebo condition. However, this does mean that the study provides knowledge of the level of improvement (or lack of it) that a patient with type 2 diabetes could expect in the early afternoon of normal daily living.

Conclusion

In contrast to much of the literature in young healthy individuals and despite a statistically significant and physiologically meaningful increase in plasma nitrite concentration, four days of beetroot juice supplementation with 6.43 mmol of nitrate did not reduce the O₂ cost of walking, improve functional capacity as determined by the 6MWT, or reduce resting BP in individuals with T2DM. The lack of effects of dietary nitrate supplementation in individuals with T2DM may be explained by increased oxidative stress and its impact on the bioavailability of NO, or an elevated ‘baseline’ plasma nitrite concentration which reduces the scope for the beneficial effects reported in other populations.

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Duality of Interest Nigel Benjamin is a director of Heartbeet ltd. Heartbeet ltd has patents granted relevant to this work (production of nitrate depleted beetroot juice).

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