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Local oxygen consumption in cycling:

The effect of chronic nitrate supplementation on muscle oxygen consumption (mVO2) during low and high intensity constant-load cycling

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Abstract

Introduction: Nitrate supplementation administered from beetroot juice has been studied more extensively in recent years due to its suggested effect on exercise efficiency by reducing the O2-cost during submaximal cycling endurance exercise. Mainly amongst trained recreational individuals, with the focus primarily on pulmonary oxygen consumption (pVO2), although physiology suggests peripheral adaptations. The purpose of this study was to use near-infrared spectroscopy (NIRS) to investigate the effect of chronic nitrate (NO_3^-) supplementation on muscle oxygen consumption (mVO2) during low and high intensity constant-load cycling. To examine this, we used vastus lateralis and tibialis anterior. **Methods:** 9 healthy recreational active men performed two constant-load cycling tests pre and post (6 days) of placebo or nitrate supplementation, each of 7 min duration, at 50% of the work rate found to elicit blood lactate levels of 4 mmol·l -1 during incremental exercise (OBLA), and at 70% maximal aerobic power (MAP). Pulmonary gas exchange (pVO2), heart rate and NIRS measurements of the muscles vastus lateralis (VL) and tibialis anterior (TA) were obtained continuously through both tests, while blood pressure, blood lactate and RPE was measured at specific time intervals. **Results:** The main findings of the present study were that chronic NO_3^- supplementation, quite surprisingly, did not significantly affect pVO_2 and blood pressure at 50% OBLA and 70% MAP in recreational active subjects. Hence, since we could not provoke an effect on pVO_2, as was expected, the corresponding absent of effect on mVO_2 in the VL muscle and in the TA muscle seems rather reasonable. Although NO_3^- treatment showed trends towards lower O_2 cost compared to PL in the VL muscle at 50% OBLA and 70% MAP. **Discussion:** The protocol is comparable with others that did find an effect on pVO_2 (and to some extent on mVO_2), so is dose and population. Results suggests that nitrate might not be as efficient as an ergogenic aid as previously depicted. The rational for the absence of effect of nitrate on mVO2 during both low and high intensity cycling in the present study is rather unclear, despite the corresponding lack of effect in pVO_2 . Factors such as intensities and fiber type characteristics might be of importance. **Conclusion:** Chronic NO_3^- supplementation did not affect mVO_2 in the VL muscle or in the TA muscle, pVO_2 or blood pressure at low (50% OBLA) and high (70% MAP) intensity cycling in recreational active subjects.

Key words: Nitrate supplementation, Near-infrared spectroscopy, cycling, pVO_2, mVO_2, vastus lateralis, tibialis anterior.

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Preface

The research described in this master thesis was conducted as part of a bigger project at the Department of Neuroscience, Faculty of Medicine, NTNU. The experimental procedures for the first phase of this project, i.e. the present study, took place during autumn 2015 and winter 2016. Data analysis were done during winter/spring 2016. The next phase of this project will include female subjects as well, and will extend the measurements to multiple muscles in both arm and leg. In parallel, a similar but simplified study design will be used to investigate the acute effects of nitrate supplementation, with only a single dose of nitrate-rich beetroot juice. When results indicate that the effects are, indeed, detectable in the peripheral muscle tissue, the project will be extended and approval will be sought to measure other subpopulations as well (e.g. elderly, patients).

Table of contents

1. Introduction

In recent years, dietary nitrate (NO_3^-) has received a lot of attention as an ergogenic aid in the world of sports. The increased interest from an exercise physiology perspective originated from a paper that found nitrate supplementation to improve exercise efficiency during submaximal endurance exercise amongst trained recreational individuals (1). This improvement in efficiency manifested itself by a reduced O_2-cost, which in in self is quite surprising due to the fact that it is commonly accepted that the O_2-cost for a given increase in work rate is relatively fixed (2). Nevertheless, Larsen, et al. (1) reported a reduced O_2-cost of around 5% following three days of nitrate supplementation. Also, due to the fact that it can be administered in an easy manner by drinking beetroot juice that naturally contains a relatively large quantity of nitrate (ca 110mg/100g), the practicality of this ergogenic aid seems to be only one of its many asset, in addition to a vasodilate effect on blood vessels reducing pressure blood pressure (3, 4) and the aforementioned reduced O_2 cost.

Although, for some time, the common consensus has been that nitrate (NO_3^-) and nitrite (NO_2^-) are simply byproducts of the L-arginine nitric oxide (NO) synthesis pathway (5). Which is O_2 reliant utilizing NOS (nitric oxide synthase) enzymes to oxidize the amino acid L-arginine to form NO (6). NO, on its turn, is believed to mediate physiological processes during exercise at muscular level. Lately, the consent regarding nitrate and nitrite has received opposition where both nitrate and nitrite are proposed to offer an alternative pathway for the generation of NO, without the relicense of L-arginine and O_2, and that nitrite can be recycled back to bioactive NO again in blood and tissues (7). This pathway is particularly interesting from an endurance exercise point of view, explicitly because during exercise, contracting skeletal muscles experience hypoxic tissue milieus (6). Which implies that NO generation thru the L-arginine nitric oxide (NO) synthesis pathway will be compromised. Therefore, the alternative pathway is favorable, not being O_2 dependent, with initial ingestion of e.g. beetroot juice containing NO_3^- that will reduce to NO_2^- in the oral cavity as NO_3^- is actively taken up by salivary glands, defecated in saliva, and reduced by commensal bacteria into NO_2^- (6). Furthermore, when NO_2^- is swallowed and reaches the acid environment of the stomach, it will again reduce to NO. The remaining NO_2^- in the stomach then goes into the circulating plasma after being absorbed and increases its levels of NO_2^-. The latter can be converted to NO throughout the body, especially in hypoxic tissue milieus, e.g. as contracting skeletal muscles, and is thought to be responsible for physiological

alterations related to exercise performance. The mechanisms behind the reduced O_2-cost are not yet understood, but have been suggested to be related to the influence of NO on muscle function, i.e. lowering the muscular demand of O_2 (8). The lower demand is assumed to derive from improvements in ATP – resynthesizing (i.e. mitochondrial alterations) (9) and/or muscle contractile function (10) (i.e. P/O ratio and force or work/VO_2 respectively). This is the rationale for the beetroot juice supplementation leading to the nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO) pathway, which in turn influences O_2-cost, which implies that when performing O_2 restricted work during submaximal endurance exercise, nitrate supplementation can potentially improve exercise efficiency. In addition to alterations seen in the blood flow amongst patient groups (3) and older adults (4). Apparently, NO also seems to have a vasodilated effect on blood vessels so that blood pressure reduces significantly with nitrate thru beetroot juice supplementation.

Given these properties of nitrate, the sports world as well as the scientific community have shown great interest in this natural ergogenic aid. Where several studies have examined the effect of nitrate on pVO_2, mainly in recreational individuals, but also in patients and elderly and athletes. A reduced O_2 cost (3-14 %) amongst trained recreational individuals (VO_{2peak} of 50-60 ml $kg^{-1}min^{-1)}$, after short-term chronic (3-6 days) dietary NO_3^- supplementation (~5.5-6.5 mmol) NO_3^- salts or beetroot juice) have been verified when performing steady state submaximal cycling (2), running (11), knee extensor exercise (10) and running at moderate intensity (5). Moreover, exercise tolerance has improved by 3-25 % during time to exhaustion tests for cycling (2) and running (11), in addition to improvements in performance by 1.2 -3 % during time trial tests in cycling (12-14). The lack of improvement after nitrate supplementation, found by others, seems merely to be related to the type of exercise (incremental exercise to exhaustion) (15, 16) or the study population, i.e. trained athletes (5, 17-20), due to that these studies involving athletes (60-82 ml $kg^{-1}min^{-1}$) in fact utilized similar dose (~5.5-6.5 mmol) and duration (~1-8 days) as recreational, and tests were performed at steady state submaximal levels. The pVO_2 adaptions proposes that the same effect will be prominent also at muscular level, knowing that the effect of nitrate suggests peripheral adaptations in the muscle tissue. Therefore, we wanted to investigate if this manifestation would be evident in the muscles during cycling. By using vastus lateralis, which is one of the main power producers during cycling (21), in addition to tibialis anterior, a muscle less activated than the former, which function is to flex the foot up towards the shin

(dorsiflexion), we might see how different muscles with different functions reacts to nitrate supplementation. Some other studies (2, 22-24) have investigated the local muscle oxygen response in relation to nitrate supplementation on account of that the lower O_2 cost can be interpreted as a higher fraction of oxygenated hemoglobin in working muscles (2). This has been done by using NIRS, predominantly in the vastus lateralis muscle at lower and higher intensities; however, no one has included the tibialis anterior, or examined nitrate's effectiveness during elicited arterial occlusion (AO) when cycling. AO are utilized to retrieve quantitative mVO_2 values from NIRS-signals. NIRS (near infrared spectroscopy) enables measurements of local muscle oxygen consumption via continuous monitoring of relative changes in the concentrations of oxy- and deoxyhemoglobin/myoglobin within the tissue, and has been used earlier in other studies (25, 26). Moreover, measurement of muscle oxygen consumption using NIRS has proven to be reliable at rest (26), as well as during exercise (27).

Against this background, when the effect of nitrate seems to be manifested locally in the muscles in recreational subjects, as described earlier, we hypothesize that the effect from nitrate supplementation, I.E a reduced O_2 cost, will be evident in the working muscles as well, and that this effect will be more prominent at higher intensities, due to a hypoxic state. Therefore, the purpose of this study was to use near-infrared spectroscopy (NIRS) to investigate the effect of chronic nitrate supplementation on muscle oxygen consumption (mVO2) during low and high intensity constant-load cycling. This was done by using vastus lateralis and tibialis anterior.

2. Methods and materials

2.1 Subjects

Nine healthy recreational active men participated voluntarily in this study. None of the subjects used tobacco or ingested dietary supplements. All participants gave their written informed consent before commencing in the study, after receiving explanation of the experimental procedures, associated risk, and possible benefits. On test days, the subjects were instructed to arrive at the laboratory in a rested and hydrated state, minimum 3 h postprandial, and to avoid strenuous exercise in the 24 h preceding each testing session. In addition, instructions were given to abstain from alcohol or caffeine intake 24 and 6 h before the tests, respectively. All tests were performed at the same time of the day. The study was approved by the Norwegian Regional Ethics Committee.

2.2 Study design

In order to investigate the effect of nitrate supplementation on muscle oxygen consumption (mVO_2), a double-blind placebo-controlled cross-over design with random distribution in a counterbalanced order was used. The participants were randomly assigned to either nitrate-rich or nitrate-depleted concentrate, with the order switched after an 8 days washout period. Both supplements were identical in color, taste and packaging to ensure that the participants would not be able to identify which supplement they had ingested. Supplementation consisted of 6 days with daily 2 x 70 ml nitrate-rich beetroot concentrate (2 x 450-500mg nitrate/2 x 6.5mmol) (Beet It, James White Drinks, Ipswich, UK) or 6 days of placebo with daily 2 x 70 ml nitrate-depleted beetroot concentrate (0.7-2.52 mg nitrate/0.006-0.02mmol) (Beet It, James White Drinks, Ipswich, UK). The subjects were informed of the possible side effects manifesting in beeturia (red urine) and red stools (2).

The present study consisted of one visit prior to the two supplementation phases. Both supplementation phases consisted of a pretest and a posttest, separated by a 8 days washout period. All tests were performed over a period of 4 weeks. Prior to the two supplementation phases, an incremental cycling-exercise was performed (preliminary test), to determine each participant's work intensity for the following tests by reaching an onset of blood lactate accumulation (OBLA), VO_2-peak/max and cycling power (watts). Each of the supplementation phases were preceded and followed by identical tests (done before and after supplementation) , i.e. a pre-test and a post-test on test day 1 first phase and test day 3 second phase. The same test-

apparatus was repeated for only one single chronic post-test on supplementation day 6 for both phases (test day 2 first phase/test day 4 second phase). These experimental test-trials consisted of resting vascular occlusion test for arm and leg and submaximal cycling test.

2.3 Experimental protocol

The study consisted of a total of four test days over two supplementation phases, in addition to the pretest for preliminary measurements. On test day 0 (preliminary test), the subjects were informed about the study and signed the informed consent. Height, weight and body composition (length, circumference, skinfold thickness) were measured as well. Moreover, a lactate threshold test and cycling VO2max test were performed, in order to define the intensity level for the submaximal tests and the maximum capacity for oxygen uptake and (see *Preliminary measurements*). Supplementation period one included test day 1 and 2, whereas period two included test day 3 and 4. Test day 1 and 3 were the pre-test and post-test days containing two of each of the following tests: a resting vascular occlusion test for arm and leg and submaximal cycling test. The same and identical test-apparatus was repeated for only one single chronic post-test session on test day 2 and 4 (day 6 of supplementation) Test day 2 and 4 were the post-test days (to investigate chronic effect) and were done immediately following the supplementation phases. Both supplementation phases were separated by a 8 days washout period to ensure complete elimination of supplementation effects.

Preliminary measurements

A schematic overview of the incremental cycling exercise; lactate threshold and VO_{2max} test is presented in figure 1. A 5-10 min warm-up period at low intensity (75-100 Watt) preceded this two step-test protocol. The lactate threshold-test began at 100 W with a freely chosen cadence between 80-100 rpm with duration of 4 min, followed by increments of 25W every 4^{th} min until OBLA occurred, a set value of 4mmol. Blood lactate and RPE were taken immediately after each period. Them, after OBLA occurred, restitution/active recovery for 5 min at 75-100 W followed, before the VO_{2max} -test was performed. The VO_{2max} -test began at the last intensity minus 25 W from the previous test, with increments of 25 W/min. Cadence maintained between 80-100 rpm, as subjects were instructed to retain cadence above 80rpm. VO2max was defined as the highest intensity until exhaustion where the highest VO_2 over 30 sec was measured, and ended when rpm fell under 80rpm. Blood lactate and RPE were taken immediately after. Criteria to define whether exercise was close to maximum, were; a RER value above 1.15, blood lactate levels above 8.0

mmol/l, heart rate at near maximum values and rate of perceived exertion (RPE) at peak values. The total duration of lactate threshold and VO_{2max} test was approximately 30-45 min, depending on how many stages there were until OBLA was reach, and when exhaustion occurred during the VO_{2max}.

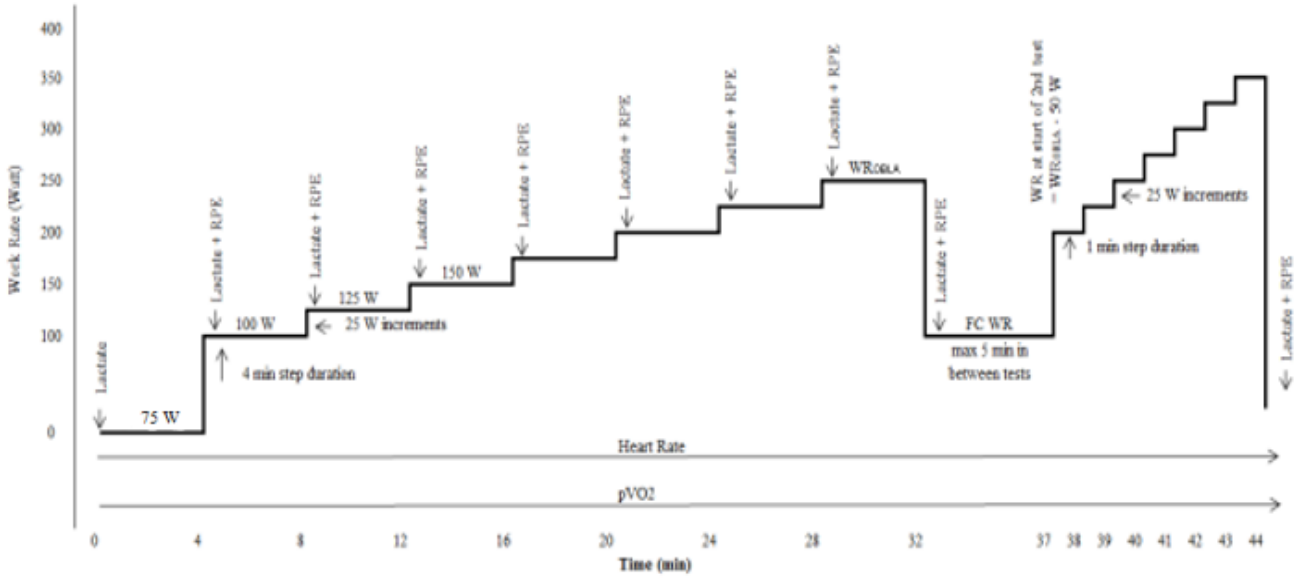


Figure 1: Schematic representation of both incremental tests. FC = freely chosen, RPE = rating of perceived exertion, WR = work rate, WROBLA = work rate that elicited blood lactate level of 4 mmol or higher, pVO2 = pulmonary oxygen uptake. Heart rate and pVO2 were measured continuously throughout the test.

Test apparatus day 1-4

An arterial occlusion test on test days 1-4 was performed under completely resting conditions and in a comfortable half-supine position on a bed. After connecting the NIRS fibres, the test started with a 5 min baseline measurement prior to the first vascular occlusion. In total the test existed of 2 periods of vascular occlusion followed by 2 recovery periods, using an arm cuff tighten around the proximal region of humerus and a thigh cuff around the upper femur. The first vascular occlusion lasted 1 min and was meant to familiarize the subject with the feeling of vascular occlusion. This occlusion was followed by 5 min of recovery. The second vascular occlusion lasted 10 min with the purpose of defining the maximum deoxygenation/saturation level and obtain resting values for muscle oxygenation to be used during analysis. Following the last occlusion, there was a final recovery period of 10 min. NIRS signals were measured throughout the whole test.

The submaximal cycling test (see a schematic overview in figure 2) consisted of two

cycling periods of 7 minutes duration separated by 10 min recovery. A 5-10 min warm-up period at low intensity (75-100 Watt) preceded the test. Lactate and RPE was taken at start of warm up. The submaximal test began with the first submaximal exercise period at moderate intensity (50% of OBLA) (< lactate threshold) with a freely chosen cadence between 80-100 rpm. Followed by the 10 minutes recovery period, i.e. resting 6min rapid AO (data not used), and 4min freely chosen watts cycling, and a second submaximal exercise period at high intensity (70% of MAP) (> lactate threshold). Cadence was instructed to be 80-100 rpm. From each 4.40 min interval of each 7 min period, a 20 sec arterial occlusion was applied by a pneumatic cuff around the thigh, rapidly inflated and rapidly deflated after 20 seconds to measure mVO_2 in the vastus lateralis and tibialis anterior, whilst subjects continued cycling. Blood lactate and RPE were taken immediately after each period whilst subjects had stopped cycling, reflecting the La- levels and subjective perception of the intensity. The right leg remained in a non-weight-baring position resting on a brick with an approximate angle of 90 degrees in hip and knee during the 6min rapid AO. NIRS, HR, and pulmonary VO_2 were continuously measured throughout the test.

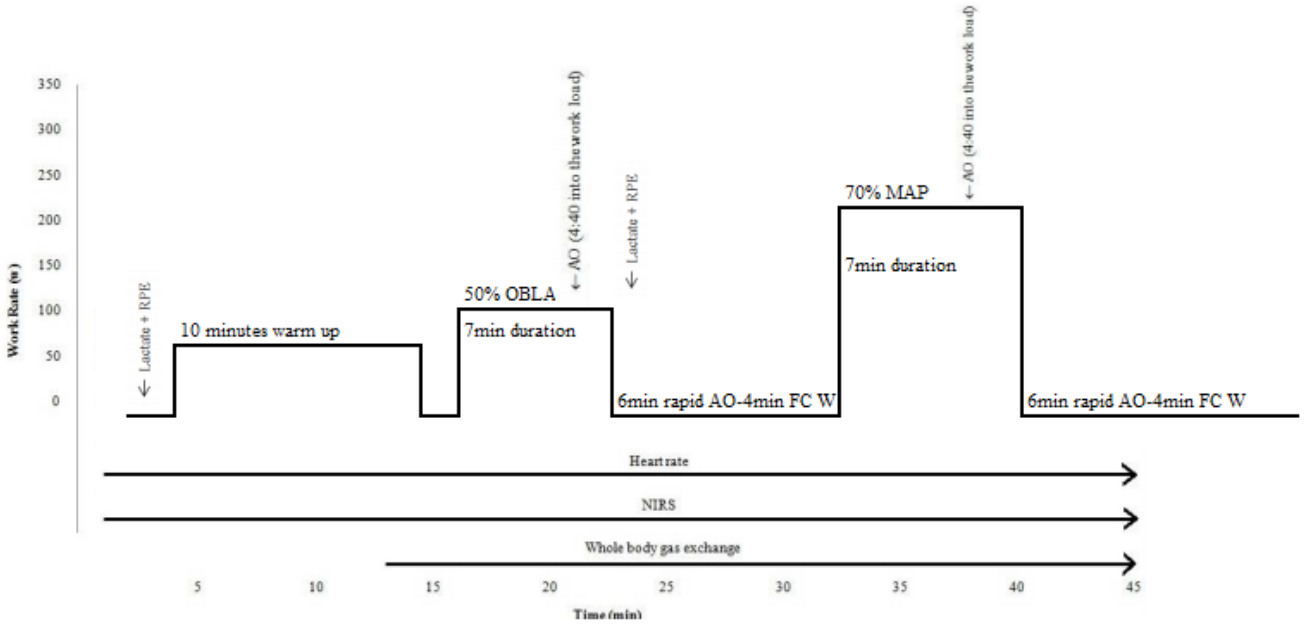


Figure 2: : Schematic representation of the submaximal cycling test. AO = arterial occlusion, RPE = rating of perceived exertion, OBLA = onset of blood lactate accumulation, MAP = maximal aerobic power. Heart rate, NIRS and pVO2 were measured continuously throughout the test.

2.4 Instrumentation

All exercise tests were performed at the laboratory of the Department of neuroscience (Faculty of Medicine). Cycling was conducted on a cycle ergometer with a computer controlled electro-magnetic brake mechanism (Velotron, Racermate inc, Washington, USA). Work rate was controlled by means of the velotron software package. Heart rate was monitored continuously during cycling using a heart rate monitor (Polar RS800, Polar Electro OY, Kempele, Finland). Pulmonary gas exchange and ventilation were measured continuously during cycling using open-circuit indirect calorimetry (Oxycon Pro, Jaeger GmbH, Hoechberg, Germany) with a sample rate 0.1Hz. The gas exchange equipment was calibrated each day of testing, using a gas of known concentration (16.0% O2 and 5.85% CO2, Riessner-Gase GmbH & Co, Lichtenfels, Germany) and a 3-liter syringe (Hans Rudolph Inc., Kansas City, MO, USA). Also, blood pressure measurements were taken at the left upper arm using an automatic cuff inflator/deflator prior to each test session on all 4 test days with a standardized blood pressure device (OSZ 5 easy Welch Allyn, Jungingen, Germany). Blood lactate measurements were taken from the fingers of the right hand during the lactate threshold test and VO2max-test, and before and immediately after each cycling exercise period (Lactate Pro LT-1710, ArkRay Inc, Kyoto, Japan). Plasma nitrate (NO_3-) and nitrite (NO_2-) concentrations were measured four times (total of 6 for each subject) by means of venous blood sample according to standard procedure. I.E. prior and 2h after supplementation on the pre-test measurements days, as well as prior an 2h after supplementation on day 6 of the supplementation period on the chronic post-test measurement days. In order to measure the skinfold thickness at the site of NIRS optodes, in addition to iliac crest, subscapular, triceps and mid-thigh, a skinfold caliper device was used (Holtain Tanner/Whitehouse skinfold caliper, Holtain Ltd, Crymych, Wales). The percentage of body fat was calculated by means of four skinfold sites: iliac crest, subscapular, triceps and mid-thigh (28). Furthermore, skeletal muscle mass was estimated according to (29) with the use of of arm, thigh and calf circumference and triceps, mid-thigh and calf skinfold thickness.

2.5 Near-infrared spectroscopy

Local changes in muscle oxygen consumption (mVO_2) were measured noninvasively and continuously with near-infrared spectroscopy (Oxymon MKIII and Portamon, Artinis Medical Systems, the Netherlands) using wavelengths of 766 and 856 nanometer for Oxymon, and 841 and 762 for Portamon. Near-infrared spectroscopy (NIRS) is an optical technique that is based on

the relative transparency of the tissue for light in the near-infrared region and on the oxygen-dependent absorption changes of hemoglobin and myoglobin (26). The sampling depth of the NIRS signal was one-half of the interoptode distance (4.0 cm). Due to the penetrating abilities and absorption by hemoglobin and myoglobin these wavelengths are utilized. Hemoglobin and myoglobin have an identical absorption spectra, and thus make impossible to contrast between the two. The present study utilized a modified Lambert-Beer law to study relative concentration changes in oxyhaemoglobin (O_2Hb) and deoxyhaemoglobin/myoglobin (HHb), owing to that O_2Hb and HHb have unlike absorption spectra, making this possible (30).

In order to retrieve quantitative mVO_2 values from NIRS signals, an arterial occlusion (AO) method was applied using an automatic cuff inflator/deflator in combination with an external air source (Hokanson E20 Rapid Cuff Inflator + a pressurized gas bottle), set to a pressure of 300mmHg. The location of the NIRS optodes was on the middle region of the muscle belly of vastus lateralis of the right thigh and tibialis anterior on the right leg. We did not measure in cm the distance from e.g. patella, although optode placement were ~ more proximal than distal and can have varied some between subjects. In addition, to minimize interference from cycling movements and signal noise, the optodes and wires were fastened with adhesive and regular tape, and a support bandage, done with caution in purpose of not occlude blood flow. The optodes were attached on top of the muscle belly and covered by a black lycra textile, and NIRS signals were measured continuously from the start of the test sessions until the last test. A distance of 35mm was set between the light source and detector with data sampled at a rate of 10Hz.

2.6 Blood sampling procedures and plasma-analysis

Authorized health personnel took blood samples on two occasions (before and after NIT/PL supplementation) on test day 1 and 3 and on two occasions (before and after NIT/PL supplementation) on test day 2 and 4. Each sample were drawn into 2 citrate reagent tubes and 1 EDTA reagent tube, after which they were instantly tilted to prevent coagulation. Within 2 min of collection the tubes were centrifuged at 3000 rpm (1000*g)* at room temperature for 10 min to obtain plasma. Plasma was subsequently extracted into smaller tubes and centrifuged again, before EDTA plasma instantaneously was frozen at -80°C before placed in a freezer and frozen at -32°C, whilst citrate plasma was slowly frozen directly at -32°C. Further analysis of $NO3^-$ and NO_2^- levels in plasma samples was performed England.

2.7 Data analysis

Due to movement artifacts, influencing the raw NIRS signals, caused by rhythmic contractions/relaxations phases of the muscles during cycling, a low pass Butterworth filter (1-Hz cut-off frequency) was utilized to filter out these artifacts. During AO periods, the time of the linearly decrease in O_2Hb and Hb_{diff} ($Hb_{diff} = O_2Hb - HHb$) concentration declines under increasing workrate as a function of elevated O2 consumption in the tissue. Thus, to calculate mvo2 throughout AO, start/end markers were checked manually and regression values were checked over the filtered NIRS signals using the occlusion markers and a fixed regression period. By using the regression data, mVO2 was calculated from the slope of the Hb_{diff} signal derived from the linear decrease in Hb_{diff} during AO (26). Correlation coefficients of mVO2, reflecting the fit of the regression line, were set to be equal or above 98 %. A delay of period 0.5sec defined the delay between the actual AO start marker and the beginning of the regression period, the latter being 2 sec. Markers were also set for exercise events.

Pulmonary VO_2 data from gas exchange measurements were averaged over 60 seconds during the first 5 min prior to occlusion of each cycling workload period. Heart rate were calculated for each cycling period and averaged over 5 min. Blood pressure was averaged from the two measurements taken at each test day. Lactate was measured three times during each test day, as was RPE. Skinfold thickness was divided by 2 to obtain ATT values. The VO2max was calculated by taking the average of the final 3 measurements during the last 30s before exhaustion.

2.8 Statistics

All results are expressed as means ± SD. A repeated measures 2-way or 3 way ANOVA was used to examine the effect of supplementation on mVO_2, pVO_2, heart rate, lactate, RPE and blood pressure. Were there any significant differences between time points or work-loads (rest, 50% OBLA and 70% MAP), post-hoc analysis using pairwise comparison were applied, using α-level adjusted via a Bonferroni correction. Adjustment of significance using the Greenhouse-Geisser method was assessed if sphericity was violated. A one-way ANOVA was used to compare watts, $\%VO_2max$ and %HRmax between tests and treatments. The level of significance was set at $P > 0.05$. The data were analyzed using Matlab, Excel and SPSS for Windows.

3. Results

All subjects completed all tests. The beetroot juice in the dosage that was utilized in this study was generally well-tolerated and led only to non-harmful side-effects such as beeturia (red urine) and red stools, as expected and consistent to a previous study (2).

The general subject characteristics are presented in table 1.

Table 1:Subject characteristics

	Mean	SD	Min	Max
Height (cm)	182.0	7.7	164.7	190.6
Weight (kg)	83.6	11.8	68.8	99.8
Age (y)	32.3	8.7	21.0	45.0
HRmax (bpm)	182.2	5.7	176	197
VO2max (ml/min/kg)	49.3	5.1	41.9	57.7
Skeletal muscle mass (kg)	35.5	5.9	28.2	43.0
Bodyfat (%)	20.5	4.4	14.4	27.8
Lactate threshold (W)	186.1	13.2	175.0	200.0
Maximal aerobic power (W)	327.7	36.3	275.0	375.0
ATT (Right VL) (mm)	5.0	2.7	2.5	7.3
ATT (Right TA) (mm)	4.1	2.1	2.0	7.1

ATT = adipose tissue thickness, TA= tibialis anterior, VL =vastus lateralis, HRmax = maximal heart rate and VO_2max= maximum whole body oxygen consumption obtained during the test. N = 9

3.1 Validation parameters of protocol

To ensure that the protocol was executed as planned and at similar intensities for both the nitrate supplementation period and the placebo period, we compared power output between all tests, % of VO_2max and % of HRmax between pretests, and between pretest and posttest for PL and NIT respectively (table 2, 3, 4). We also did data analysis for pVO_2, by means of comparing pretest PL with pretest NIT. The data of plasma levels of $NO3^-$ and NO_2^- has unfortunately not yet been analyzed. Therefore, we are not in possession of any results that could validate that plasma levels of $NO3^-$ and NO_2^- increased as a consequence of beetroot supplementation.

Watts, %VO_2max, %HRmax

Table 2: Exercise intensity expressed as power output (Watt)

	Pretest	**Posttest**
Power output at 50% OBLA (Watt)		
PL	101.1 ± 19.0	101.1 ± 19.0
NIT	101.1 ± 19	101.1 ± 19.0
Power output at 70% MAP (Watt)		
PL	223.8 ± 21.0	223.8 ± 21.0
NIT	225.5 ± 20.3	223.8 ± 21.0

Mean values in watt ± SD for pretest and posttest with PL or NIT at 50% OBLA and 70%.MAP. N = 9..

Table 3: Exercise intensity expressed as % of VO2max

	Pretest	**Posttest**
% of VO2max at 50% OBLA		
PL	41.2 ± 7.5	41.8 ± 7.9
NIT	39.5 ± 8.7	41.2 ± 7.1
% of VO2max at 70% MAP		
PL	70.1 ± 10.5	69.2 ± 8.9
NIT	70.6 ± 11.9	70.2 ± 9.1

Mean values in % of VO_2max ± SD of 5min cycling for pretest and posttest with PL or NIT at 50% OBLA and 70% MAP.N = 9. Percent VO_2max is expressed relative to the corresponding VO_2max measured in the pretest (Table 1).

Table 4: Exercise intensity expressed as % of HRmax

	Pretest	**Posttest**
% of HRmax at 50% OBLA		
PL	57.7 ± 7.2	56.3 ± 7.2
NIT	58.9 ± 8.4	56.9 ± 6.3
% of HRmax at 70% MAP		
PL	76.8 ± 8.4	73.8 ± 6.5
NIT	77.1 ± 8.8	77.1 ± 8.7

Mean values in % of HRmaxx ± SD of 5min cycling for pretest and posttest with PL or NIT at 50% OBLA and 70% MAP. N = 8, missing = 1. Percent HRmax is expressed relative to the corresponding HR_2max measured in the pretest (Table 1).

An overview of the mean power output, % of VO2max and % of HRmax between all tests are shown in table 2, 3, 4 respectively. Mean power output showed similar W for all tests. % of VO2max and % of HRmax showed similar relative pVO2 values and similar relative HR values, respectively, between pretests (baseline). Overall, there were no significant differences (all $p > 0.05$).

3.2 Pretest vs pretest

We compared pVO_2 for pretest 1 (PL) with pretest 2 (NIT) in order to test the baseline conditions were similar. No main effect of pretest PL vs pretest NIT was found for pVO_2 at 70% MAP ($F(1, 8) = 0.50$, $p=0.828$). The main effect of time was ($F(1626, 13.0) = 59.292$ $p<0.001$), and the contrasts showed significant increase in pVO_2 in the initial phase of exercise ($p=0.000$) and from min 3 to 4 ($p=0.038$). However, no interaction between supplementation and time was found ($F(1.7, 13.7 = 0{,}403$, $p=0{,}645$). The effect of pretests at 50% OBLA showed similar patterns as for 70% MAP. For 50% OBLA, no main effect of pre test PL vs pretest NIT was found ($F(1, 8.) = 0.630$, $p=0{,}450$), but a main effect time ($F(1.671, 13.369) = 55.5$, $p<0.001$). Contrasts revealed significant increase in pVO_2 from min 1 to 2 ($p<0.001$). Tendencies towards significant interaction effect between test and time ($F(1.2, 9. = 4{,}300$, $p= 0{,}06$) was evident.

Taken as a hole, one can therefore say that work rate was identical over all tests, and that baseline conditions (pretest vs pretest) were similar. The protocol was executed as scheduled at identical exercise intensity levels for both pre tests.

When considering the effect of group, no significant difference in pVO_2 was found between those who received PL first or nitrate first for 50% OBLA ($F(1, 7) = 0.003$, $p=0.995$) and 70% MAP ($F(1, 7) = 1.589$ $p=0.248$).

3.3 Lactate and RPE (Borg scale) response to supplementation

In addition, we tested whether subjective lactate and RPE showed a similar pattern as the above (see Fig. 3 A-D). Lactate measurements taken at rest/during warm up at 75 watts and immediately after 50 % OBLA and 70% MAP, showed no significant alterations from pre to post in placebo or nitrate states, no effect of PL vs NIT, but a main effect of intensity. A three-way ANOVA (suppl x intensity x test (pre/post)) showed no main effect of PL vs NIT ($F(1, 8.) = 0.002$ $p=0.966$), no main effect of test (pre vs post) ($F(1, 8.) = 0.058$ $p=0.816$ and main effect of intensity levels ($F(1.052, 8.416) = 237.874$, ($p<0.001$). Contrasts exposed higher lactate from rest to 50% OBLA ($F(1, 8) = 22.283$, $p=0.002$ and from 50% OBLA to 70% MAP ($F(1, 8.)$

=214.367, (p<0.001). No interaction effects were found between treatment and test, treatment and intensity, test and intensity, nor treatment x intensity x test) (all p > 0.05).

For RPE, no main effect of PL vs NIT (F (1, 8.) =0.326 p=0.584), no main effect of test (pre vs post) (F (1., 8.) =1.703 p=0.228 and main effect of intensity levels (F (1.246, 9.966) =294.006, (p<0.001) was found. Contrasts reviled higher RPE from rest to 50% OBLA (F (1, 8) =133.244, p=0.000 and from 50% OBLA to 70% MAP (F (1, 8.) =130.572, p=0.000). No interaction effects was found between treatment and test, treatment and intensity, test and intensity, nor treatment x intensity x test (all p > 0.05).

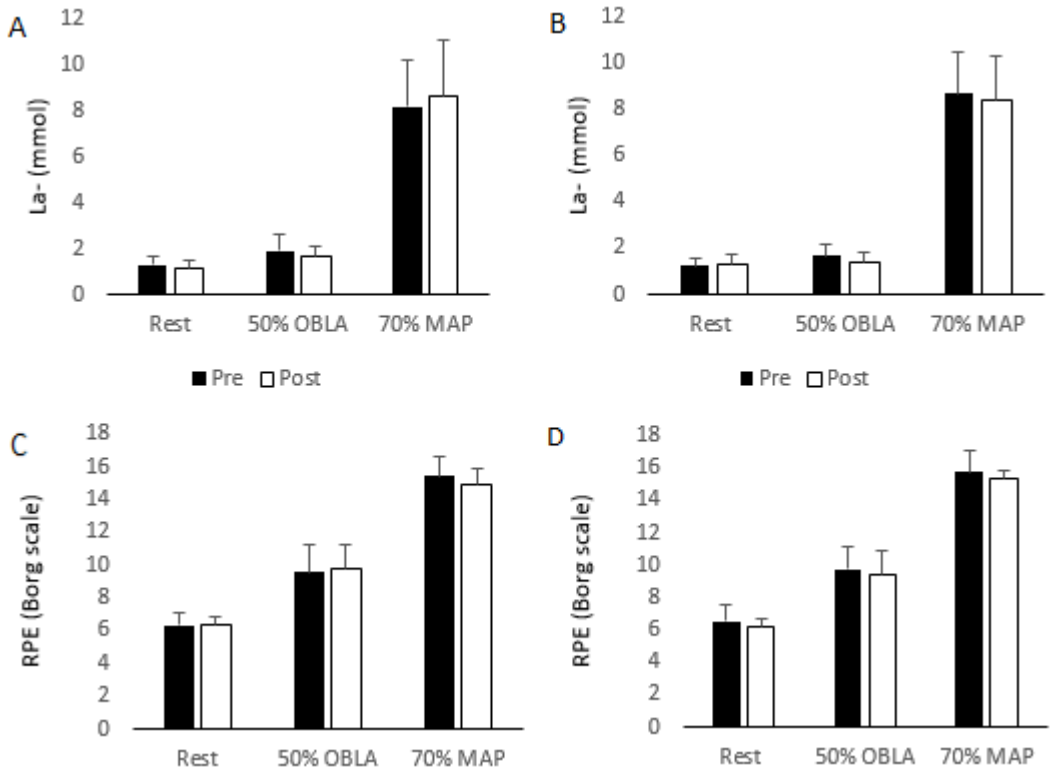


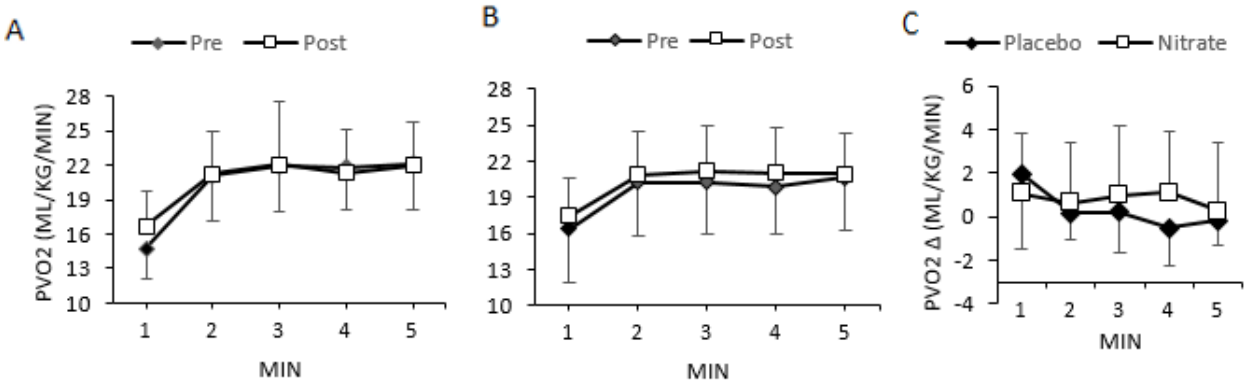
Figure 3: Lactate (A-B) and RPE (C-D) in pre vs posttests for placebo (A and C) and nitrate (B and D). SD is shown as bars.

3.4 pVO_2 response to supplementation

Group mean responses for pVO_2 during 50% OBLA (A-C figure 4) and 70% MAP (D-F figure 4) for placebo (A and D in figure 4) and nitrate (B and E in figure 4) respectively, and comparison (using delta (Δ) (i.e. Δ(pVO2) = (pVO2)post – (pVO2)pre) (C and F in figure 4)) between the two treatments are presented in figure 4. It can be seen in figure 4 that pVO_2 at 50% OBLA increases in the initial phase of exercise and is roughly stable after 2-3 minutes. (in comparison

with 70% MAP where steady state is more unclear/occurs later). These responses were roughly similar for PL and NIT, and for pre and post, though a slightly higher post pVO_2 was seen in NIT throughout the whole time period. A two-way ANOVA (suppl x time) on the delta values between pre and post pVO2 values showed indeed, as we expected based on the figures of pVO_2, no main effect of treatment (F (1., 8.) = 0.203, P = 0.664), no main effect of time (F (2.234, 17.872) = 2.597, P = 0.098), and no interaction effect between treatment and time (F (1.536, 12.508) = 1.420, P = 0.272).

Group mean responses for pVO2 at 70% MAP for placebo (D in figure 4) and nitrate (E in figure 4) respectively, and comparison (F in figure 4) (using delta (Δ) (i.e. Δ(pVO2) = (pVO2)post - (pVO2)pre) between the two treatments are presented in figure 4 D-F. Whereas statistics was conducted on delta (Δ) values only. Figure 4 shows that pVO2 values at 70% MAP in the placebo treatment increased in pre and post conditions on average until min 4, indicating that pVO2 did not reach a steady state before the 4. min. In the nitrate condition, pVO2 also increased in the initial phase between min 1 and 2, and from min 3 to 4. Statistics on the delta values showed no main effect of treatment (F (1.000, 8.000) =0.102 p=0.757), no main effect time (F (1.552, 12.173)=0.248 p=0.725) and no interaction effect between treatment and time (F (1.651, 13.207)=0.263 p=0.731). Neither placebo nor nitrate had an effect on pVO2 at 70 % MAP during cycling.



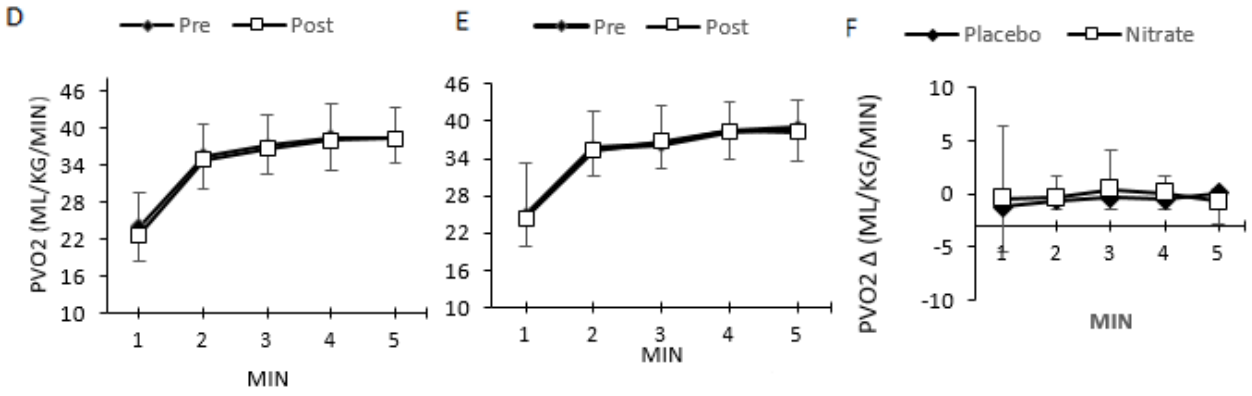


Figure 4: pVO_2 responses at 50% OBLA (A-B) and 70% MAP (D-E), pre (black squares) vs post (white squares) treatment for placebo (A, D) and nitrate (B, E), and (Δ) values at 50% OBLA (C) and 70% MAP (F) for placebo (black squares) and nitrate (white squares) as a function of min. SD is shown as bars.

3.5 Blood pressure

Group mean responses for systolic and diastolic BP are shown in figure 5 A and B respectively. Figure 5 A shows a greater reduction in systolic BP from pre to post with placebo supplementation than with nitrate, in addition to the higher pre value. Small reductions and no major differences can be seen in diastolic (Fig. 5B). A two-way ANOVA (suppl x pre/post test) analysis of systolic BP comparing placebo with nitrate showed no main effect of treatment on systolic BP ($F(1.000, 8.000) = 4.462$ $p=0.068$), i.e no difference between treatments. A nearly significant main effect of test (pre vs post) was found ($F(1.000, 8.000) = 5.176$ $p=0.052$), and there was an interaction effect between treatment and test ($F(1.000, 8.000) = 7.024$ $p=0.029$). Indicating that a reduction was evident. Further contrasts showed a nearly significant decrease in systolic BP from pre to post ($F(1.000, 8.000) = 5.176$ $p=0.052$), and this reduction was evident in interaction with treatment ($F(1.000, 8.000) = 7.024$ $p=0.029$). The effect seems come from a reduction from pre to post of 135.5 to 128.5 mmHg in the placebo state, as compared to nitrate, as seen in (Fig 5A). Suggesting that the test (pre to post) had a stronger influence on systolic BP than treatment, since the main effect of test was significant, and treatment was not. This relationship tells us that the effect which is present is contrary to that expected (i.e reduction in NIT).

Comparison of diastolic BP between PL and NIT, using a two-way ANOVA (suppl x pre/post test) analysis showed no main effect of treatment ($F(1.000, 8.000) = 2.970$ $p=0.123$), no

main effect of test (F (1.000, 8.000) =0.672 p= 0.436) and no interaction effect between treatment and test (F (1.000, 8.000) =0.026 p=0.875).

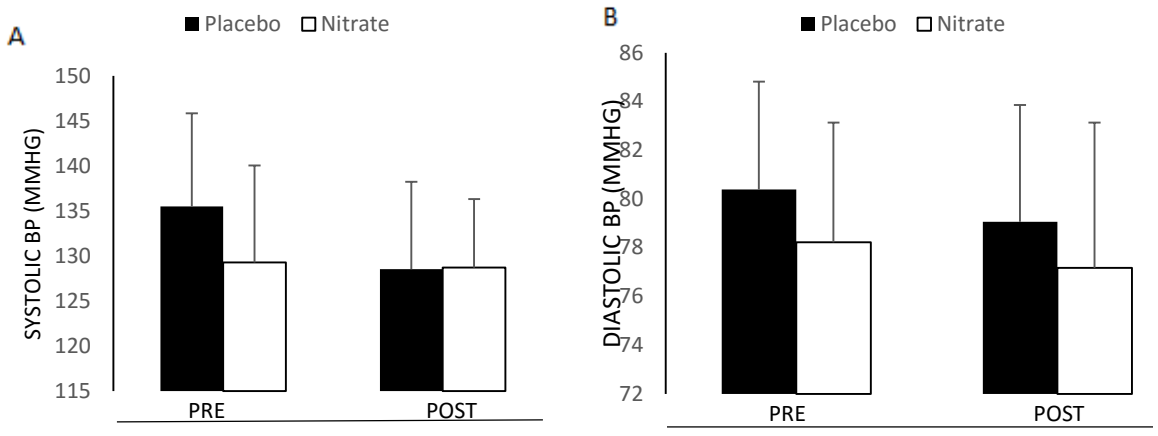


Figure 5: Systolic (A) and diastolic (B) blood pressure and comparison between pre and post treatment with either placebo or nitrate. SD is shown as bars.

3.6 Heart rate

Group mean responses for HR during during 50% OBLA (A-C Fig. 6) and 70% MAP (D-F Fig. 6) for placebo (Fig. 6A + D) and nitrate (Fig. 6B + E) respecttively, and comparison (using delta (Δ) (i.e. Δ(HR) = (HR)post – (HR)pre) (Fig. 6C + F)) between the two treatments are presented in figure 6. Whereas statistics was conducted solely on delta (Δ) values. Figures and analysis are conducted on 8 subjects due to missing HR values for one subject. Figure 6 shows that HR increased from the first to the second minute at 50% OBLA for both PL and nitrate, and for pre and post, whereas only minor changes can be observed afterwards. HR at pre seemed slightly higher for both PL and NIT. At 70% MAP, an increase in HR is evident between min 1 to 2, as during 50% OBLA, and HR did increase some for the rest of the period. These responses were roughly similar for PL and NIT, and for pre and post. In addition, HR in the placebo treatment increased slightly more than nitrate treatment from min 2 to min 4. A two-way ANOVA analysis comparing placebo and nitrate (in Δ - values) showed no main effect of treatment (placebo vs nitrate) on HR at 50% OBLA (F (1, 7) =0.107 p=0.754), no main effect of time on HR (F (4, 28) =1.923 p=0.134) and no interaction effect between treatment and time (F (4, 28) , 32=0.736 p=0.575).

At 70 % MAP, analysis comparing placebo and nitrate (in Δ - values) showed no main

effect of treatments (F (1.000, 7.000 =1.978 p=0.202), no main effect of time (F (4, 28) =1.400 p=0.260), and no interaction effect between treatments and time F (2.196, 15.374) =2.286, p=0.132). Taken together, the similar pattern in HR from pre to post in placebo and nitrate for 50% OBLA and 70 % MAP respectively, indicates that nitrate had no effect on HR during cycling.

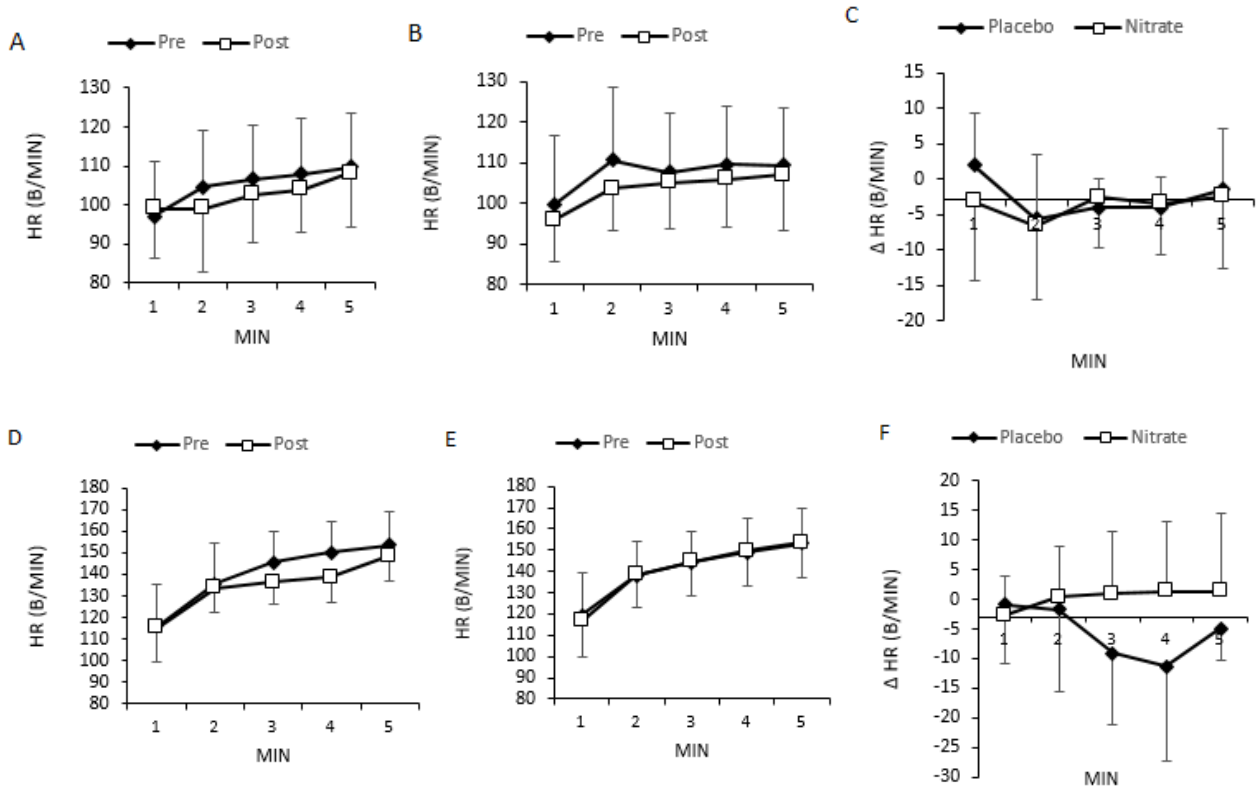


Figure 6: HR responses at 50% OBLA (A-B and 70% MAP (D-E), pre (black squares) vs post (white squares) treatment for placebo (A, D) and nitrate (B, E), and (Δ) values at 50% OBLA (C) and 70% MAP (F) for placebo (black squares) and nitrate (white squares) as a function of min. SD is shown as bars.

3.7 Near-infrared spectroscopy

An example showing the filtered data from AOs at the two different intensities, 50% OBLA (left) and 70% MAP (right) in Vastus lateralis, from a single subject is presented in figure 7. As can be seen in figure 7, a steeper decrease in the Hbdiff occurred when the work rate increases. A steeper decrease in the Hbdiff indicates higher mVO2 with increasing work rate. Apart from that, it can also be seen that the time in which the decrease in O2Hb and the Hbdiff stays linear,

decreased with increasing work rate. The tHb has in this example been blood volume corrected; consequently, the actual response of tHb is not visible here.

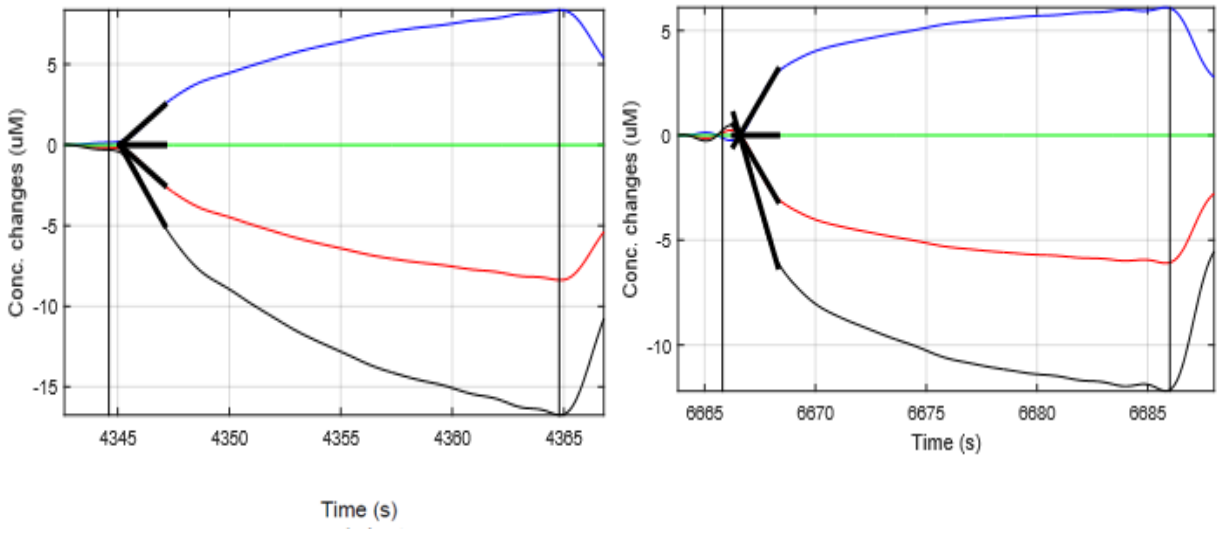


Figure 7 Lines indicate concentration of O2Hb (red line), HHb (blue), tHb (green) and Hbdiff (black). Vertical lines indicate start and stop of AO. Bold black lines indicate fixed regression period.

3.8 Local muscle oxygen consumption

Vastus lateralis

The group mean values for mVO_2 in VL during rest and cycling at 50% OBLA + 70% MAP for placebo (Fig. 8A) and nitrate (Fig. 8B) pre and posttests are shown in Fig. 8. The changes from pre to post for placebo and nitrate are shown as well (using delta (Δ) (i.e. Δ(mVO_2) = (mVO_2)post - (mVO_2)pre) (Fig. 8C)). Statistics was conducted exclusively on delta (Δ) values. It can be seen in Fig. 8A that the mVO_2 response was similar from pre to post in the placebo treatment, both showing increase in mVO2 from rest to 50 % OBLA (1.088 ml/min/100g), and from 50 % OBLA to 70 % MAP (0.236 ml/min/100g). Suggesting a higher demand of O2 at higher intensities. In the nitrate treatment (Fig. 8B), mVO_2 showed parallel responses as PL from pre to post in terms of increased mVO_2 at higher intensities, from rest to 50 % OBLA (1.052 ml/min/100g), and from 50 % OBLA to 70 % MAP (0.317 ml/min/100g). However, mVO_2 responses at 50 % OBLA and 70% MAP seemed to be somewhat lower from pre to post in the nitrate treatment (9.1% for 50% OBLA, 13.3% for 70% MAP). Indicating a possible effect of nitrate treatment.

When we compared PL and NIT using (Δ) values (Fig. 8C), no main effect of treatment (PL vs NIT) (F (1, 8) = 2.248 p=0.172), and no main effect of intensity (rest, 50% OBLA, 70% MAP) was found for mVO_2 (F= (2, 16) 1.161 p=0.338). The interaction effect between treatment and intensity were also non-significant (F= (2, 16) 0.996 p=0.391). In addition, a 3-way ANOVA (treatment x intensity x test (pre/post)) on the mVO_2 values was done, resulting in similar effects (all $p > 0.05$), apart from a main effect of intensity (F= (2, 16) 44.682 $p<0.001$).

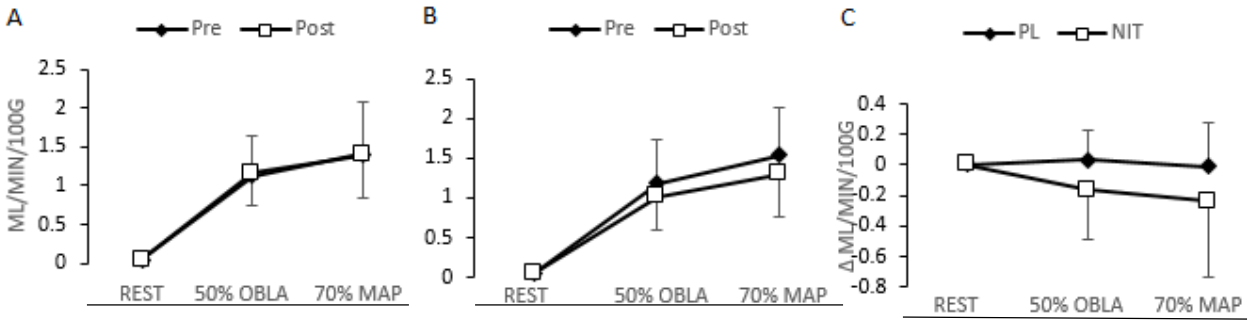


Figure 8: Group mean (± SD) values for local muscle oxygen consumption measured by NIRS in the VL muscle pre (black squares) vs post (white squares) treatment for placebo (A) and nitrate (B), and Δ-values (C) for placebo (black squares) and nitrate (white squares) as a function of intensity. SD is shown as bars.

Tibialis anterior

The group mean values for mVO_2 in VL during rest and cycling at 50% OBLA + 70% MAP for placebo (Fig. 9A) and nitrate (Fig. 9B) pre and posttests are shown in Fig. 9. The changes from pre to post for placebo and nitrate are shown as well (using delta (Δ) (i.e. $\Delta(mVO_2) = (mVO_2)post - (mVO_2)pre$) (Fig. 9C)). Statistics was conducted exclusively on delta (Δ) values. The general response for both PL and NIT from pre to post tests, as seen in Fig. 9 A + B, was an increase in mVO_2 from rest to 50 % OBLA and only a slight increase from 50 % OBLA to 70 % MAP, although most evident in the post treatment for PL and pre for NIT. mVO_2 seems to level off at cycling from 50% OBLA to 70% MAP, indicating a plateau in mVO_2 consumption in the TA muscle at high intensities, and lesser degree of activity during cycling. An overall lower mVO_2 consumption (peaking at ~1 ml/min/100g) can also be seen in figure 9 A-B, as compared to VL (peaking at ~1.5 ml/min/100g) in Fig. 8 A-B.

When we compared PL and BR using (Δ) values (Fig. 8C), no main effect of treatment (PL vs BR) (F (1, 8.) = 0.50 p=0.829, no main effect of intensity (rest, 50% OBLA, 70% MAP) (F= (2, 16) 0.189, p=0.830) and no interaction effect between treatment and intensity was found

for mVO_2 (F= (2, 16) 0.830 p=0.411). In addition, a 3-way ANOVA (treatment x intensity x test (pre/post)) on the mVO_2 values was done, resulting in similar effects (all $p > 0.05$), and a main effect of intensity (F= (1.1, 9.1) 33.4 $p<0.001$).

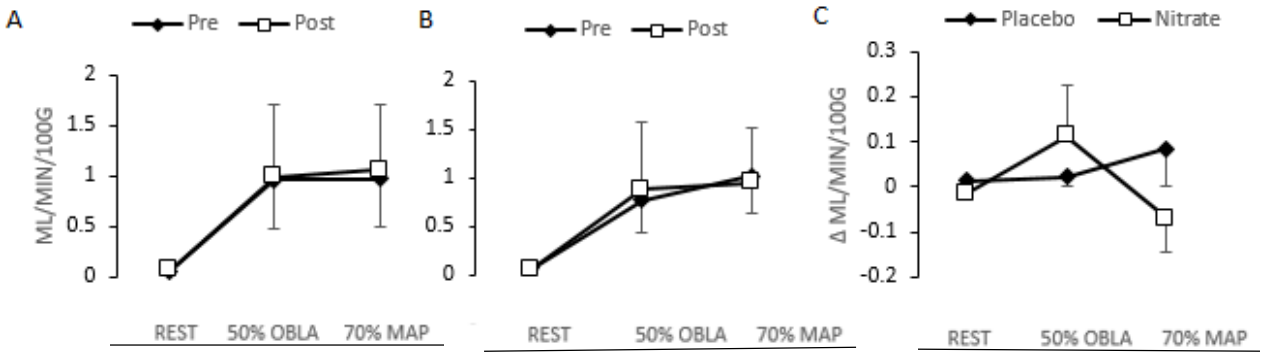


Figure 9: Group mean (± SD) values for local muscle oxygen consumption measured by NIRS in the TA muscle pre (black squares) vs post (white squares) treatment for placebo (A) and nitrate (B), and Δ-values (C) for placebo (black squares) and nitrate (white squares) as a function of intensity. SD is shown as bars.

4. Discussion

The main purpose of this study was to investigate the effect of chronic 6 days NO_3^- supplementation on mVO_2 during low and high intensity constant-load cycling, using NIRS. The main findings of the present study were that chronic NO_3^- supplementation did not significantly affect pVO_2 and blood pressure at 50% OBLA and 70% MAP in recreational active subjects. Therefore, since we could not provoke an effect on pVO_2, as was expected, the corresponding absent of effect on mVO_2 in the VL muscle and in the TA muscle at 50% OBLA and 70% MAP seems rather reasonable. Albeit, the NO_3^- treatment showed trends towards lower O_2 cost compared to PL in the VL muscle at 50% OBLA and 70% MAP. No such tendencies was seen in TA. Heart rate, lactate and RPE were unaffected by nitrate supplementation. Although other studies have found NO_3 to lower the pVO2 cost at submaximal intensities, and some have shown parallel responses in muscle oxygenation (2, 22), and we hypothesized that the effect on pVO2 would be evident in mVO_2, at least at higher intensities, due to the suggested physiological pathways, neither a lower pVO_2 nor mVO_2 was found. The lack of effect on the systemic scale could be on account of supplementation, protocol, subjects and/or intensity levels.

4.1 Effect on whole body oxygen consumption (pVO2)

Contrary to literature, our results showed that 6 days with daily 2 x 70 ml nitrate-rich beetroot concentrate (2 x 450-500 mg/(2 x 6.5 mmol)) (Beet It, James White Drinks, Ipswich, UK) did not affect pVO_2 (see Fig. 4). This is in contrast to other studies which have revealed an effect (reduction of 3-14%) using a similar or even lower supplementation dose and duration (~5.5-6.5 mmol for 1-6 days - NO_3^- salts or beetroot juice). (2, 5, 10, 11, 14). Supplementation similar to ours has also been found to increase acute plasma nitrite (NO_2^-) levels in a dose-dependent fashion with peak changes occurring at approximately 2-3h (14). However, due to un-analyzed data of plasma samples in the present study, a conclusion regarding $NO3^-$ and NO_2^- plasma levels cannot be drawn. Furthermore, Wylie, et al. (14) found that the dose-response and pharmacodynamics affiliations reviled that a dose of 1 x 70 ml (4.2 mmol) beetroot concentrate did not alter the physiological responses to exercise, while 2 x 70 ml (8.4 mmol) and 4 x 70 ml (16.8 mmol) beetroot concentrate reduced the acute steady-state oxygen uptake during moderate-intensity exercise. This indicates that the (relatively) high dose and duration (6 days) utilized in this present study, similar to the implementations of previous studies, should have been adequate to provoke a response.

Another possible reason for our findings could be due to the protocol used. Our protocol showed that pVO_2 did not differ between pretests. i.e. both baseline measures were equal both at 50% OBLA and 70% MAP, indicating similar exercise intensity levels. Therefore, lower or higher pVO2 in either PL baseline or NIT baseline can be out ruled, and thus, this has not influenced our results. In addition, pVO2 expressed as % of VO_{2max}, was similar between pretests for each respective intensity (see Table 3), as was % of HR_{max} (see Table 4). Watts were also alike when comparing pretests, posttests, and pretest with posttest for PL and NIT respectively (see Table 2). Consequently, the power output was not a bias factor. Moreover, lactate and RPE measurements showed no differences between pretests, further reflecting that we used identical tests, and intensity levels that were set were correct throughout the study. In addition, HR showed no differences neither (see Fig. 6).

In comparison with those studies that did find an effect, our protocol is comparable, where we utilized 5 min cycling exercise at low (50% OBLA – mean W 101.1, mean % $VO2_{max}$ 39.5-41.8 (all tests), % HR_{max} 56.3-58.9 (all tests) and high intensities (70% MAP – mean W 223.8-225.5 (all tests), mean % VO2max 69.2-70.6 (all tests), % HR_{max} 73.8-77.1 (all tests)). The intensity levels used here reflects other studies which have used % Δ (70% of the difference between the power output at the GET and $VO_{2\ peak}$, severe exercise), % of VO2max/peak or % of GET (gas exchange threshold – occurring approximately at lactate threshold). Moreover, our protocol is comparable with other studies in terms of exercise (cycling), exercise time interval, submaximal levels and subject population (recreational men, mean VO_{2max} 49.3 ml $kg^{-1}min^{-1}$). Owing that others have investigated nitrates influence in recreational men with similar VO_2peak/max of 50-60 ml $kg^{-1}min^{-1)}$, finding lower O_2 cost. Porcelli, et al. (5) e.g. used four repetitions of 6-min submaximal constant load running exercise on a motorized treadmill at approximately 80% of gas exchange threshold. A lower O_2 cost of 7-10% was found amongst men with VO_{2peak} 45-60 ml $kg^{-1}min^{-1}$ and for those below 45 ml $kg^{-1}min^{-1}$ (7-13%), and non in those over VO_{2peak} 60 ml $kg^{-1}min^{-1}$. The intensity level of 80% of gas exchange threshold can be translated to ~148.9 W in our first intensity level, using 80% of OBLA instead of GET. This indicates that O_2, at least at 70% MAP (mean W 223.8-225.5), should have provoked a response to NIT supplementation, albeit we used cycling and not running, knowing running to be more strenuous on pVO_2. Nevertheless, others, which have used cycling as exercise with similar population, have also found reductions in O_2 consumption (2, 14). Both utilized a 5-6 min step

exercise tests from a 20W baseline to moderate (e.g 93 ± 11 W) and severe-intensity work rates (e.g. 258 ± 23 W) (Watt-data from (Wylie, et al. (14)), performing one or two session of moderate-intensity exercise and one session of severe-intensity exercise on day 4 and 5. On day 6 the severe-intensity exercise was sustained until task failure to measure exercise tolerance. Wylie, et al. (14) found that 8.4 mmol NO_3^- and 16.8 mmol NO_3^- (140 and 280 ml) reduced the steady-state oxygen (O_2) uptake during moderate-intensity exercise by 1.7% and 3% respectively (N=10). The steady-state $\dot{V}O_2$ measured over the final 30 s was reduced (by ~50 ml/min) only by 16.8 mmol NO_3^-. Bailey, et al. (2) showed reduction at moderate (80% get) and severe (70% Δ (70% of the difference between the power output at the GET and VO_{2peak}, severe exercise)) after 5.5 mmol NO_3^-/day (N=8). At moderate, a reduction from 10.8 $ml \cdot min^{-1} \cdot W^{-1}$ (PL) to 8.6 $ml \cdot min^{-1} \cdot W^{-1}$ (NIT) in functional gain (i.e., the ratio of the increase in O_2 consumed per minute to the increase of external power output) was evident. The final 30 s VO_2 was also reduced. Furthermore, the gross O_2 cost of exercise (involving resting metabolic rate, i.e. the O_2 cost of moving the limbs during baseline cycling, and the O_2 cost of muscle contraction to meet the enforced work rate) was reduced by ~5%. During severe intensity, the amplitude of the VO_2 slow component was reduced with NIT. Both these studies used breath-by-breath and were very similar with each other and to ours. E.g. Wylie, et al. (14) used 93 ± 11 W at moderate and 258 ± 23 W at severe-intensity work rates, comparable to our 101.1 ± 19 W (41% VO_{2max} and 223.8-225.5 ± 21 W (70% VO_{2max}) at respective work rates. So, due to that our protocol was executed correctly for all tests, and is comparable with others that did find an effect, and dose and subjects also is comparable, we can say that our study most likely should have elicited pVO_2 responses. The reasoning for the lack of effect of NIT on pVO2 during both low and high intensity cycling in the present study is, therefore, unclear. Although, the unit of pVO_2 here was ml/kg/min, whereas others have reported l/min. Still, this was the unit of choice due to the range in VO_{2max} (41,96-57,76 ml/min/kg) and in skeletal muscle mass (28.2-43kg).

However, in consistency with our results, others have also failed to find any effect, though acute, of 8 mmol NO_3^- (140ml) on pVO2 in 8 recreational males (mean VO_{2peak} 46 ± 3 $ml \cdot kg^{-1} \cdot min^{-1}$) cycling for 60 min at similar intensity (here 69.2-70.6 % of $VO2_{max}$ at 70% MAP) (comparison 65% of VO_2 peak) (31). Neither did Puype, et al. (32) find any long-term chronic (8 weeks) effect of a relative small dose (NO_3^- (0.07 mmol/kg bw/day) (~500 mL) in the form of BR (700 mg NO_3^-/L) starting 4 days before pretest in moderately trained recreational individuals

(~VO_{2max} 60 (ml/min/kg), ~21.5 years) when investigating if NO_3^- supplementation could enhance the effects of 6 weeks cycling training program at simulated hypoxia (~4000 m) on endurance exercise performance at sea level. A possible rationale for the lack of effect for the two aforementioned and our results could be that the effect of NO_3^- on O_2 cost is affected by aerobic fitness level. I.E. as demonstrated by that those having a higher VO_{2max} and can be categorized as athletes (5, 17-20) (60-82 ml $kg^{-1}min^{-1}$), are already well-adjusted to the physical demands of the specific sport, meaning that there most likely, and almost unavoidably will be fewer weak links for NO_3^- to induce influence on (8). In comparison to recreational individuals. As these studies would indicate (5, 17-20), on the basis of finding no effect on O2 cost, even though receiving similar dose (~5.5-6.5 mmol) over similar duration (~1-8 days) as recreational. Some of these week links are thought to be related be related to aspects such as greater NOS activity, improved muscle oxygenation and mitochondrial efficiency, and/or a lower portion of type II fibers in the muscles of highly endurance trained compared with moderately trained subjects (33). E.g., an increased capillary density as a result of training (34) will preserve muscle oxygenation in most conditions, except the extreme at higher intensities, such that the need to reduce NO_2^- to NO would probably be abbreviated. Despite displaying elevated plasma levels, which may already be high (enough) due to extensive NO_3^- dietary intake as athletes through several meals a-day containing NO_3^-rich food (we encouraged participants to refrain from NO_3^-rich food). Although, in our study, the consideration that the subjects included are physically adapted similar to athletes, can with some certainty be ruled out, owing the lower mean VO_{2max} (49.3 ml $kg^{-1}min^{-1)}$ (± 5,1, 41,96-57,76), despite one subject displaying a relatively high.VO_{2max} of 57.76 ml $kg^{-1}min^{-1}$. Another possible factor for our results may be that antibacterial mouthwash could have altered the microflora of the oral cavity, thus affecting the NO3 – NO2 – NO pathway, yet unlikely when subjects were asked to refrain from utilizing antibacterial mouthwash. Therefore, other explanations must be considered. Some clarity may be found when looking closer on the local muscle oxygenation in relation to capillary density and fiber type characteristics as a possible explanation.

4.2 Effect of supplementation on mVO2

Coinciding with the lack of effect of NIT on the systemic variable, we did not find an effect on local metabolism, reflected in the means of mVO_2 in the VL muscle or in the TA muscle at 50% OBLA and 70% MAP. Although, the NO_3^- treatment showed trends towards lower O_2 cost

compared to PL in the VL muscle at 50% OBLA and 70% MAP (see Figure 8). No such tendencies was seen in TA. (see Figure 9). The seemingly effect in VL may have been influenced by the lower number of subjects (N=9), though similar numbers have been utilized by those who did find an effect. In addition, the relative difference between pre and post with NIT of 9.1% for 50% OBLA, 13.3% for 70% MAP can be interpreted as a possible influence by $NO3^-$. Furthermore, the seemingly difference between PL and NIT (Figure 8c) further suggests that alterations in oxygenation locally in the muscles might be manifested despite of lacking effects on a systemic scale, even though there was no main effect of treatment and interaction effect between treatment and intensity, showing far from sig. values. This difference was not accompanied by increase in lactate concentration, suggesting a more efficient energy production. The mean VL mVO_2 resting values was 0.05 for both pre and post PL(± 0.02 pre and post) ml·min-1 ·100g-1 and similar values for NIT. Whilst TA values was 0.05 for pre and 0.07 post PL(± 0.02 pre ± 0.02 post) ml·min-1 ·100g-1 and 0.07 for pre and 0.06 post NIT(± 0.06 pre ± 0.02 post) ml·min-1 ·100g-1. The VL mVO_2 values at 50% OBLA and 70% MAP for pre PL were 1.1 and 1.4 respectively (± 0.4 , ± 0.6) ml·min-1 ·100g-1 and similar for post. For NIT, pre mVO_2 was 1.2 and 1.5 (± 0.5 , ± 0.6) ml·min-1 ·100g-1, and for post 1.02 and 1.3 (± 0.4 , ± 0.5) ml·min-1 ·100g-1 . This is, both for PL and NIT, lower values than those (4.05 ± 0.55 ml·min-1 ·100g-1) reported by Skovereng, et al. (35) at 155W (i.e. between our two workrates) and 90rpm in VL, although, without NIT supp. Our findings is in contrast with other studies that investigated the effect of 6-9 days with 5.5-8.4 mmol NO_3^-/day on local muscle oxygenation with NIRS as well, i.e. only in VL in recreational subjects (2, 22-24). These used comparable time intervals (4-20min) and intensity levels. The similarity between these studies is the use of deoxyhemoglobin (HHb) and oxyhemoglobin (HbO_2) as indexes of muscle oxygen consumption, as in contrast to the present study which used AO method to measure the quantitative mVO_2 values derived exclusively from a 20s AO. HHb response reflects the balance between local O_2 delivery and utilization, and not the actual oxygen consumption (mVO_2) when blood flow and thus O_2 delivery is completely rendered. By using an AO, one must consider the possibility of the AOs (in combination with high workrate) impact on mVO_2 (hence nitrates influence due to hypoxia) when both promotes hypoxic states in muscle tissue. Also, we gathered data on concentration changes, which might be of interest, although these were not included here and must therefore be interpreted at a later stage. However, the present study is, as far as the author is aware of, one of

very few that have examined nitrate's effectiveness during elicited AO when cycling. The lack of studies on TA response after $NO3^-$ ingestion during cycling makes it somewhat difficult to compare our results to others, but the interpretation of TA is an asset of the present study. Results showed, that mVO2 seemed to level off at cycling from 50% OBLA to 70% MAP, indicating a plateau in mVO_2 consumption in the TA muscle at high intensities, and lesser degree of activity during cycling. An overall lower mVO_2 consumption (peaking at ~1 ml/min/100g) can also be seen in figure 9 A-B, as compared to VL (peaking at ~1.5 ml/min/100g) in figure 8 A-B. Most likely owing to that TA`s function is dorsal flexion in the ankle joint, consequently working at the end of the pedal stroke and perhaps to a lesser "muscle memory" automatic degree in non-professional cyclists. Additionally, VL is one of the main agonist of the quadriceps of the knee joint, which is activated during cycling (21).

Masschelein, et al. (22) investigated 15 young recreational active men (21yr, VO_2peak: 61.7 ± 2.1 $ml \cdot kg^{-1} \cdot min^{-1}$) performing a 20-min constant-load exercise bout at a workload of ~45% of sea-level VO_2peak (comparable to our ~40 % VO2max), corresponding with ~70% of the VO_2peak measured at simulated 5,000 m altitude. Results showed that NIT reduced VO_2 by ~4%, possibly reflecting a lower amount of VO_2 by active muscles. Furthermore, TOI in VL was higher in hypoxic NIT than in hypoxic PL, in addition to oxygenated hemoglobin (%SpO_2) whilst a lower exercise-induced increase in muscle deoxyhemoglobin content (Δ HHb was evident. The authors suggestively explained this by improved coupling of mitochondrial respiration with oxidative phosphorylation (raised phosphate/O_2 ratio) (9) and by a reduced ATP cost for a given rate of muscle contractions (10).

Bailey, et al. (2) revealed an effect of nitrate supplementation on indexes of muscle oxygenation, using NIRS (optodes placed ~middle on thigh), during 6min moderate (80% GET) cycling exercise amongst 8 recreationally men aged 19–38 ($\dot{V}O_{2max}$ 49 ± 5 $ml \cdot kg^{-1} \cdot min^{-1}$). They found a lower muscle fractional O_2 extraction in the VL during moderate exercise, indicated by a reduction of 13% in the HHb amplitude after NO3 supplementation (PL: 88 ± 38 vs. NIT: 78 ± 34. An increase was seen in the HbO_2 at baseline and after 2min, yet this difference was not evident at the end of the 6min exercise. The Hb_{tot} was higher only at baseline. During severe intensity (70% Δ (70% of the difference between the power output at the GET and $\dot{V}O_{2\ peak}$,), performed after a 25 min passive recovery from moderate periode, no alterations was observed after NO3 supplementation. Breese, et al. (24) used a similar protocol, only GET was set at 90%

for 9 recreationally active subjects (four male and five female), and subjects started cycling 3min at 15 W, then going directly to 4min moderate intensity period, which was immediately followed by a severe period. Results showed faster pVO kinetics and HHb time constant kinetics (PL: 20 ± 9 vs. NIT: 10 ± 3 s going from moderate to severe, but not from unloaded to moderate. Both these studies found effects, Bailey, et al. (2) only at moderate and Breese, et al. (24) at severe, may be due to that Bailey, et al. (2) used a 25min brake in-between periods, and Breese, et al. (24) continued the protocol onto severe. The latter could explain why we found no such (significant) effect at 70 % MAP, owing the 10min brake (6min sitting still, 4min recovery cycling). The former, however, did find effect even at moderate intensity, which could be, compared to the present study, on part of that 80% GET will be at higher intensity, i.e. ~148.9 W, than 50OBLA (101W) if we were to translate 80% GET to our study. Whereas maybe a less hypoxic milieu will be present, compared to the higher work rate at 80% GET. Moreover, an important aspect by Breese, et al. (24) is that after NO_3^- supplementation, faster pVO_2 and HHb time constant kinetics were only seen in moderate to severe intensity. Which can be explained by that this transition would be expected to recruit fast-twitch fibers (type II) to a greater extent (than type I, more pronounced at lower intensities) (36). Hence, this implicates that NIT supplementation might prompt specific physiological responses within and at microvasculature surrounding type II muscle fibers, as proposed in rodent models by (37). Therefore, nitrates influence on muscles may perhaps be more prominent during very high pedal cadence, as compared to low (24). Whereas we used rpm from 80-100, perhaps too low to provoke responses. This was investigated by Bailey, et al. (23) using a severe-intensity step cycle tests starting with 4min at 20W, and then directly going to 80%Δ at pedal cadences of 35 rpm or 115 rpm for 7 male recreational subjects (aged 21yr). The intensity levels were set at 80% of the difference between the VO_2 at the GET and $\dot{V}O_{2\,peak}$ (80%Δ), optodes fastened 20 cm above the fibular head. They found no differences between NIT and PL at 35 rpm in muscle oxyhemoglobin concentration [O_2Hb], phase II VO_2 kinetics, or exercise tolerance. But, at 115 rpm, muscle [O_2Hb] was higher at baseline and throughout exercise without alterations in Hb_{tot}, phase II VO_2 kinetics was faster (47 ± 16 s vs. 61 ± 25 s) and exercise tolerance was greater (362 ± 137 s vs. 297 ± 79 s) with NIT than with PL. These results suggests that NIT possibly increased muscle O_2 delivery at 115 rpm enabling faster phase II $\dot{V}O_2$ kinetics (23). The increased muscle O_2 delivery in 115rpm –NIT, may have reduced the divergence between muscle O_2 delivery and

muscle O_2 utilization at the higher pedal cadence. Influencing a faster adjustment of phase II VO_2 kinetics and better exercise tolerance in 115rpm NIT compared to 115rpm PL (23). This high rpm might serve as a possible explanation for the results we provided.

As emphasized above, our study is comparable with others that did find an effect, in terms of exercise time, somewhat the intensities, the dose, and subjects, but not in AO methods. The rational for the lack of effect of NIT on mVO_2 during both low and high intensity cycling in the present study is, therefore, rather unclear. Although protocol, fiber types and intensity seems to be of importance.

4.3 Muscle composition

Further consideration regarding the muscles utilized here may be of importance. A possible rationale for the non-significant effect in the present study can be find in the distribution of fiber types and their relation to capillary density. Staron, et al. (38) studied muscle fiber type composition taken with biopsies from the VL muscle (~16 cm proximal of the superior edge of the patella), collected from young untrained (no participation in any consistent exercise program for the last 6 months before enrolling) men and women over 10 years. They found that the VL of both men and women had roughly 41% type I slow-twitch oxidative fibers, 31% type IIa fast-twitch oxidative fibers and 20% IIb fast-twitch glycolytic fibers. Owing that Ferguson, et al. (37) have found type IIb fibers to be more responsive to nitrate in form of increased blood flow and vasodilation due to the more hypoxic milieu, this suggests that the more pronounced type I fibers in VL will preserve their oxygenation from increased capillary density, and thus may enlighten our findings. Moreover, the fiber type composition in the TA have been shown to have a similar distribution, with 39.8% % type I, 31.2% type IIa and 29.2% type IIb fibers (39). However, there was a wide range for the percentage of Type I fibers for both the men (17.6-65.6%) and the women (16.5-97.4%) found by Staron, et al. (38), so no clear consensus can be drawn. Also, VL is one of the main power producers during cycling, hence most likely to achieve hypoxic milieus.

Due to the relatively small area of NIRS measurement (3 – 4 cm in the present study) using a oxymon or a portamon device, one must anticipate the possible regional differences in muscle oxygenation within the single muscle when interpreting the results. Kennedy, et al. (40) demonstrated such differences in the distal and proximal region of the right leg VL muscle at low intensity cycling, but with less or no such differences of muscle oxygenation or differences between individuals during high intensity cycling exercise. At low intensities (25 and 50% of

VO2peak), the distal region had significantly smaller muscle oxygen values than the proximal, proposing an area of type II fibers. I.e if the muscle bulk area which we investigated consisted mainly of type I fibers, with sufficient capillarity density providing the fibers with $O_{2,}$ this could have influenced our results. Knowing that supposedly type II fibers predominately will be effected by nitrate (37). In that case, this would have been evident at the low intensity (50% OBLA). At higher intensities, such as 70% MAP, the lack of differences between regions may highlight the recruitment of a larger proportion of the muscle or the whole muscle itself.

4.4 Body composition

Various body composition measurements were taken in the present study. The reason being that adipose tissue thickness (ATT) is a confounding factor for NIRS measurements (25) and should, therefore, always be measured when utilizing NIRS. In the present study the average ATT at the site of NIRS measurement were 5 and 4.1mm for VL and TA respectively. This could have influenced our results, i.e. by the light from the NIRS probe not measuring the whole muscle tissue area in its range, but also in counting some signals from fatty tissue. Compared to similar studies, Masschelein, et al. (22) were the only one reporting ATT, being 3.9mm (i.e. lower than our ATT) in relation to NIRS-measurements. In addition to the tissue thicknesses that were measured directly on top of the muscles of interest (i.e. measured with NIRS), an approximation of the percentage of body fat (20.5% BF) and skeletal muscle mass (35.5kg) were assessed. Due to that body composition might effect NIRS muscle oxygenation/saturation characteristics in a different manner, as compared to the direct confounding impact of ATT. E.g. an endurance-trained muscle has other characteristics than a “normal” muscle or hypertrophic strength-trained muscle, i.e. in terms of mitochondrial content, capillaries and capacity of oxidative phosphorylation. Hence, it can be assumed that these alterations in body composition also affect the oxidation/saturation features derived from a standardized arterial occlusion test. Considering the range in skeletal muscle mass from 28.2 to 43kg, one may not out rule such influences. The possible impact of ATT and %BF can be said to be of less importance, taking to account that subjects were their own control. A lacking aspect in this study were, however, that we did not control for ATT in our analysis, albeit, comparable studies have not reported any data on this parameter.

4.5 Effect of supplementation on blood pressure

Blood pressure has been shown in several studies (2-4, 14) to be significantly reduced as an effect of NO_3^-, the mechanisms thought to be related to endothelium releases NO_3^- (endogenously: NO synthase enzymes or NO_3^- pathway), controlling the vasodilation and blood flow by relaxing smooth muscles (41). However, this was not the case in our study; on the contrary, placebo treatment reduced systolic BP from pre to post, but not nitrate, while no effect was found for diastolic BP. We measured BP on two occasions (using the mean value) at the beginning of each session whilst subjects were resting in a semi-elevated bed, without the perhaps sufficient resting time for such measures, due that subjects had only been resting for approximately 5 min. The varying and short timeframe for resting may have contributed to the placebo effect, i.e. if the placebo measurements were taken after a longer resting time. Wylie, et al. (14) reported peak reductions in systolic BP of ~5, ~10, and ~9 mmHg after acute supplementation of 4.2, 8.4, and 16.8 mmol inorganic NO_3^- respectively. In addition to peak reductions in diastolic BP of ~3 and ~4 mmHg after 8.4 and 16.8 mmol NO_3^-. Subjects had been resting 10min before four measurements were taken, using the mean of the last three. Similar reductions was found by Kapil, et al. (3) after 6.4 mmol NO_3^- /day for 4 weeks in in 64 (aged 18-85) hypertensive patients (some using antihypertensive drugs). Clinic systolic BP and diastolic BP was sig decreased compared to baseline with mean 7.7 and 2.4 mmHg respectively, with no changes in placebo. Twenty-four-hour ambulatory (walking, sitting) systolic and diastolic BL showed 7.7 and 5.2 mmHg reduction, and remained lower both day and night. During the first week, home systolic and diastolic BP was reduced with beetroot juice and not in placebo, and lasted throughout the intervention; systolic reduction compared to placebo of 8.1 and diastolic 3,8 mmHg. It can be argued that our subjects were not hypertensive or elderly, so that the effect would be abbreviated on that account. Although, the subjects are comparable in terms of age, and the dosage for both Kapil, et al. (3) and Wylie, et al. (14) resembles those in the present study.

5. Conclusion

This study assessed the effect of 6 days with NO_3^- supplementation on mVO_2 during low and high intensity constant-load cycling, using NIRS. The present study is, as far as the author is aware of, one of very few that have examined nitrate's effectiveness during elicited AO when cycling. This is an area in the literature which lack of several consistent interventions. The main findings of the present study were that chronic NO_3^- supplementation, quite surprisingly, did not significantly affect pVO_2 and blood pressure at 50% OBLA and 70% MAP in recreational active subjects. Hence, since we could not provoke an effect on pVO_2, as was expected, the corresponding absent of effect on mVO_2 in the VL muscle and in the TA muscle at 50% OBLA and 70% MAP seems rather reasonable. This results proposes that nitrate might not be as efficient as an ergogenic aid as previously depicted. Albeit, in agreement with other studies, the NO_3^- treatment showed trends towards lower O_2 cost compared to PL in the VL muscle at 50% OBLA and 70% MAP, suggesting that adaptations in oxygenation locally in the muscles may be manifested despite of lacking effects on a systemic scale. No such tendencies was seen in TA. The rational for the absence of effect of NIT on pVO_2 and somewhat mVO_2 during both low and high intensity cycling in the present study is rather unclear; yet, factors such as intensities and fiber type characteristics might be of importance, due to the present study`s implementation of similar supplementation dosage and timeframe as reported in the literature. Finally, future research investigating mVO_2 in relation to nitrate is required to verify whether an effect exists, and in case, how to elicit it. This might also be beneficial in revealing the yet not fully understood responsible physiological mechanisms.

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