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No effect of acute nitrate supplementation on muscle oxygen consumption in arm and leg

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Abstract

Purpose: The purpose of this thesis was to investigate the effect of acute nitrate (NO_3^-) supplementation on muscle oxygen consumption (mVO_2) in the vastus lateralis (VL) and the flexor digitorum superficialis (FDS) muscles. **Method:** In a double-blinded, placebo-controlled, crossover study, nine recreationally active men consumed an acute dose of either NO_3^--rich beetroot juice (2 x 400 mg NO_3-) or NO_3^--depleted beetroot juice (2 x 0.35 mg NO_3-) 2 h prior to a dynamic handgrip test (DHT) and a cycling test (CT). The DHT consisted of three 2-min periods of dynamic handgrip exercise at 30%, 50% and 70 % of the maximal incremental handgrip test. The CT consisted of two 5-min periods of cycling at a work rate of 50% of OBLA and 70% of MAP, where pulmonary oxygen consumption (pVO_2) was measured continuously. Near infrared spectroscopy (NIRS) was measuring tissue oxygenating continuously in FDS muscle and VL muscles. **Results:** No effect of NO_3^- supplementation were found on pVO_2, blood pressure (BP) or HR after an acute dose of NO_3-, nor any differences on mVO_2 in FDS and VL muscles during exercise were found. **Discussion:** No effect was found on pVO_2 despite the exercise protocol was similar to previously protocols that reported an effect. Even though, the right amount of NO_3^- was used and similar test conditions for the placebo period and NO_3^- period, no effect on mVO_2 was observed. **Conclusion:** At the applied dose and protocol, no effect of acute NO_3^- supplementation were found on pVO_2 and BP, nor an effect on mVO_2 in FDS muscle and VL muscle.

Key words: Nitrate, near-infrared spectroscopy, mVO_2, vastus lateralis, flexor digitorum superficiali

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Preface

This master thesis was part of a larger study at the Norwegian University of Science and Technology, therefore, specific parts of the method are selected in order to address the study aim of this thesis. Three master students collaborated with the data collection and all had their own individual study aim. In addition to an acute effect of nitrate supplementation, the project also included 6 days of chronic nitrate supplementation.

Contents

1. Introduction 1

2. Methods and materials 3

2.1 Subjects 3

2.2 Study design 3

2.3 Experimental protocol 3

2.3.1 Incremental handgrip test (IHT) 4

2.3.2 Lactate profile test and VO_{2max} test 4

2.3.3 Vascular occlusion test (VOT) 5

2.3.4 Dynamic handgrip test (DHT) 5

2.3.5 Cycling test (CT) 6

2.4 Measurements 7

2.5 Near-infrared spectroscopy (NIRS) 8

2.6 Statistics 8

3. Results 9

3.1 Study design 9

3.2 Blood pressure 11

3.4 Blood lactate concentration and rated perceived exertion (RPE) 14

3.5 Local muscle oxygen consumption (mVO_2) 14

4. Discussion 16

5. References 23

1. Introduction

The use of different dietary supplements and nutrition strategies to optimize performance in the sports world has long been in focus. One of the latest nutritional aids that has become popular is dietary nitrate (NO_3^-). The increased interest started after Larsen et al. (2007) and colleagues reported that dietary NO_3^- reduced oxygen consumption (VO_2) during submaximal cycling. The current literature shows substantial evidence that supplementation of NO_3^- rich beetroot juice (BRJ) can reduce VO_2 during exercise in recreationally active men (Bailey et al., 2009, Bailey et al., 2010, Kelly et al., 2014, Lansley et al., 2011b).

Dietary NO_3^- supplementation has been shown to increase nitric oxide (NO) levels in the human body, which it thought to be the reason for the reported effect of the improved exercise efficiency (Lundberg and Weitzberg, 2009, Bond et al., 2014). It was until recently believed that the formation of NO followed one pathway. This pathway is known as the L-arginine pathway and it uses nitric oxide synthase enzymes to oxidize the amino acid L-arginine to form NO (Lundberg et al., 2011). This reaction also causes production of NO_3^- and nitrite (NO_2^-), which was believed to be byproducts. However, it was discovered that NO_3^- and NO_2^- could be recycled *in vivo* and reused in an alternative (Lundberg and Govoni, 2004). In contrast to the L-arginine pathway, the nitrate-nitrite-NO pathway is oxygen (O_2) independent and active under hypoxic states. By ingesting BRJ, NO_3^- is reduced to NO_2^- by anaerobic bacteria in the oral cavity. Some NO_3^- will not be reduced in the first place, but is absorbed in the upper stomach and intestinal tract and out to the bloodstream (Lundberg et al., 2008). Circulating NO_3^- is taken up by the salivary gland and is reduced to NO_2^- by anaerobic bacteria. The saliva transports NO_2^- down to the stomach, where it reacts with the acidic conditions and reduces to NO (Lundberg et al., 2008).

The exact mechanism(s) behind an improved exercise efficiency remains unclear. However, based on the suggested theories it is probably related to a lower muscular demand of O_2. Theories suggest that NO increases the mitochondrial oxidative capacity (Larsen et al., 2011) and reduces the ATP cost during muscle contractions (Bailey et al., 2010), which may lead to an improved exercise efficiency. In addition, it is thought that NO causes an increased blood flow to exercising muscles, which may be favorable due to greater O_2 delivery to active muscles (Thomas et al., 2001). Lately, studies have been investigating the effect of NO_3^- supplementation on muscle metabolism by using near infrared spectroscopy (NIRS). It has been found that deoxyhemoglobin (HHb) are decreased during exercise for the vastus lateralis (VL) muscle and gastrocnemius muscle

after NO_3^- supplementation, indicating a reduced O_2 extraction (Bailey et al., 2009, Breese et al., 2013, Kenjale et al., 2011, Thompson et al., 2014).

Based on the reported effects of NO_3^-supplementation on a reduced pulmonary gas exchange during cycling exercise (Bailey et al., 2009, Bailey et al., 2010, Kelly et al., 2014, Lansley et al., 2011b), a logical assumption may therefore be, if whole body gas exchange is reduced, an effect may also be apparent in the most active muscles as well. The VL muscle was therefore chosen for the known involvement in cycling exercise (Ericson et al., 1985) and based on the reported effect of NO_3^- supplementation on muscle metabolism during cycling (Bailey et al., 2009, Breese et al., 2013, Thompson et al., 2014). In addition to the VL muscle, we are also investigating the effect of NO_3^- supplementation on isolated muscle work, in terms of activating the flexor digitorum superficialis (FDS) muscle during a handgrip exercise. This type of exercise focus on local metabolic demands without the influence of systemic variables, which makes it an interesting field of research. The effect of NO_3^- supplementation on small isolated muscle groups when local metabolic demands are required is currently unknown. NIRS was measuring local tissue oxygenation for both muscle continuously during exercise in order to see if local muscle metabolism were changed after NO_3^- supplementation.

The purpose of this study is, therefore, to investigate the effect of acute NO_3^- supplementation on mVO_2 in the FDS and the VL muscles.

2. Methods and materials

2.1 Subjects

Nine healthy recreationally active men (mean ± SD, age; 32,3 ± 8,7 years, height 182,0 ± 7,6 cm, body mass 83,5 ± 11,7 kg, VO_{2max} 49,3 ± 5,1 ml/min/kg) volunteered to participate in this study. The subjects were instructed to avoid strenuous exercise 24 hours before the tests and arrived at the laboratory in a rested and hydrated state. All subjects were right handed. The study was approved by the regional medical ethical committee. The subjects gave their written informed consent prior to commencement of the study, after explaining the associated risks and benefits of participation. All tests were performed at the same time of the day (± 1 h).

2.2 Study design

The subjects were required to report to the laboratory on 3 occasions, over a 3-week period**.** During the first visit, the subjects went through various anthropometric measurements, an incremental handgrip test and a lactate profile test followed by a VO_{2max} test. Visit 2 and 3 consisted of day 1 and day 2 of the placebo-controlled cross-over design. It started with a pre-test consisting of a vascular occlusion test (VOT) followed by a dynamic handgrip test (DHT) and a constant-load cycling test (CT). Immediately after the pre-test was completed, a venous blood sample was collected, before the subjects consumed two shots of either NO_3^--rich BRJ (2 x 450 mg NO_3-; Beet It; James White Drinks Ltd) or NO_3^--depleted BRJ (2 x 0.35 mg NO_3-; Beet It; James White Drinks Ltd). The assignment of the juice was randomized and double-blinded. A 2-hour break separated the pre-test from an identical post-test. On returning to the laboratory, a new blood sample was taken prior to the post-test. The given break is needed to allow blood NO_3^-levels to rise (Wylie et al., 2013). After having finished the first supplementation phase, the subjects switched to a new juice to ensure that they had completed a period of both NO_3^- and placebo (PL). PL and NO_3^- periods were separated by an 8-days wash-out period.

2.3 Experimental protocol

At the first visit, anthropometric measurements were done followed by a handgrip test, a lactate profile test and a VO_{2max} test.

2.3.1 Incremental handgrip test (IHT)

Prior to the IHT, maximal voluntary contraction (MVC) handgrip force was measured using a handgrip dynamometer. MVC measurement consisted of two contractions, each lasting for maximum 3 sec, 1 min break separated the contractions. MVC was defined as the absolute highest force value of the two trials.

The MVC test was followed by an IHT performed with a custom-made handgrip dynamometer to the limit of tolerance. The test started with 1 min with rhythmic handgrip exercise at a load of 6 kg, after which the load was increased with 250 g per 30 sec until the subject could not keep up with the rhythm of the metronome (60 beats per minute) or reached the limit of tolerance**.** Force was measured continuously. Maximal dynamic handgrip force was defined as the highest load (kg) achieved during IHT (maxIHT). maxIHT was used to define the different workloads for the DHT, and the weight was calculated as the percentage of the final load of the IHT.

2.3.2 Lactate profile test and VO_{2max} test

The lactate profile test was performed on a cycle ergometer and started with a 10 min warm-up period at 100 watt (W). All subjects started the test with a workload of 100 W, each load lasted for 4 min before the load was increased by 25 W. At the end of each 4-minute stages, a blood sample was taken at the tip of the finger to measure blood lactate. The test was terminated when the subject reached 4 mmol/L (OBLA) or higher. The lactate profile defined the low-intensity workout rate for the CT at 50 % of OBLA for the main part of the placebo-controlled cross-over design.

There was a 5 min break before the subjects performed a ramp incremental exercise test to estimate VO_{2max}. The individual starting load for the VO_{2max} test was calculated based on the final load (W) of the lactate profile test subtracted by 25 W. The load was increased with 25 W each minute until volitional exhaustion. VO_{2max} was defined as the average of the last 30 sec before exhaustion. Pulmonary oxygen consumption (pVO_2) and heart rate (HR) were continuously measured during both tests. The VO_{2max} was used to define the high-intensity workout rate at 70% of maximal aerobic power (MAP) for the CT.

2.3.3 Vascular occlusion test (VOT)

Before the VOT started, an inflatable pneumatic cuff was placed around the upper arm and leg on the right side of the body in order to induce the arterial occlusion (AO).

The VOT (figure 1) started with 5 minutes of baseline measurements of the NIRS signal, during this period, blood pressure (BP) was measured twice. The baseline measurements were then followed by an AO that lasted for 1 min with the intention to familiarize the subject with the feeling of an occlusion. A 5-minutes recovery period separated the second occlusion of 10 min duration. The purpose of the 10 min occlusion was to calculate resting mVO_2 for both muscles simultaneous. The VOT was terminated after 10- minutes of recovery.

2.3.4 Dynamic handgrip test (DHT)

The same custom-designed handgrip dynamometer that was used for the IHT was also used for the DHT**.** After the VOT was finished, the air supply to the pneumatic cuff on the right leg was disconnected. The DHT (figure 1) consisted of 2 minutes of rhythmic contraction-relaxation exercise at 3 different workloads (30%, 50% and 70% of maxIHT), each period was separated by 5-minutes with recovery. Immediately after completing a 2-minutes handgrip period, an AO was applied for 45 sec. The pace of the contractions were guided by a metronome, which was set at 60 bpm. NIRS was measured continuously during the VOT and the DHT.

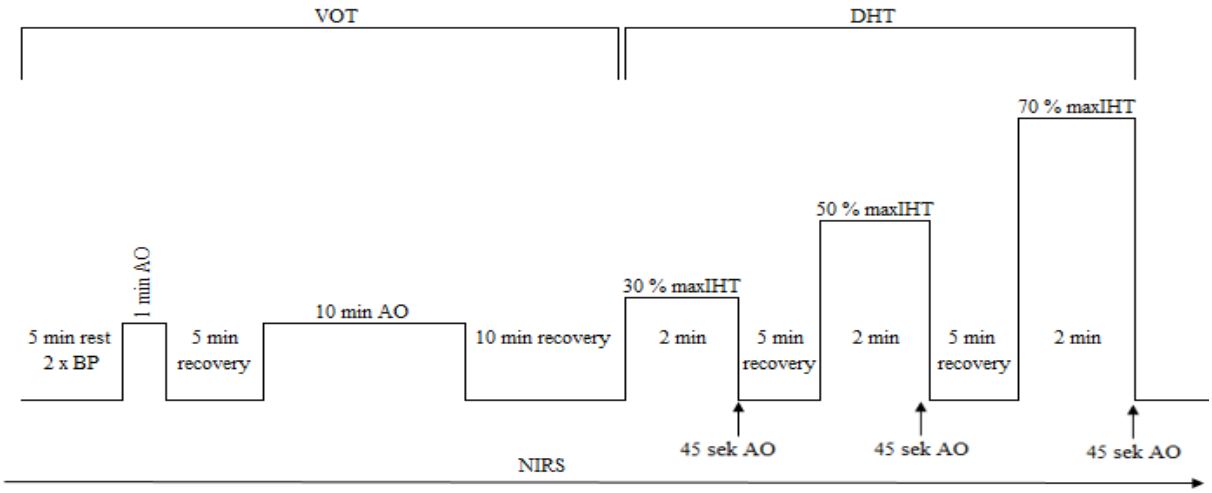

Figure 1. Schematic illustration of the vascular occlusion test (VOT) and dynamic handgrip test (DHT). AO = arterial occlusion, maxIHT = maximal dynamic handgrip force, BP = blood pressure.

2.3.5 Cycling test (CT)

A period of 10 min separated the DHT from the CT. The subjects switched from bed to cycle ergometer without disconnecting the NIRS equipment. The test started with a measurement of blood lactate and rate of perceived exertion (RPE) at rest (figure 2). The CT preceded with a warm-up period of 5 minutes at 75W, followed by 1 minute with rest, before two different cycling periods of 7 minutes duration were performed. After 4 min and 40 sec of cycling, a 20 sec AO was applied while the subject continued cycling. At the end of each work rate, blood lactate and RPE were measured. The two work periods were separated by 10 minutes of rest where the first 6 minutes consisted of repeated occlusions (RO), which is beyond the scope of this thesis, followed by 4 minutes of active recovery at 75 W before the next workload started. In addition to the RO, the periods of min 5 to min 7 of cycling were beyond the focus of this thesis as well. The subjects used a freely chosen cadence within a range of 80-100 RPM during the test. NIRS, HR and whole body gas exchange measurements were continuously measured during the CT.

After the pre-test was completed, a venous blood sample was drawn from the subjects forearm before supplementation of juice. 2 h later, a new blood sample was collected before the post-test was performed.

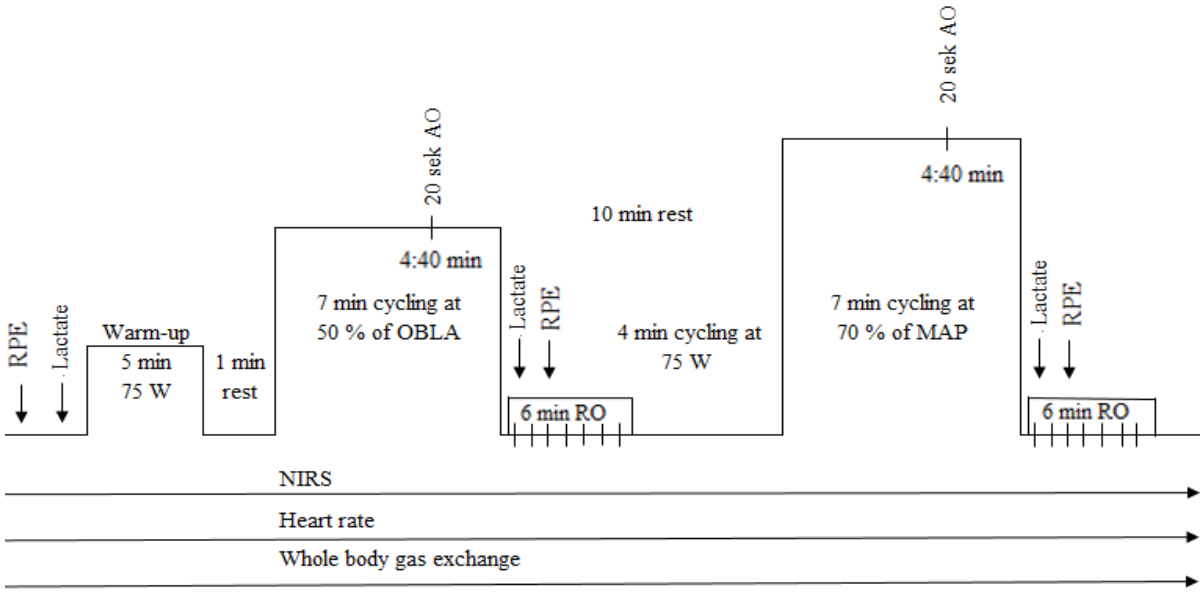

Figure 2. Schematic illustration of the cycling test. AO = arterial occlusion, RO= Rapid occlusion, RPE = rate of perceived exertion.

2.4 Measurements

Before each test session, NIRS optodes were fastened on the top of the VL muscle (Oxymon, MKIII , Artinis Medical Systems, the Netherlands) and the FDS muscle (Portamon, Artinis Medical Systems, Netherlands). Tissue oxygenation was measured continuously during the DHT and CT. The handgrip tests were performed in a semi-supine position on a treatment table so that the subjects got back support and could straighten their legs. The MVC test was performed with a handgrip dynamometer (Lafayette Instruments, Model No. 5030L1, Indiana, USA). A custom-designed handgrip dynamometer was used to perform the IHT and the DHT. The dynamometer was adjusted to address the height of the treatment table. The subject`s forearm rested on the handgrip dynamometer with an upward angle at 30° and the upper arm positioning at heart level to prevent venous pooling of the blood. All tests were performed with the dominant hand. During the IHT, force was measured continuously by using a force transducer (Model 9363, 50 kg capacity, Revere Transducers, California, USA). The force was measured in kg. In a rested state, measurements of the BP (OSZ 5 easy, Welch Allyn, Jungingen, Germany) were conducted. To apply the AO, a pneumatic cuff was placed around the upper arm and leg. The cuffs were connected to an automatic inflation system (Hokanson E20 Rapid Cuff, Marcom Medical ApS, Denmark + external air source) that put a pressure at 300 mmHg to induce AO. All cycling was performed on an electronically braked cycle ergometer (Velotron, Racermate Inc, Washington, USA). During cycling, cadence was measured continuously**.** In addition, a heart rate monitor (Polar RS800, Polar Electro OY, Kempele, Finland) was used to measure HR throughout the CT. Pulmonary gas exchange was measured using open circuit calorimetry with a mixing chamber (Oxycon Pro, Jaeger GmbH, Hoechberg, Germany). The system was calibrated 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). Blood samples were taken at the tip of the finger to measure lactate accumulation (Lactate Pro LT-1710, ArkRay Inc, Kyoto, Japan). During subsequent testing the subjects were asked to rate their perceived exertion (RPE). Skinfold thickness was measured at the site of the NIRS optodes placement, by a skinfold caliper (Holtain Tanner/Whitehouse skinfold caliper, Holtain Ltd, Crymych, Wales) and was divided by 2 to determine the adipose tissue thickness (ATT). The optode placement was marked with a pen to ensure identical placement of the NIRS devices at the next visit. Skeletal muscle mass was calculated according to the method of Lee et al. (2000) and the percentage of body fat according to

Peterson et al. (2003). Venous blood samples were drawn into one EDTA tube and two citrate tubes and were immediately centrifuged at 3000 rpm for 10 minutes at 18°C in order to separate plasma from red blood cells. Plasma were then extracted from the tubes and centrifuged a second time at the same program. Plasma that was drawn on EDTA tube was quick frozen at - 80° C and citrate tubes were slowly frozen at - 32°C for later analysis of NO_3^-.

2.5 Near-infrared spectroscopy (NIRS)

NIRS is a noninvasive method that provides continuous real-time *in vivo* changes in local tissue oxygenation. In the present study, we used wavelengths at 764 ± 2.2 and 848 ± 10.6 nanometer. These specific wavelengths enables NIRS to separate oxyhemoglobin (O_2Hb) and HHb. By applying the modified Lambert-Beer law with an incorporated differential path-length factor of 4.0, we can study relative changes in O_2Hb and HHb, due to the different absorption spectra between the two. In the present study, an interoptode distance was set at 35 mm. The optodes were covered with black cloth in order to ensure that no natural light could interfere the NIRS signal. Data was sampled with a frequency of 10 Hz. To remove movement artifacts from the raw NIRS signal during the rhythmic contraction/relaxation phases, a Butterworth filter was applied in order to filter out movement data. Concentration changes were converted from $\mu M \cdot s^{-1}$ to $ml O_2 \cdot min^{-1} \cdot 100g^{-1}$, by using a value of 22.4 L molar volume of gas and value of 4 molecules of O_2 per hemoglobin. For muscle density a value of 1.04 $kg \cdot L^{-1}$ was applied. O_2 consumption was calculated by using the intial linear rate of decrease in O_2Hb and Hb_{diff} ($Hb_{diff} = O_2Hb - HHb$) after the start of the AO.

2.6 Statistics

All data was checked for normality by applying a Shapiro-Wilk test before further analysis. Delta (Δ) values of the different variables were expressed as the changes from pre to post**.** The data was tested with a one-way, two-way or three-way ANOVA. A one-way ANOVA was used to test if the work rate was similar between the four test sessions on weight, % of maxIHT , % of MVC and W. A two-way ANOVA was used to test the effect of NO_3^- supplementation or to see if the pre-tests were similar on % of VO_{2max} % of HR_{max}, BP, Δ pVO_2, Δ HR Δ mVO_2. A three-way ANOVA was used to test the effect of NO_3^-supplementation on RPE and lactate. If significant differences were detected, a within-subject contrast was applied in order to explore the differences. When the

assumption of sphericity was violated, the results were adjusted according to the Greenhouse-Geisser correction. All results are expressed as mean ± SD. Statistical significance was accepted when $P < 0.05$. Data analysis were performed with Matlab R2015a (MathWorks Inc. Natic, USA) and SPSS 21.0 (SPSS, Chicago, USA).

3. Results

In total, 9 subjects completed the DHT and the CT. One subject completed the first supplementation phase and dropped out because of illness. Due to missing HR data of one subject, eight subjects were included in the analysis of HR (figure 5). Unfortunately, we did not get the opportunity to do statistics on plasma NO_3^- and NO_2^-, because of the ongoing analysis of the blood plasma in England. Table 1 shows the characteristics of the subjects.

Table 1. Subject characteristics.

Variables	Mean	SD	Min	Max
HR_{max} (bpm)	182.0	6.0	174	196
MAP (W)	327.7	36.3	275	375
Lactate threshold (W)	186.1	13.7	175	200
MVC (kg)	62.2	8.5	44.0	75.2
Incremental HG (Kg)	11.9	1.4	10.2	14.7
ATT (Right VL) (mm)	5.0	1.4	2.5	7.3
ATT (Right FDS) (mm)	2.3	0.5	1.5	4.0
Body fat (%)	20.5	4.3	14.4	27.85
Skeletal muscle mass (SM in kg)	35.5	5.8	28.8	40.8

MAP = maximal aerobic power, ATT = adipose tissue thickness, VL = vastus lateralis, FDS = flexor digitorum superficialis. HG = handgrip, MVC =maximal voluntary contraction. Values are means (± SD).

3.1 Study design

In order to check if workload (W) was similar over all test session for the CT, we tested the work rate variable (Table 2). A one-way ANOVA analysis was used to test if W was similar over all tests.

The result showed that that the workload was not significant ($p = 0.05$), indicating that the workload was similar over all test sessions.

To see if both pre-tests (PL and NO_3^-) had similar baseline at the start of the phases (Table 2), we applied a two way ANOVA with factors specified as test (PL-pre and NO_3^--pre) and time

(1-5 min). The result showed that % of VO_{2max} and % HR_{max} at 50% of OBLA and 70% of MAP was not significant ($p = 0.005$)**,** indicating that relative VO_2 and relative HR was similar within both pre-tests.

Table 2. Shows the control variables for the cycling test.

Cycling test	Placebo (pre-test)	Placebo (post-test)	Nitrate (pre-test)	Nitrate (post-test)
50% of OBLA				
Watts	101.1 ± 19.0	101.1 ± 19.0	101.1 ±19.0	101.1 ± 19.0
% of VO2max	41.1 ± 7.0	42.8 ±5.1	39.5 ± 7.5	43.0 ± 5.6
% of HRmax	57.2 ± 5.9	59.8 ± 5.6	57.4 ± 5.6	60.7 ± 6.7
70% of MAP				
Watts	225. 7 ± 20.6	225. 7 ± 20.6	224.1 ± 21.3	225. 7 ± 20.6
% of VO2max	70.3 ± 12.4	68.2 ± 13.1	70.0 ± 12.8	68.8 ± 13.8
% of HRmax	76.7 ± 9.3	78.0 ± 8.9	76.4 ± 9.4	78.6 ± 8.6

Values are means (± SD).

In order to check if the workload were similar over all four test sessions for the DHT, we tested the workload variables (Table 3). A one-way ANOVA analysis was used to test if the weight, % of maxIHT and % of MVC was similar over all tests.

The result showed that weight, % of maxIHT and % of MVC at 30, 50 and 70 % of maxIHT were not significant ($p = 0.05$), indicating that workloads on the DHT were similar over all four test sessions.

Table 3. Shows the control variables for the dynamic handgrip test.

Dynamic handgrip test	Placebo (pre-test)	Placebo (post-test)	Nitrate (pre-test)	Nitrate (post-test)
30% of maxIHT				
Weight (kg)	3.8 ± 0.4	3.8 ± 0.4	3.7 ± 0.4	3.6 ± 0.4
% of maxIHT	32.2 ± 3.5	32.4 ± 3.6	31.1 ± 2.1	30.7 ± 2.2
% of MVC	6.42 ± 1.4	6.4 ± 1.4	6.1 ± 0.7	6.1 ± 0.7
50% of maxIHT				
Weight (kg)	6.4 ± 0.9	6.4 ± 0.8	6.1 ± 0.7	6.1 ± 0.6
% of maxIHT	54.1 ± 6.3	54.3 ± 6.8	51.8 ± 0.9	51.7 ± 0.9
% of MVC	10.8 ± 2.7	10.8 ± 2.8	10.2 ± 1.5	10.2 ± 1.5
70% of maxIHT				
Weight (kg)	9.1 ± 1.1	9.1 ±1.1	8.8 ± 1.0	8.8 ± 1.0
% of maxIHT	77.2 ± 8.4	77.4 ± 8.8	74.6 ± 1.6	74.1 ± 1.6
% of MVC	15.4 ± 3.6	15.4 ± 3.7	14.7 ± 1.9	14.9 ± 1.9

MVC = maximal voluntary contraction, maxIHT = maximal intremantal handgrip test. Values are means (± SD).

3.2 Blood pressure

Group mean (± SD) BP values are shown in figure 3. In general, the post-tests tended to be lower at the pre-tests for both systolic and diastolic BP. At the NIT period the pre-tests shows a tendency to be lower compared with PL period**.** BP changes were tested with a two-way ANOVA with factors specified as treatment (PL vs. NO_3^-) and test (pre- vs. post-tests).

A significant main effect of treatment was observed for systolic BP ($F(1,8) = 8.179$, $p = 0.021$). A contrast revealed that systolic BP was significantly lower at the NO_3^- period ($F(1,8) = 10.181$, $p = 0.013$). In addition, a significant main effect of test was found ($F(1,8) = 6.658$, $p = 0.033$). A contrast revealed that systolic BP was significantly lower at the post-tests ($F(1,8) = 6.658$, $p = 0.033$). However, no interaction effect was found between treatment and test for systolic BP ($F(1,8) = 0.012$, $p = 0.917$) , indicating that NO_3^- supplementation had no effect on systolic BP (figure 3 A).

There was not found a significant main effect of treatment for diastolic BP ($F(1,8) = 0.010$, $p = 0.923$). However, a significant main effect of test was observed found ($F(1,8) = 21.631$, $p = 0.002$). A contrast revealed that diastolic BP was significantly lower at the post-tests ($F(1,8) = 5.735$, $p = 0.044$). Overall, there was a non-significant interaction between treatment and test for diastolic BP ($F(1,8) = 3.151$, $p = 0.114$), indicating that NO_3^- supplementation had no effect on diastolic BP (figure 3 B).

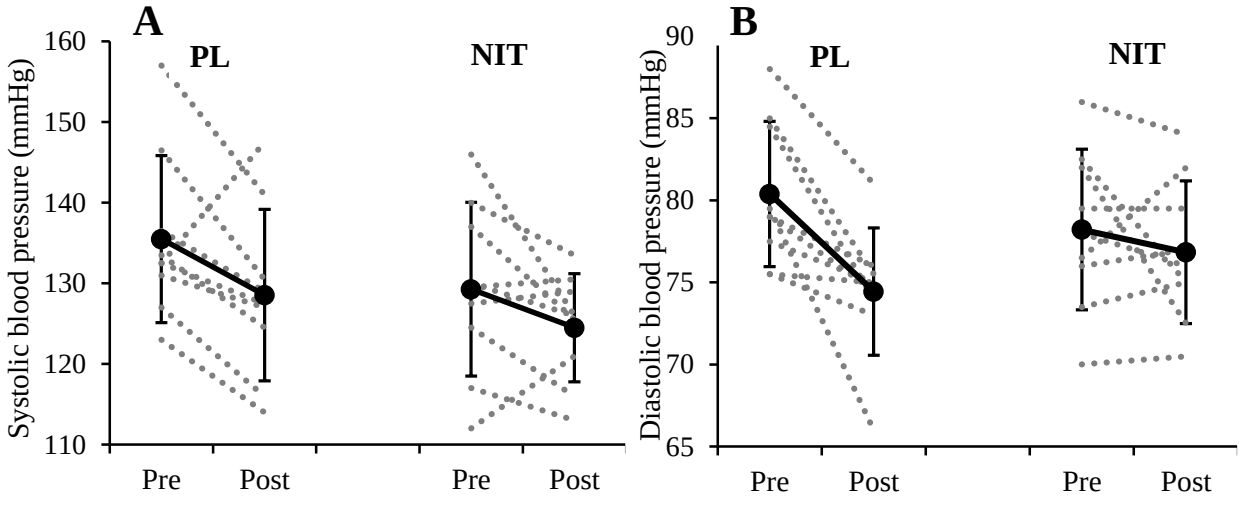

Figure 3 – Systolic blood pressure (A) and diastolic blood pressure (B) after an acute dose of NO3- -rich BRJ and PL juice. Mean (± SD).

3.3 Systematic variables

Group mean (± SD) pVO_2 values are shown in figure 4. As figure 4 A (PL) shows, the pre- and post-test are roughly the same apart of the initial phase where pre pVO_2 values are lower, while B (NO_3^-) shows that pVO_2 had a tendency to be higher after NO_3^-consumption. Consequently, the Δ-values shows a tendency to be higher at the NO_3^- period. Δ pVO_2 was tested with a two-way ANOVA with factors specified as treatment (PL vs. NO_3^-) and time (1-5 min). Δ-changes at 50% of OBLA (figure 4 C) and 70% of MAP (figure 4 F) are used in the analysis.

No main effect of treatment was found for Δ pVO_2 between the PL and NO_3^- at 50 % of OBLA ($F(1,8) = 0.715$, $p = 0.442$). A significant main effect of time was observed ($F(4,32) = 4.953$, $p = 0.003$. A contrast revealed that the difference in Δ pVO_2 was largest between the first and second minute, but similar during the remainder of the exercise ($F(1,8) = 7.412$, $p = 0.026$). However, no interaction effect between treatment and time for Δ pVO_2 was found ($F(4,32) = 2.324$, $p = 0.153$), indicating no effect of NO_3^- supplementation on the response in pVO_2 from PL to NO_3^- (figure 4 C).

Figure 4 D (PL) and E (NO_3^-) shows a roughly similar response for pVO_2 during cycling at 70 % of MAP (start- end- pre- post). Not surprisingly, statistics showed no main effect of treatment or time, neither no interaction effect between treatment and time for Δ pVO_2 during cycling at 70% of MAP ($p < 0.05$), indicating that pVO_2 was similar between the treatments (figure 4 F).

Group mean (± SD) HR values are shown in figure 5. As seen in figure 5 A (PL) and B (NO_3^-) a tendency shows a higher HR at the post-tests when cycling at 50% of OBLA. Δ HR was tested with a two-way ANOVA with factors specified as treatment (PL vs. NO_3^-) and time (1-5 min). Δ-changes at 50% of OBLA (figure 5 C) and 70% of MAP (figure 5 F) are used in the analysis.

No main effect of treatment was observed for HR during cycling at 50% of OBLA ($F(1,7) = 0.216$, $p = 0.654$), nor a main effect of time ($F(4,28) = 0.798$, $p = 0.460$). Overall, there was a non-significant interaction between treatment and time for Δ HR ($F(1,7) = 2.087$, $p = 0.917$) (5 C).

As can be seen in figure 5 D (PL), HR was consistent between the tests when cycling at 70% of MAP, the same can be seen in figure E (NO_3^-). No main effect was observed of treatment for HR at 70% of MAP ($F(1,7) = 0.485$, $p = 0.508$). Neither was it found a main effect of time ($F(4,28) = 0.410$, $p = 0.588$). Overall, there was a non-signifcant interaction of treatmeant and time for Δ HR ($F(1,7) = 0.332$, $p = 0.861$), indicating that HR was similar between the treatments (figure 5 F).

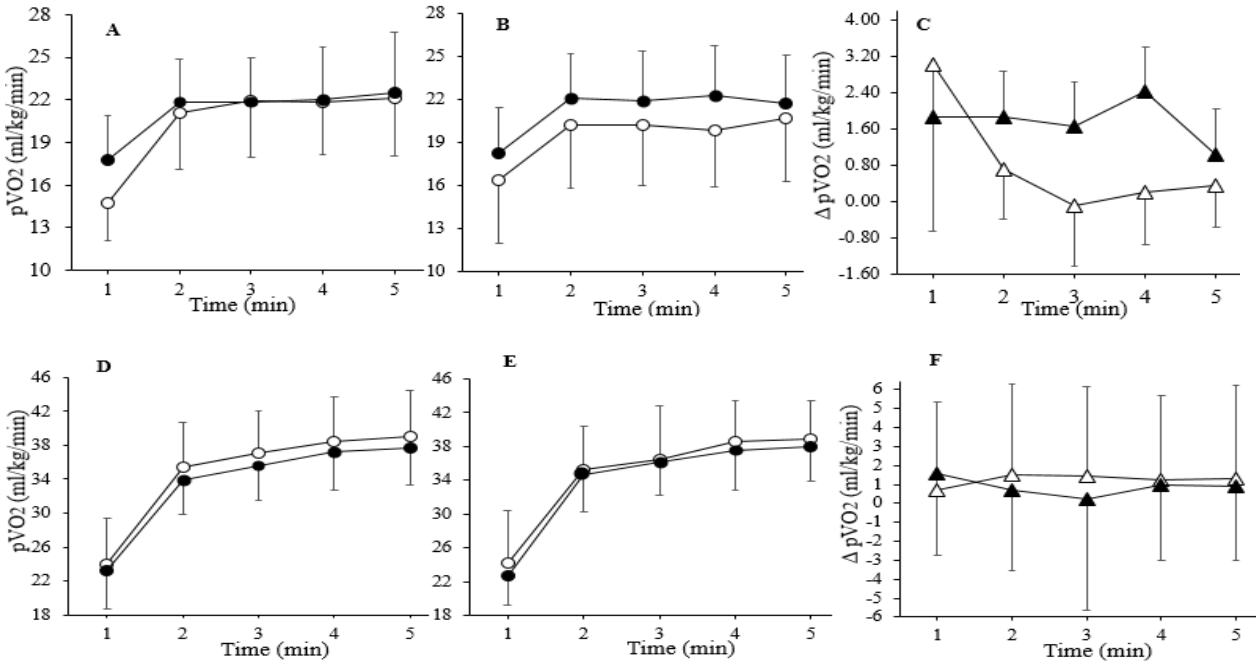

Figure 4 – Group mean (± SD) pVO_2 values during cycling at 50% of OBLA after ingestion of (A) PL juice and (B) NO_3^--rich BRJ. Open circles represent pre-test data, closed circles represent post-test data. Figure C shows ΔpVO_2 values between the treatments. Open triangles represent PL - data, closed triangles represent NO_3^- data. Figure D-F shows the same, but are for 70% of MAP. N= 9.

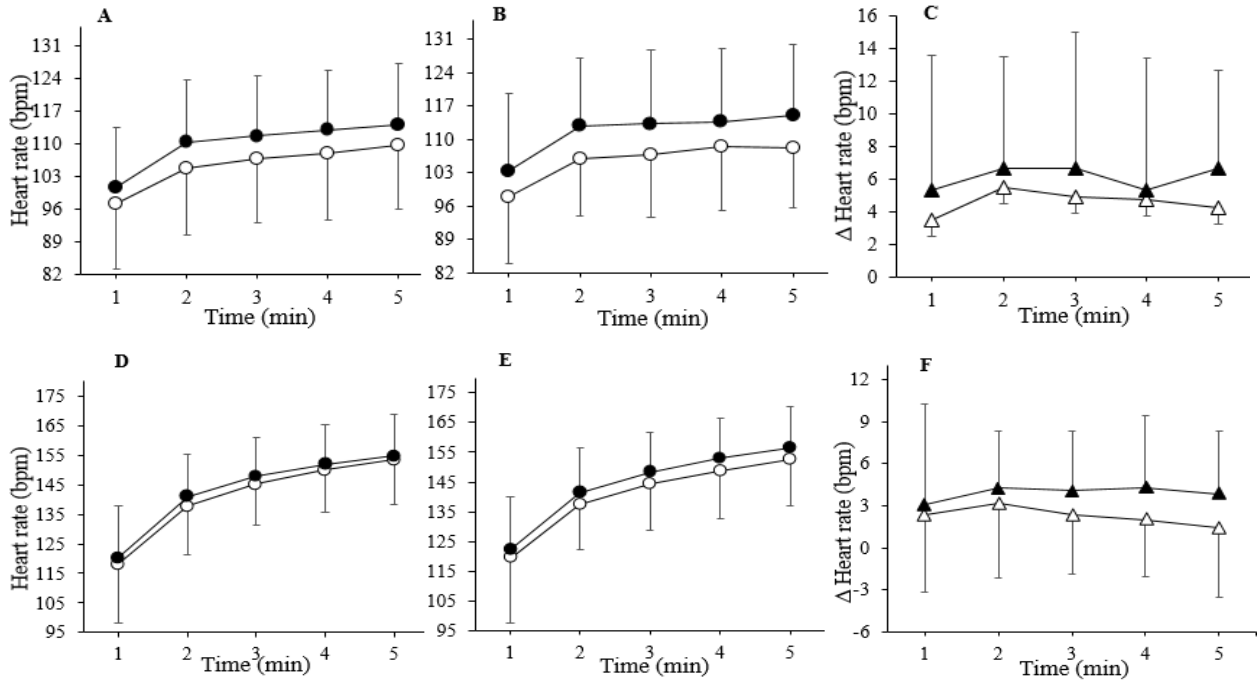

Figure 5 – Group mean (± SD) HR values during cycling at 50% of OBLA after ingestion of (D) PL juice and (E) NO_3^--rich BRJ. Open circles represent pre-test data, closed circles represent post-test data. Figure F shows ΔHR values between the treatments. Open triangles represent PL - data, closed triangles represent NO_3^- data. Figure D-F shows the same, but are for 70% of MAP. MAP. N=8.

3.4 Blood lactate concentration and rated perceived exertion (RPE)

Group mean (± SD) lactate and RPE values are shown in table 4. A 3-way ANOVA with facors specified as treatmeant (PL vs. NO_3^-), intensity (rest -50% of OBLA -70% of MAP) and test (pre- vs. post-tests) were applied in order to investigate if lactate or RPE were different between the treatments.

No main effect of treatment on lactate was found ($F(1,8) = 1.557$, $p = 0.247$), nor a main effect of tests ($F(1,8) = 2.063$, $p = 0.189$) and no interaction effect of treatment × intensity × test ($F(2,16) = 0.841$, $p = 0.450$), indicating that NO_3^- supplementation had no effect on the lactate response.

No main effect of treatment on RPE was found ($F(1,8) = 0.364$, $p = 0.563$). Neither was a main effect of test found ($F(1,8) = 2.588$, $p = 0.146$). Overall, there was no interaction effect of treatment × intensity × test ($F(2,16) = 1.659$, $p = 0.221$), indicating that perceived exertion was similar between the treatments.

Table 4. Lactate and RPE mesuerd during the cycling test after ingestion of PL and NO_3^-.

	Lactate				RPE			
	Placebo		Nitrate		Placebo		Nitrate	
	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test
Rest	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.2	1.1 ± 0.2	6 ± 0.7	6 ± 1.0	6 ± 0.4	6 ± 0.5
50% of OBLA	1.9 ± 0.7	1.8 ± 0.6	1.7 ± 0.5	2.0 ± 0.9	10 ± 1.6	11 ± 1.5	10 ± 1.3	10 ± 1.3
70% of MAP	8.2 ± 2.0	8.3 ± 1.6	8.7 ± 1.8	8.5 ± 1.6	15 ± 1.1	15 ± 1.2	16 ± 1.3	16 ± 1.4

RPE = rate of perceived exertion. Values are means (± SD).

3.5 Local muscle oxygen consumption (mVO_2)

Figure 6 shows group mean (± SD) values for local mVO_2 in VL muscle during cycling at 50% of OBLA and 70% of MAP. Δ-changes are illustrated in figure (6 C).

In general, a slightly higher mVO_2 at the post-tests for both workloads can be seen in figure 6, except at 70 % of MAP on the NO_3^- period where the pre and post tended to be similar. The Δ-values for the VL muscle was tested with a two-way ANOVA with factors specified as treatment (PL vs. NO_3^-) and intensity (rest - 50% of OBLA - 70% of MAP).

The results for Δ mVO_2 showed no main effect of treatment at 50% of OBLA ($F(1,8) = 1.366$, $p = 0.276$). A significant main effect of intensity for Δ mVO_2 was observed ($F(2,16) = 4.719$, $p = 0.025$). A contrast revealed a significant difference between rest and 50% of OBLA and between 50% of OBLA and 70% of MAP ($p < 0.05$). However, no interaction effect was found

between treatment and intensity for Δ mVO_2 (F(2,16) = 0.246, p = 0.783), indicating that there was no difference in Δ mVO_2 during exercise in VL muscle between the treatments (figure 6 C).

Figure 7 A (PL) and B (NO_3^-) illustrates mVO_2 at different workloads during dynamic handgrip exercise, and Δ-changes mVO_2 are illustrated in figure (7 C). As can be seen in both figure 7 A and B, mVO2 is at the highest at a work intensity at 50% of maxIHT. In addition, a tendency towards a reduced mVO_2 at 70 % of maxIHT compared with 50% of maxIHT can also be seen in the figures. Δ-values for the FDS muscle was tested with a two-way ANOVA, with factors specified as treatment (PL vs. NO_3^-) and intensity (rest - 30 - 50 – 70% of maxIHT). Δ-values are used in the analysis.

The results for Δ mVO_2 showed no main effect of treatment (F(1,8) = 0.060, p = 0.812). Neither was it found any main effect of intensity for Δ mVO_2 (F(3,24) = 1.413, p = 0.273). Overall, no interaction effect was found between treatment and intensity for Δ mVO_2 (F(3,26) = 0.164, p = 0.970), indicating that, similar as for the VL muscle during cycling, no effect of NO_3^- supplementation on mVO_2 of the FDS muscle during handgrip exercise (figure 7 C).

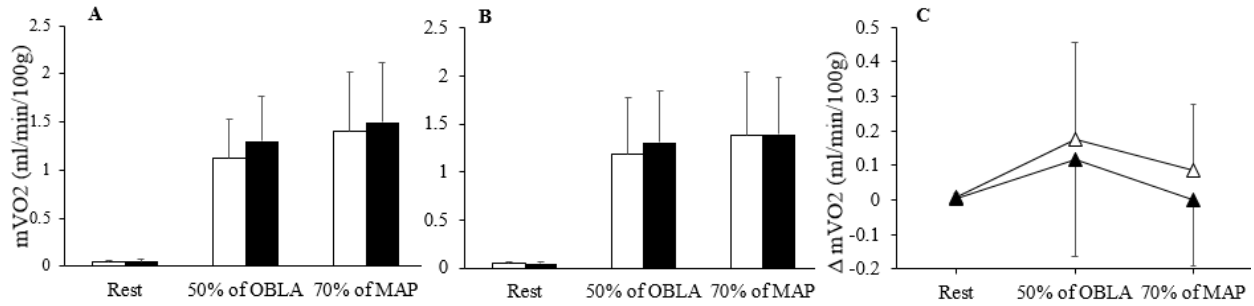

Figure 6 – Group mean (± SD) mVO_2 during the CT at rest and 2 different workloads after ingestion of (A) PL juice and (B) NO_3^--rich BRJ. White bars represent pre-test data, black bars represent post-test data. Figure C shows Δ mVO_2 changes between the treatments. Open triangles represent PL data, closed triangle represent NO_3^- data.

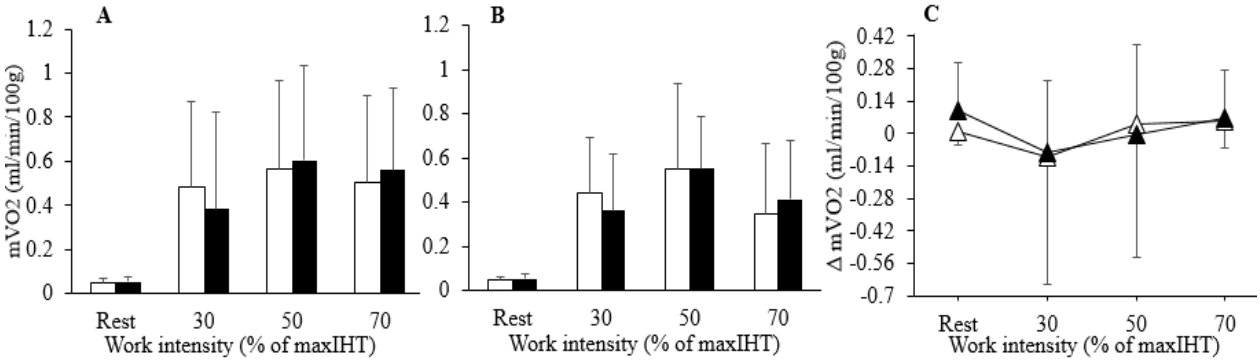

Figure 7 – Group mean (± SD) mVO_2 during the DHT at rest and 3 different workloads after ingestion of (A) PL juice and (B) NO_3^--rich BRJ. White bars represent pre-test data, black bars represent post-test data. Figure C shows Δ mVO_2 changes between the treatments. Open triangles represent PL data, closed triangles represent NO_3^- data.

4. Discussion

The aim of this study was to examine if there were any effects of acute NO_3^- supplementation on local metabolism measured as mVO_2 in the VL and FDS muscles during dynamic exercise in healthy recreationally active men. The main findings of this study showed that an acute dose of NO_3^- had no effect on pVO_2 during moderate- and high intensity exercise nor on the BP at rest. Neither was it found any effects on mVO_2 in FDS and VL muscles during exercise.

That we did not found any effect of NO_3^- supplementation on pVO_2 during cycling at 50% of OBLA and 70% of MAP was surprising, because the literature shows great evidence that VO_2 are reduced during exercise in recreationally active men after NO_3^- supplementation (Bailey et al., 2009, Bailey et al., 2010, Kelly et al., 2014, Lansley et al., 2011b). However, in support of our findings, one recent study performed on recreationally active males showed that an acute dose of NO_3^- had no effect on VO_2 during exercise, although they found a significant increased plasma NO_3^- (Betteridge et al., 2016). Additionally, one study found that VO_2 during exercise remained unchanged after NO_3^- supplementation in older adults (Kelly et al., 2013). The results from this study showed that the post-test tended to be slightly higher after NO_3^- supplementation compared with the pre-test at 50 % of OBLA (figure 4 B), this may be explained by the low pVO_2 at the pre-test (table 2). Consequently, this cause the Δ-values to be higher at the NO_3^- period compared with PL period (figure 4 C). Additionally, it can be thought that if there was an effect of NO_3^- supplementation, the effect could be minimized because of the low pre-test. However, when looking at table 2, it shows that post-test at the NO_3^- period had the highest value of pVO_2 over all four test sessions, and therefore, unlikely to have an effect of NO_3^- supplementation anyway. In addition, no differences were found when analyzing Δ pVO_2 at 70 % of MAP (figure 4 F). If there was an effect of NO_3^- supplementation this is where we expected it to be, based on the reporting ergogenic effect of NO_3^- supplementation during hypoxia and high–intensity exercise (Masschelein et al., 2012, Bond et al., 2012). In addition, statistics showed that relative VO_2 and relative HR were similar within the pre-tests for both 50% of OBLA and 70% of MAP, indicating that the PL period and NO_3^- period had similar baseline at the start of the phases (table 2). No differences were neither found when analyzing the workload (W) between the four test sessions (table 2). Overall, there is no reason to believe that the amount of effort differed between the tests.

The exact physiological mechanism behind the reported effect on VO_2 has not yet been resolved, but one of the mechanisms suggested is that NO_3^- supplementation reduce the proton

leakage across the inner mitochondrial membrane. Consequently, an increased P/O ratio (i.e., the amount of ATP molecules produced per unit O_2 consumed) occurs, probably due to a lower activity of adenine nucleotide translocase which is associated with contributing to proton leakage, explaining an improved mitochondrial efficiency (Larsen et al., 2011). Another possible mechanism is that NO downregulates the activity to the enzymes actomyosin-ATPase and Ca^+ ATPase, two enzymes that are responsible for the breakdown and reformation of ATP. This causes a decreased accumulation of ADP, PCr and P_i, indicating a reduced ATP cost in contracting muscles (Bailey et al., 2010). In addition, one theory suggest that NO causes an increased blood flow to exercising muscles, because of the ability NO have to vasodilate arteries and veins. This may be favorable due to greater O_2 delivery to active muscles (Thomas et al., 2001).

One possible explanation of lack of effect on pVO_2 might be that plasma NO_3^- levels did not reached a peak before the post-test began. In general, it is considered that plasma NO_3^- and NO_2^- peaks in the blood 2-3 hours after supplementation of NO_3^- rich BRJ (Webb et al., 2008, Kapil et al., 2010). Therefore, it is common to wait 2-3 hours before the tests are performed, because it is believed that a higher value of plasma NO_3^- favors the production of NO, thus, have the strongest physiological effect. However, one study that was performed on trained-cyclists found that plasma NO_3^- peaked 1.5 hour and NO_2^- peaked at 1 hour after ingesting 2 x 60 ml NO_3^--rich gel (~ 8.1 mmol) (Muggeridge et al., 2015). This suggests that the absorption of NO_3^- into the blood stream occurs more rapidly when ingesting NO_3^--rich gel compared with supplementation of NO_3^--rich BRJ. However, the current literature are limited concerning NO_3^- gel, and therefore too early to conclude that this is a fact. As mentioned previously, we did not get the opportunity to do statistics on plasma NO_3^- and NO_2^-, and can therefore, not quantify if this was the case in the present study. However, until these data are analyzed, this might be a possible explanation for the lack of findings, due to the time between supplementation and the testing exceeded 2-hours.

The lack of effect are unlikely related to the applied dose. In the present study, the subjects consumed 2 x 70 ml sport shots equal to 13 mmol NO_3^-. It has previously been reported that a NO_3^- dosage of 5-8 mmol significantly reduces VO_2 during exercise (Bailey et al., 2009, Lansley et al., 2011c, Vanhatalo et al., 2011). In addition, Wylie et al. (2013) and colleagues found that an acute dose of 4.2 mmol NO_3^- did not influence the O_2 cost in physical active men, however, a dose of 8.4 mmol and 16.8 mmol NO_3^- significantly reduced VO_2 during moderate-intensity exercise. Interestingly, a dose of 16.8 mmol NO_3^- was related to a further decrease in VO_2 (~ 3.0%)

compared with a dosage of 8.4 mmol (~ 1.8%). Based on the above-mentioned literature and the dose used in this study, it is unlikely that the dose might be the cause for the lack of finding on pVO_2.

Several studies reports that well-trained individuals have no effect of NO_3^- supplementation on VO_2 during exercise (Bescos et al., 2012, Peacock et al., 2012, Bescos et al., 2011). However, the lack of effect on pVO_2 are unlikely to be related to the training status of the subjects in this study, because the subjects were not well-trained (VO_{2max} 49,3 ± 5,1 ml/min/kg). Based on the literature, studies shows that VO_2 are reduced after NO_3^- supplementation on a study population that tested a group mean of 50 ml/min/kg or higher on a VO_{2max} test (Muggeridge et al., 2014, Larsen et al., 2007, Lansley et al., 2011c, Muggeridge et al., 2013, Cermak et al., 2012a). Therefore, no reason to believe that the training status was a confounding factor.

Regarding the applied exercise protocol in the present study, Larsen et al. (2007) and colleagues found that sodium NO_3^- supplementation reduced VO_2 during 5 min cycling at 45, 60, 70, 80 and 85% of VO_{2peak}. Compared with this study, a workload of 45% of VO_{2peak} represent roughly the same as a workload of 50 % of OBLA and a workload of 70% of VO_{2peak} are representative for a workload of 70% of MAP (table 2). In addition, the mentioned study was performed on well-trained individuals (VO_{2peak} 55 ± 3,7 ml/min/kg). However, it should be noted that the mentioned study used sodium NO_3^- and 3 days of supplementation. One study, found that an acute dose of NO_3^--rich BRJ reduced VO_2 during 5 min of cycling at 40%, 60% and 80% of VO_{2peak} (Bond et al., 2014). Based on these studies, the exercise protocol seems not to be the cause for lack of findings on pVO_2 in this study.

That NO_3^- supplementation had no effect on the systolic BP was not as expected, considering that the literature shows great evidence on a reduced systolic BP after NO_3^- supplementation (Vanhatalo et al., 2010, Lansley et al., 2011a, Webb et al., 2008). The literature suggest that a reduced BP are caused by NO which enables the arteries and veins to vasodilate, allowing the blood flow to circulate with less vascular resistance (Lundberg et al., 2008). However, not all studies found an effect on systolic BP, but these studies were performed on well-trained individuals (Wilkerson et al., 2012, Cermak et al., 2012b). Concerning the diastolic BP, studies shows that the results differ between studies. Some reports that the diastolic BP are unaffected after NO_3^- supplementation which is in agreement with the present study (Bond et al., 2014, Coles and Clifton, 2012) and others reports an effect NO_3^- (Larsen et al., 2007, Sobko et al., 2010, Larsen et

al., 2010). A meta-analysis that included in total 12 studies examined the effect of NO_3^- supplementation on the BP. The daily amount of NO_3^- ranged from 5.1 and 45 mmol and the duration of the supplementation phase was stratified as < 3 days. The result showed that consumption NO_3^- rich BRJ significantly reduced the systolic BP, but not the diastolic BP (Siervo et al., 2013). Even though no effect on the systolic- and diastolic BP was found in this preset study, a main effect revealed that both systolic and diastolic BP were significantly reduced on the post-tests. In addition, a significant main effect showed that the systolic BP was significantly lower at the NO_3^- period compared with the PL period. This may be because of the low pre-test on the NO_3^- period, and therefore, an overall lower systolic BP.

One possible explanation for the lower systolic and diastolic BP from the pre- to post-test at the PL period (figure 3) may due to the subjects stress level. One study demonstrated that BP was increased during a stressful situation (Hjortskov et al., 2004). Since 6 out of 9 subjects started with PL supplementation, it can be that the subjects were nervous during the first visit. In addition, the subjects could have felt discomfort during the setup of the equipment. As can be seen in figure 3, the post- test at the PL period are more similar to the pre-test at the NO_3^- period. This may indicating that that the subjects were more familiarized with the procedure and was more comfortable.

Another possible explanation for the lack of effect on the BP can be that the duration of rest was too short before the measurements were performed. Common for those studies that reported an effect of NO_3^- supplementation on the systolic BP, is that they used a protocol with a duration of 10 min of rest before BP measurements (Bailey et al., 2010, Lansley et al., 2011a, Wylie et al., 2013). In addition, these studies also recorded four measurements of the BP where they used the mean of the final three in the data analysis. In contrast, the present study used a protocol consisting of two BP measurement over a 5 min period. One study that were performed on individuals with hypertension reported that 5 min of rest could lead to overestimation of the BP. In addition, they found that 10 min of rest significantly reduced the BP compared with 5 min of rest. They concluded that 10 min of rest gave a more valid measurement of the true BP (Nikolic et al., 2014). Therefore, it may be that a longer duration of rest could prevent the differences between the pre- and post -test at the PL period (figure 3), because of a more stabilized BP. If a longer duration of rest could cause a reduced BP as an effect of NO_3^- supplementation remains, however, unknown.

That HR was similar between PL and NO_3^- period (figure 5) was as expected and these finding are consistent with the literature. The same applies lactate and RPE (table 4) (Wilkerson et al., 2012, Cermak et al., 2012b, Bescos et al., 2011).

When it comes to mVO_2, no differences were found in the FDS muscle and the VL muscle during exercise after NO_3^- supplementation. There are currently no studies that have been investigated the effect of NO_3^- supplementation on mVO_2. However, there are some studies that report a decreased HHb during exercise for the VL and the gastrocnemius muscle, indicating a reduced O_2 extraction (Bailey et al., 2009, Breese et al., 2013, Kenjale et al., 2011, Thompson et al., 2014, Masschelein et al., 2012).

It has previously been proposed that exercising during a hypoxic state favor the NO production due to activation of the nitrate-nitrite-NO pathway, which may be a possible explanation for a change in exercise efficiency (Lundberg et al., 2008, Bond et al., 2012). This may explain why Breese et al. (2013) did not found an effect of NO_3^- supplementation during moderate-intensity exercise, but found a decreased HHb during severe-intensity exercise. In addition, one study that was performed during hypoxic conditions found that 20 min of cycling at 45% of VO_{2peak} and a maximal incremental exercise test decreased HHb (Masschelein et al., 2012). In contrast to these findings, Bailey et al. (2009) found that HHb was significantly reduced during moderate-intensity exercise, but no change was observed during severe-intensity exercise. This may suggest that hypoxia or high-intensity exercise not are an absolute requirement in order to find an effect of NO_3^- supplementation on muscle metabolism. Hypothetical, a workload at 50% of OBLA and 70% of MAP should therefore be sufficient in order to find an effect on muscle metabolism.

One possible explanation for lack of finding for the VL muscle can be due to ATT at the site of the optode placement, which is considered to be a confounding factor when using NIRS (van Beekvelt et al., 2001). A large ATT can prevent the infrared light to reach muscle tissue. Van Beekvelt et al. (2001) found that subjects with a high value for ATT had a lower mVO_2 compared with those subjects with the leanest ATT. The range of the infrared light is thought to be approximately one-half of the interoptode distance (Ferrari et al., 2004), which was set at 35 mm in the present study. Not all studies that have been investigating the effect of NO_3^- on muscle metabolism reports ATT (Kenjale et al., 2011, Breese et al., 2013, Bailey et al., 2009). However, one study that found an improved muscle metabolism status in VL, reported that ATT for the VL muscle on average was 3.9 mm (range: 2.4–5.5). Additionally, they used an interoptode distance

of 4 cm (Masschelein et al., 2012). Based on they had a lower ATT for the VL muscle compared with our study (average: 5.0 mm, range: 2.5 – 7.3) and used a interoptode distance of 4 cm it can be that they where able to get a more representative value for tissue oxygenation, explaining an improved muscle metabolism.

In addition to the VL muscle, we also studied the FDS muscle during dynamic handgrip exercise. This type of exercise focus on isolated muscle work and local metabolic demands, without the influence of systemic variables. To our knowledge, this is the first study that investigated the effect of NO_3^- supplementation on mVO_2 in the FDS muscle, and the result showed that an acute dose of NO_3^- did not affected mVO_2 during exercise. Statistics showed that the applied weight was similar at 30, 50 and 70 % of maxIHT between all test sessions. Not surprisingly, the same was found at % of maxIHT and % of MVC. Based on these results, there is no reason to believe that the amount of effort during the tests were different between the four test sessions.

One possible reason for lack of finding for the FDS muscle may due to the muscle fiber composition. One study found that the muscle fiber composition for FDS muscle mainly consisted of type I (54%) and IIA (36%) and almost absent of type IIX fibers (Moreno-Sanchez et al., 2008). Jones et al. (2016) and colleagues suggested that since type II fibers have a lower microvascular O_2 pressure during exercise compared with type I fibers. That this could promote the formation of NO_3^- to NO and thereby increasing local blood flow to type II fibers. This theory is supported by studies that have been performed on rats. They found that NO_3^- supplementation increased blood flow to type IIX fibers, but not in type I fibers (Ferguson et al., 2013, Ferguson et al., 2015). Thus, an ergogenic effect of NO_3^- supplementation may be more apparent when a greater % of type IIX fibers is activated during exercise. One theory suggest that well-trained individuals do not have an effect of NO_3^- supplementation due to a great proportion of type I fibers and mitochondria, hence a better muscle oxygenation (Bescos et al., 2012, Christensen et al., 2013). Sine Type I fibers have a great oxidative capacity, it may be that the effect of NO are limited. However, the Δ-values for O_2Hb reveals a higher deoxygenated status in the FDS muscle at 70% of maxIHT (Δ O_2Hb -16.2 ± 7.8) compared to 70% of MAP in the VL muscle (Δ O_2Hb -7.6 ± 7.8). This indicating a much more hypoxic state in the FDS muscle than in VL muscle. Since the effect of NO_3^- supplementation seems to have the greatest effect during hypoxia, this may suggest that if there was an effect it would be more apparent in the FDS muscle than in the VL muscle.

In conclusion, this is the first study that investigated the effect of acute NO_3^- supplementation on mVO_2 in the FDS and VL muscles during exercise. The main findings of the present study showed that we could not support the reported effect of NO_3^- supplementation on pVO_2 and BP. Neither did we found an effect of NO_3^- supplementation on mVO_2 in the FDS and VL muscles during exercise. Since the current research, concerning the effect of NO_3^- supplementation on muscle metabolism is limited, and considering that, no other studies have been investigating the effect of NO_3^- supplementation during isolated muscle work. Further research is, therefore, needed in order to conclude whether NO_3^- supplementation can have an effect on muscle metabolism during exercise, and if isolated muscles during isolated muscle work can benefit of dietary NO_3^-.

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