UNIVERSITA' DEGLI STUDI DI MILANO

Department of Biomedical Sciences for Health
PhD Course in Integrative Biomedical Research

XXXI cycle

Effects of enhanced nitric oxide bioavailability on exercise tolerance in different conditions

PhD Supervisors:
Prof. Antonio La Torre
Dott. Simone Porcelli

Letizia Rasica

LIST OF PUBBLICATIONS

Rasica L, Porcelli S, Marzorati M, Salvadego D, Vezzoli A, Agosti F, De Col A, Tringali G, Jones AM, Sartorio A, Grassi B. Ergogenic effects of beetroot juice supplementation during severe-intensity exercise in obese adolescents. *Am J Physiol Regul Integr Comp Physiol* 315(3):R453-R460, 2018. doi: 10.1152/ajpregu.00017

Mastropietro M, Porcelli S, Cadioli M, **Rasica L**, Scalco E, Gerevini S, Marzorati M, Rizzo G. Triggered Intravoxel Incoherent Motion MRI for the Assessment of Calf Muscles Perfusion during Isometric Exercise. *NMR Biomed* 31(6):e3922, 2018. doi: 10.1002/nbm.3922

Zuccarelli L, Porcelli S, **Rasica L**, Marzorati M, Grassi B. Comparison between slow components of HR and $\dot{V}O_2$ kinetics: functional significance. *Med Sci Sports Exerc* 50(8):1649-1657, 2018 . doi: 10.1249/MSS.0000000000001612

Porcelli S, **Rasica L**, Marzorati M. Commentary on viewpoint: Principles, insights and potential pitfalls of the non-invasive determination of muscle oxidative capacity by near-infrared spectroscopy. *J Appl Physiol* 124(1):249-255, 2018. doi: 10.1152/japplphysiol.00857.2017

Porcelli S, Pugliese L, Reich E, Pavei G, Bonato M, Montorsi M, LaTorre A, **Rasica L**, Marzorati M. Effects of a Short-Term high-Nitrate Diet on Exercise Performance. *Nutrient* 8:E534, 2016. doi: 10.3390/nu8090534

Porcelli S, Bellistri G, Pugliese L, **Rasica L**, Marzorati M, Pavei G. Commentaries on Viewpoint: Can elite athletes benefit from dietary nitrate supplementation? *J Appl Physiol* 119:762-769, 2015. doi: 10.1152/japplphysiol.00640.2015

Porcelli S, Ramaglia M, Bellistri G, Pavei G, Pugliese L, Montorsi M, **Rasica L**, Marzorati M. Aerobic fitness affects the exercise performance responses to nitrate supplementation. *Med Sci Sports Exerc* 7(8):1643-51, 2015. doi: 10.1249/MSS.0000000000000577

CONGRESS COMMUNICATIONS

- **Rasica L**, Porcelli S, Salvadego D, Mrakic-Sposta S, Gelmini F, Beretta G, Marzorati M, ""BEet On Alps": ergogenic effects of dietary nitrate supplementation on cycling and arm-cranking during a prolonged exposure to high altitude"
  - Oral presentation 10th International Conference on the Biology, Chemistry and Therapeutic Applications of Nitric Oxide Oxford 19/09/2018
    *NO Society Travel grant awarded

- Porcelli S, **Rasica L**, Salvadego D, Giovanelli N, Floreani M, Adami A, Lazzer S, Marzorati M, "Linear relationship between muscle oxidative capacity determined by nirs and fitness level in young adults"
  - Poster presentation ACSM's 2018 Integrative Physiology of Exercise Conference San Diego 07/09/2018

- **Rasica L**, Porcelli S, Salvadego D, Mrakic-Sposta S, Gelmini F, Beretta G, Marzorati M, ""BEet On Alps": ergogenic effects of dietary nitrate supplementation on cycling and arm-cranking during a prolonged exposure to high altitude"

- Oral presentation 23th annual Congress of the European College Sport Science (ECSS) Dublin 03/07/2018

❖ Pedrali M, Zuccarelli L, Biasutti L, Azzini V, **Rasica L**, Marzorati M, Porcelli S, Sechi A, Grassi B, "Exercise tolerance in late-onsetPompe disease patients: positive effects of physical training and high-protein diet"
- Poster presentation 23th annual Congress of the European College Sport Science (ECSS) Dublin 05/07/2018

❖ Pilotto A, **Rasica L,** Scalise G, Pedrali M, La Torre A, Marzorati M, Porcelli S, "Assessment of lactate anaerobic threshold in flat- water kayakers"
- Oral presentation 23th annual Congress of the European College Sport Science (ECSS) Dublin 03/07/2018

❖ **Rasica L**, Porcelli S, Salvadego D, Mrakic-Sposta S, Gelmini F, Beretta G, Marzorati M, ""BEet On Alps": ergogenic effects of dietary nitrate supplementation at high altitude"
- Invited speaker 7th international congress Montain, Sport & Health (MSH) Roveretp 09/11/2017

❖ **Rasica L**, Porcelli S, Salvadego D, Mrakic-Sposta S, Gelmini F, Beretta G, Marzorati M, ""BEet On Alps": ergogenic effects of dietary nitrate supplementation at high altitude"
- Oral presentation IX Congresso Nazionale Società Italiana delle Scienze Motorie e Sportive (SISMES) Brescia 30/09/2017

❖ Zuccarelli L, **Rasica L**, Marzorati M, Porcelli S, Grassi B, "The slow component of HR kinetics occurs at a lower work rate and is more pronounced than the slow component of $\dot{V}O_2$kinetics"
- Oral presentation IX Congresso Nazionale Società Italiana delle Scienze Motorie e Sportive (SISMES) Brescia 30/09/2017

❖ Pilotto A, Scalise G, Aliprandi D, Baglioni A, Zuccarelli L, **Rasica L**, Marzorati M, Porcelli S, "Assessment of individual anaerobic threshold in flat- water kayakers"
- Oral presentation IX Congresso Nazionale Società Italiana delle Scienze Motorie e Sportive (SISMES) Brescia 30/09/2017

❖ Porcelli S, Pugliese L, Rastelli F, **Rasica L**, Mrakic-Sposta S, Moretti S, Vezzoli A, LaFortuna C, Marzorati M, "Effects of sodium nitrate supplementation on muscle blood flow and skeletal muscle oxidative metabolism during dynamic forearm exercise"
- Poster presentarion Italian Physiologica National Society Congress (SIF) Pavia 07/09/2017

❖ Zuccarelli L, Rasica L, Marzorati M, Porcelli S, "Non-invasive evaluation of cardiovascular and metabolic responses to arm and leg exercise in elite kayakers"
- Oral presentation 22th annual Congress of the European College Sport Science (ECSS) MetropolisRuhr 05/07/2017

❖ Porcelli S, **Rasica L**, Salvadego D, Mrakic-Sposta S, Gelmini F, Beretta G, Marzorati M, ""BEet On Alps": Ergogenic Effects Of Dietary Nitrate Supplementation During Prolonged Exposure To Hypobaric Hypoxia"

- Poster Presentation American College Sport Medicine Annual Meeting (ACSM) Denver 31/06/2017

❖ Salvadego D, Marzorati M, **Rasica L**, Porcelli S, ""BEet On Alps": Effect of Dietary Nitrate Supplementation on Skeletal Muscle Oxidative Capacity during Prolonged Exposure to
- Hypobaric Hypoxia"
- Thematic Poster Presentation American College Sport Medicine Annual Meeting (ACSM) Denver 02/06/2017

❖ **Rasica L**, Porcelli S, Ferguson BS, Kavazis AN, McDonald JR, Mowry F, Sun Y, Hogan MC, Grassi B, Gladden B, "Nitrate infusion does not alter isolated in situ canine muscle oxidative metabolism during hypoxia with normal convective O_2 delivery"
- Oral presentation VIII Congresso Nazionale Società Italiana delle Scienze Motorie e Sportive (SISMES) Roma 7/10/2016

❖ Zuccarelli L, **Rasica L**, Marzorati M, Porcelli S, Grassi B, "Importance of slow components of HR and V'O_2 kinetics during submaximal cycling on exercise intensity prescription"
- Mini-oral presentation 21th annual Congress of the European College Sport Science (ECSS) Vienna 07/07/2016

❖ **Rasica L**, Salvadego D, Porcelli S, Marzorati M, Montorsi M, Sartorio A, Grassi B, "Dietary nitrate supplementation and exercise tolerance in obese adolescents"
- Mini-oral presentation 21th annual Congress of the European College Sport Science (ECSS) Vienna 06/07/2016

❖ Porcelli S, Pugliese L, Rastelli F, **Rasica L**, Mrakic-Sposta S, Moretti S, Vezzoli A, Lafortuna CL, Marzorati M," Sodium nitrate effects on muscle blood flow and oxidative metabolism during forearm exercise"
- Poster presentation American College Sport Medicine Annual Meeting (ACSM) Boston 31/05/2016

❖ **Rasica L**, Porcelli S, Ferguson BS, McDonald JR, Mowry F, Sun Y, Hogan MC, Grassi B, Gladden B, " Effects of nitrite infusion in hypoxia on oxygen consumption and mitochondrial respiration in canine skeletal muscle"
- Oral presentation Annual Meeting of Young Researchers in Physiology (SIF) Magnano Riviera 5/05/2016

❖ Ferguson BS, McDonalnd JR, Mowry F, Sun Y, **Rasica L**, Porcelli S, Hogan MC, Grassi B, Gladden B, "Effects of hypoxia with matched convective O_2 delivery on fatigue and V'O_2 on-kinetics in canine skeletal muscle *in situ*"
- Poster presentation Experimental Biology 2016 (EB) San Diego 2/04/2016

❖ **Rasica L**, Salvadego D, Porcelli S, Marzorati M, Montorsi M, Sartorio A, Grassi B, "Effects of dietary nitrate supplementation on exercise tolerance in obese adolescents"
- Poster presentation VII Congresso Nazionale Società Italiana delle Scienze Motorie e Sportive (SISMES) Padova 4/10/2015
  * SISMES best poster award winner

CONTENTS

1. ABSTRACT *1*

2. INTRODUCTION

2.1. Nitric oxide: molecule and synthesis pathways 4

2.2. Nitric oxide bioavailability and exercise performance in healthy individuals 8

2.2.1. Mechanisms of action 8

2.2.1.1. *S*keletal muscle blood flow autoregulation 8

2.2.1.2. *F*orce production 9

2.2.1.3. *G*lucose homeostasis 10

2.2.1.4. *M*itochondrial respiration and biogenesis 11

2.2.2. Effects of increased NO bioavailability on exercise performance in normoxia 13

2.2.3. Effects of increased NO bioavailability on exercise performance in hypoxia 18

2.3. Nitric oxide bioavailability and exercise tolerance in disease populations 23

2.3.1. Chronic Heart Failure 24

2.3.2. Peripheral Arterial Disease 25

2.3.3. Diabetes Mellitus 26

2.3.4. Chronic Obstructive Pulmonary Disease 27

3. AIMS 28

4. MANUSCRIPTS

4.1. Increased nitric oxide bioavailability does not alter isolated canine muscle oxidative metabolism in hypoxia 29

4.2. “BEet On Alps”: ergogenic effects of dietary nitrate supplementation on cycling and arm-cranking during a prolonged exposure to high altitude 48

4.3. Ergogenic effects of beetroot juice supplementation during severe-intensity exercise in obese adolescents 68

5. FINAL CONSIDERATIONS *84*

6. REFERENCES *85*

1. ABSTRACT

Nitric oxide (NO) is a signaling molecule that influences different aspects of the cellular homeostasis and regulates several physiological processes. It is normally produced endogenously starting from aminoacid L-arginine but recent evidences suggest that it can be also increased through the ingestion of food rich in inorganic nitrate (mainly green leafy vegetables and beetroot). Indeed, ingested inorganic nitrate (NO_3^-), after been absorbed, can be converted in the oral cavity to nitrite (NO_2^-) and be finally reduced into NO in the blood. This alternative NO_3^- - NO_2^- - NO pathway seems facilitated in condition of low O_2 availability (ischemia and hypoxia) and low pH.

In the last 10 years, several studies have been conducted to investigate the effects of dietary NO_3^- supplementation on skeletal muscle function, since NO regulates microvascular blood flow, muscle contractile proprieties, glucose homeostasis, intracellular calcium handling, and mitochondrial respiration. Dietary NO_3^- supplementation is likely to elicit a positive outcome when testing endurance exercise capacity in healthy subjects and enhance exercise tolerance in patients. However, there are still unresolved questions about the mechanisms utilized by inorganic NO_3^- to affect skeletal muscle functions, the possible potentiating effect of hypoxic conditions and the effectiveness of this intervention in other disease populations. My PhD projects tried to address some of these questions.

The first study aimed to examine the effects of increased NO bioavailability on contraction economy. One of the most interesting effects of dietary NO_3^- supplementation is the reduction of muscle O_2 consumption ($\dot{V}O_2$) at a given exercise intensity, since the $\dot{V}O_2$ requested to carry out a specific exercise is generally a fixed amount regardless of sex, age and training status of the subject involved in the exercise. A lower $\dot{V}O_2$ during moderate intensity exercise has been demonstrated both in healthy subjects and disease patients after dietary NO_3^- supplementation. The mechanistic basis is not clear yet, and this effect has been related to reduced ATP cost of contraction and/or an enhanced mitochondrial coupling efficiency. In our study, we evaluated skeletal muscle contraction economy following NO_2^- infusion in hypoxic condition. Contractions of the in-vivo isolated canine muscle were obtained by direct nerve stimulation. Muscle blood flow was kept constantly high by pump-perfusion. O_2 consumption during exercise was assessed directly by Fick method. Mitochondrial respiration rates were evaluated by high-resolution respirometry from muscle biopsies. In hypoxic conditions, but in the presence of constant and normal convective O_2 delivery, NO_2^- infusion did not affect canine skeletal muscle oxidative metabolism. These evidences suggest that the effects of increased NO availability on

muscle contraction efficiency in hypoxia, if present, are likely not attributable to changes in mitochondrial respiratory efficiency.

The second study aimed to investigate the effects of increased NO bioavailability on the physiological responses to exercise after prolonged permanence at altitude. Data from Tibetan population living at altitude from generations suggest that changes in NO bioavailability may contribute to hypoxia acclimatization and their enhanced exercise efficiency. Moreover, recent studies have demonstrated that NO_3^- supplementation can limit exercise impairment following exposure to acute hypoxia. However, it is not known if Caucasian subjects exposed to several days of hypobaric hypoxia can benefits from increased NO bioavailability. Thus, we investigated the ergogenic effects of dietary NO_3^- supplementation on exercise performance at different intensities during a prolonged permanence at altitude. Cycling and arm-cranking exercises were performed in order to test possible different effects of NO_3^- supplementation in relation to a different muscle fibers recruitment pattern. Dietary NO_3^- supplementation reduced O_2 cost during moderate-intensity exercise both in cycling and arm-cranking. In cycle-ergometer exercise this effect was dependent from aerobic fitness level of the subjects, in accordance to previous results obtained in normoxia. Moreover, dietary NO_3^- supplementation enhanced severe-intensity exercise tolerance, suggesting that dietary NO_3^- supplementation can be a valid ergogenic aid to counteract exercise intolerance at altitude.

Finally, in the third study we evaluated the possible ergogenic effects of dietary NO_3^- supplementation in obese subjects. Literature shows that NO_3^- can exert significant positive effects on exercise tolerance and, as a consequence, quality of life of patients with an impaired skeletal muscle oxidative metabolism such as chronic heart failure, chronic obstructive pulmonary diseases and peripheral arterial disease patients. Obese patients are characterized by a higher O_2 cost of exercise, and therefore a reduced exercise tolerance during constant work-rate exercise compared with healthy subjects. We evaluated the effects of beetroot juice (BR, rich in NO_3^-) supplementation on the main physiological variables associated with exercise tolerance in obese adolescents. We observed a significant increase in plasma NO_3^- concentration after BR supplementation. The O_2 cost of moderate-intensity exercise was not different in BR condition versus placebo, whereas, during severe-intensity exercise, signs of a reduced amplitude of the O_2 uptake slow component were observed in BR, in association with a significantly longer time to exhaustion. Thus, exercise intolerance of obese adolescents, at least at severe-intensity, can be attenuated by short-term dietary NO_3^- supplementation. This intervention can be a useful aid to counteract early fatigue and reduced physical activity in this at-risk population.

Overall, the studies carried out during my PhD extend the current knowledge about dietary NO_3^- supplementation on physiological responses to exercise, starting from a mechanistic investigation in isolated canine muscle up to the evaluation of the ergogenic benefits of this intervention on exercise tolerance in obese adolescents.

2. INTRODUCTION

2.1 Nitric oxide: molecule and synthesis pathways

Nitric oxide (NO) was the first gas signaling molecule discovered in mammals (Culotta & Koshland 1992) and in the early 90s it was defined as "*molecule of the year"* by the journal *Science* for its importance in neuroscience, immunology and physiology. Few years later (1998) Robert F. Furchgott, Louis J. Ignarro and Ferid Murad won the Nobel Prize in Physiology or Medicine "*for their discoveries concerning nitric oxide as a signaling molecule in the cardiovascular system"*. Thus, NO is a key molecule for human physiology. Thanks to the numerous studies conducted on this topic, today we know that NO controls several fundamental biological function by interfering with neurological, cardiovascular, musculoskeletal and immunological systems. From a chemical point of view, NO (free radical nitrogen monoxide, NO•) is a gaseous, inorganic, uncharged diatomic molecule that is a poor oxidant and reducing agent under physiological conditions. The only known rapid reactions of NO with biological compounds is spin pairing with other species containing unpaired electrons, such as other radicals and transition metal ions. Therefore, despite being a free radical, NO reacts with only a few targets in cells, and this was very important for the evolution of NO into a biological mediator (Toledo & Augusto 2012). Its mechanisms of action are based on redox-related signaling events, through oxidative modifications, as well as nitrosactive compound production that underlie damage to proteins (Stamler & Meissner 2001). NO also interacts with proteins containing heme group leading to NO dioxigenation or nitrosilatyon in oxyhemoglobin/hemoglobin (HbO_2/Hb), binds to cytochrome-c oxidase (CcOx) impairing cellular respiration, and facilitates the release of cyclic guanosine monophosphate (cGMP) by interacting with soluble guanylate cyclase (sCG) (Toledo & Augusto 2012). NO is a very reactive molecule with a half-life shorter than 5 s, it cannot be stored in free form and is generally synthesized on demand with specific physiological effects (Tengan et al. 2012).

Since NO is an uncharged and almost nonpolar molecule it is reasonably soluble in water and about 10 times more soluble in hydrophobic solvents. Thanks to these characteristics, NO can be transported in the plasma and can cross the hydrophobic double layer of biological membranes by simple diffusion. This transport modality confers unique biological properties to NO among messengers. Indeed, it easily passes through cellular membranes without specialized transporters and it is not confined in cells or cell compartments but there is a net movement of NO from a more concentrated compartment to neighboring cells because simple diffusion is a random process driven by a spatial concentration gradient (Toledo & Augusto

2012). Moreover, as previous mentioned, NO also interacts with proteins containing heme group like hemoglobin that is the major protein in the blood of mammals. Both oxyhemoglobin and deoxyhemoglobin react very rapidly with NO but in different ways. The nitrosylation of the heme occurs in the case of deoxyhemoglobin and the further dissociation is so slow that the overall process can be considered irreversible. In the case of oxyhemoglobin, the reaction with NO is irreversible, and both NO and heme are oxidized at the expense of molecular oxygen. Because both atoms of O_2 are incorporated into nitrate (NO_3^-), this process is called NO dioxygenation. This reaction can collapse in either the ferric protein form and NO_3^- or dissociates to the protein ferryl form and nitrogen dioxide (NO_2•) formation. The overall yield of NO_2• is about 10%, and therefore, the reaction of oxyhemoglobin or oxymyoglobin with NO functions primarily as a NO sink. Because no functional cell in tissues is more than 100–200 μm away from a blood vessel, these reactions are a powerful and unstoppable sink for NO in vivo, which determine its dynamics and bioavailability (Toledo & Augusto 2012).

Endogenously NO is produced by three isoforms of NO synthases (NOS) enzymes: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). These isoforms were originally named from the tissues where they were first discovered (neuronal and endothelial) and the mode of activation in the case of iNOS. Today, we know that these enzymes are well distributed in human tissues and different isoforms can coexist in the same tissue (for example, all three types of NOS are expressed within skeletal muscle). eNOS and nNOS, classified as NOS I, are constitutive and regulated by Ca^{2+} and its interaction with calmodulin. On the contrary, iNOS, classified as NOS II, is calcium-independent and permanently bond to calmodulin (Toledo & Augusto 2012). The production of NO from NOS enzymes follow a pathway that is O_2 dependent, consisting in the oxidation of L-arginine thanks to four co-factors: tetrahydrobiopterin (BH4), flavin adenine dinucleotide (FAD), flavin monucleotide (FMN) and nicotinamide adenine dinucleotide phosphate (NADPH). This oxidative pathway was discovered in the 80's and leads to the production of NO and L-citrulline (Tengan et al. 2012) (**Fig. 1, upper part**). In plasma, NO is highly instable and it is almost immediately oxidized to nitrite (NO_2^-) or nitrate (NO_3^-) in case of reactions between NO and HbO_2. Almost 50 % of endogenous NO_3^- and NO_2^- in humans comes from this conversion and these metabolites can persist in the systemic circulation from 110 s to 5 – 8 h for NO_2^- and NO_3^-, respectively (Bendich et al. 2017).

However, NO can be reversely produced from NO_3^- and NO_2^- as demonstrated by two independent research groups in the 90's (Benjamin et al. 1994, Lundberg et al. 1994).

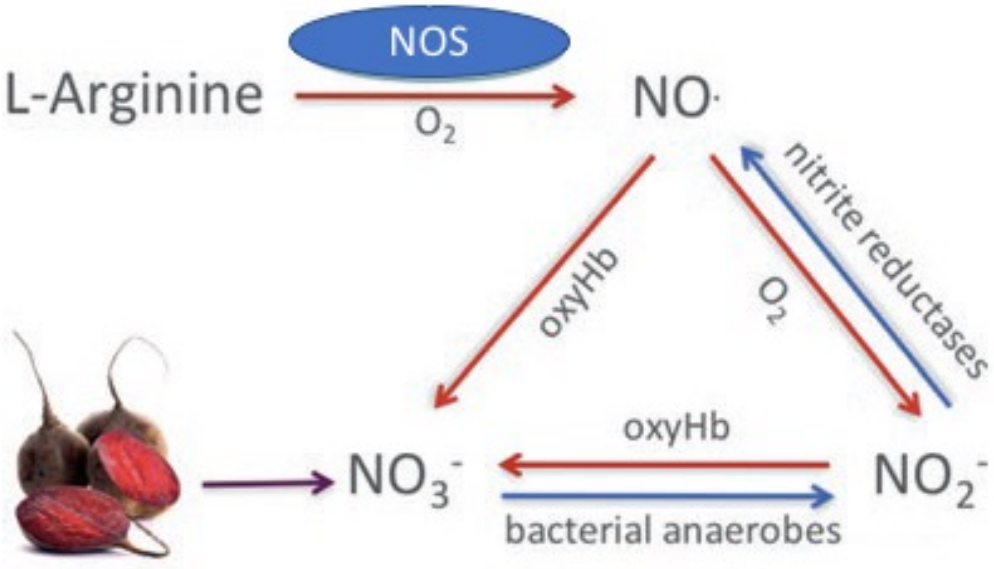

Figure 1 The L-Arginine and NO_3^- - NO_2^- - NO pathways. Nitrate and nitrite, oxidative metabolites of nitric oxide, can be sequentially reduced to produce bioactive NO. Augmentation of the nitrate-nitrite-NO could be provide by food rich in nitrate like beetroot.
NO nitric oxide, NO_3^- nitrate, NO_2^- nitrite, NOS nitric oxide synthase, O_2 oxygen, oxyHb oxyhemoglobin
(modified from Kapil "Nitrate and Nitrite in Human Health and Disease" 2017)

This alternative NO production pathway has been demonstrated to be valid at systemic level, it is NOS and O_2 independent and is based on the reduction of NO metabolites (NO_3^- in NO_2^-) in NO (NO_3^- - NO_2^- - NO) (**Fig. 1, lower part**). However, the plasma concentration of NO_3^- and NO_2^- can also be increased by ingestion of food rich in nitrate, like beetroot and green leafy vegetables, or sodium nitrate ($NaNO_3$). The ingestion of these compounds increases the concentration of NO_3^- in the upper gastrointestinal tract where they are absorbed. Approximately 25% of the absorbed NO_3^- enters in the entero-salivary circulation and stores inside the salivary gland. Then, only 5 % of the original NO_3^- ingested are reduced to NO_2^- in the oral cavity thanks to the commensal anaerobic bacteria present on the tongue surface. In the stomach, because of the acid environment, part of the swallowed NO_2^- is immediately protonated to nitrous acid (HNO_2), then decomposed in NO, nitrogen dioxides (NO_2), and dinitrogen trioxide (N_2O_3). These nitrogen oxides react with protein forming bioactive adducts, such as S-nitrosothiols and N-nitrosoamine. NO in the stomach contributes to increase the blood flow of the gastric mucosal and the mucous thickness able to ensure the normal gastric function and act as first-line defense counteracting swallowed pathogens. However, a few NO_2^- eludes the protonation in the stomach, are absorbed in the small intestine and enters the systemic circulation, reaching the peripheral organs, including skeletal muscles (**Fig. 2**) (Kobayashi et al. 2015).

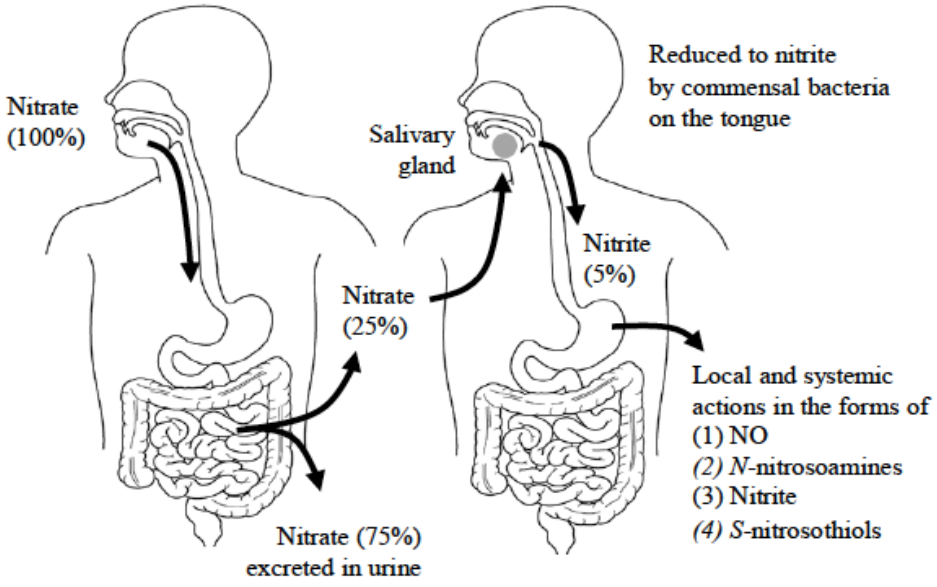

Figure 2 The entero-salivary NO_3^-- NO_2^- -NO pathway. 25 % of the ingested NO_3^- is stored in the salivary glands, and 20% of the NO_3^- in saliva is converted to NO_2^- by oral anaerobic bacteria. Approximately 5% of the originally ingested NO_3^- is swallowed into the stomach and provides NO in various forms. (1) NO for stomach vasodilation, mucus formation, and antimicrobial activity; (2) N-nitrosoamines for local carcinogenesis; (3) NO_3^- for NO_2^- pool in the peripheral tissues; (4) S-nitrosothiols for transnitrosylation in the peripheral tissues. (from Kobayashi et al. 2015)

Since the plasma levels of NO_2^- are highly dependent on the amount of salivary NO_3^- and its reduction to NO_2^-, the use of an antibacterial mouthwash decreases the reduction of NO_3^- in NO_2^- (Govoni et al. 2008, Woessner et al. 2016). The amount of dietary NO_3^- coming from vegetables could seem negligible compared with the endogenous NO_3^- production. On the contrary, one serving of NO_3^- rich vegetables contains more NO_3^- compared with those endogenously generated by NOS enzymes in humans during a full day (Lundberg et al. 2009). Vegetables account approximately 70% of the daily NO_3^- intake in a Western diet, with a significant increase of plasma NO_2^- concentration (Lundberg et al. 2008).

In the past, some concerns have been raised about the possible carcinogenic behavior of dietary intake of inorganic NO_3^-. Although NO_3^- and NO_2^- are not carcinogenic, the ingestion of high amount of NO_2^- contained in red and cured meats was demonstrated to cause carcinogenic N-nitrosamines formation due to possible reaction of these compounds with amines groups. Nevertheless, it has been demonstrated that the ingestion of vegetables and fruits containing NO_3^- but also vitamins C and E and polyphenols, combined with the acid environment present in the stomach, lead to S-nitrosothiol formation instead of carcinogenic N-nitrosamines, reducing the risk of nitrosamines formation. Moreover, compounds present in vegetables and fruits eliminate potent nitrosating agents, such as the N_2O_3 formed from NO_2^-. Consistent with this hypothesis, in the last few years several studies demonstrated no link between dietary NO_3^- and cancer (Gilchrist et al. 2010, Milkowski et al. 2010), and the ingestion of fruits and

vegetables often rich in NO_3^- (including beetroots) has been demonstrated to be inversely related to the risk of upper gastrointestinal tract, lung and colorectal cancer (Bradbury et al. 2014).

2.2 Nitric oxide bioavailability and exercise performance in healthy individuals

As previous described, NO can influence several physiological functions connected to skeletal muscle function and consequently affect exercise performance. Indeed, NO can improve skeletal muscle blood flow regulation, force production (excitation-contraction coupling), calcium handling, glucose homeostasis, and mitochondrial respiration and biogenesis (Jones 2014). Before describing the effects of increased NO bioavailability on exercise performance, a brief description of the main mechanisms of action utilized by NO to affect muscle function will be reported.

2.2.1 Mechanisms of action

2.2.1.1 Skeletal muscle blood flow regulation

Blood flow regulation is defined as the adjustment in blood supply to various organs in relation to the relative metabolic demand. It has been demonstrated that NO is involved in the regulation of muscle microvascular blood flow during contractions (active hyperemia) (Hickner et al. 1997), during hypoxia exposure (Ward et al. 1996), during vascular occlusion (reactive hyperemia) (Bjornberg et al. 1990), and in the modulation of the sympathetic reflex accompanying both resting and exercising conditions (Thomas et al. 1998, Thomas & Victor 1998). However, some studies suggest that the role of NO in enhancing blood flow during skeletal muscle contraction may modestly affect active hyperemia at higher exercise intensities (for reviews see Radegran & Saltin 1999, Tschakovsky & Joyner 2008). Indeed, in animal experiments the blockage of NOS activity had a small effect on the blood flow response during exercise at high contraction frequencies (60 contraction per minute) (Dua et al. 2009). Although more studies are needed to clearly address the role of NO in modulating microvascular blood flow during exercise, it can be stated that muscle hyperemia is probably affected by several redundant factors (Lamb et al. 2018) and NO can play a primary vasodilator role during moderate intensity exercise and at microvascular level whereas other vasodilators, like potassium or adenosine, may become relevant when NO production is blocked or exercise intensity increases (Lamb et al. 2018).

According to the role of NO on microvascular blood flow, some researchers have investigated possible effects of increased NO bioavailability on macro- and microvascular blood flow. After

dietary NO_3^- supplementation Ferguson and colleagues demonstrated an increased blood flow in active muscle of rats especially in muscles mostly composed by type 2 fibers (Ferguson et al. 2013, Ferguson et al. 2015). Conversely, different studies investigating the effects of dietary NO_3^- supplementation on blood flow muscle regulation during hand grip exercise did not observed positive effects in brachial artery blood flow after supplementation (Kim et al. 2015, Casey et al. 2015, Craig et al. 2018). Thus, it seems that increased NO bioavailability has different effects on muscle blood flow in relation to exercise intensity (moderate intensity vs severe intensity exercise) and the level at which the blood flow is evaluated (macro- vs microvascular bed).

2.2.1.2 Force production

Production of muscle force is intrinsic related to the excitation-contraction coupling and it is determined at least in part by the interaction of chemical and/or electrical processes at the cell surface producing Ca^{2+} release from the sarcoplasmic reticulum (SR). Indeed, Ca^{2+} is able to induce actin-myosin interaction and thereby contraction in muscle fibers.

NO has been demonstrated to modulate excitation-contraction coupling and Ca^{2+} bioavailability at different levels.

At first, NO modulate muscle contraction by interacting with the second messenger cGMP. Increased cGMP levels result in activation of protein kinase G (PKG) and hence in skeletal muscle myosin light chain kinase (skMLCK) pathway, leading to an increase in myosin regulatory light chain phosphorylation activity (pRLC). This would enhance Ca^{2+} sensitivity of the contractile apparatus (Coggan & Peterson 2018). cGMP activity in skeletal muscle is relatively low compared with other tissues and its effects on mechanical parameters of muscle contraction are generally modest except for shortening velocity. In animal model, NO donors increased maximum velocity of shortening of muscle in vitro between 8 % and 15 %, depending from the drug utilized (Maréchal & Beckers-Bleukx 1998).

However, In the last couple of decades, it has become increasingly recognized that NO can also influence cellular events more directly, either by itself or after nitrosylation or transnitrosylation of thiols, especially cysteine residues of proteins (Gould et al. 2013).

In skeletal muscle most of non-cGMP-mediated effects of NO are actually inhibitory, they tend to diminish muscle contractile function. Indeed, studies on single-muscle fibers showed negative effects of NO donors on force production during maximal isometric contractions (Kobzik et al. 1994, Reid 1998) as well as during submaximal tetanic contractions (Brotto &

Nosek 1996, Andrade et al. 2001), whereas NOS inhibitors increased the force output in isolated muscle fibers (Coggan & Peterson 2018).

In contrast to the inhibitory effects described above, transnitrosylation of ryanodine receptors (RyRs) may be one mechanism by which NO could enhance muscle contractile function. RyRs are Ca^{2+} channels directly connected to dihydropyridine receptors (DHPRs) and they control the levels of this ion in skeletal muscle by its release from the SR.

NO has been demonstrated to increase free [Ca^{2+}] in the cytosol and consequently to improve muscle force production at low frequency of stimulation. Finally, it has more recently been demonstrated that NO bioavailability can increase expression of calsequestrine (CASQ), in particular CASQ1, maintaining free [Ca^{2+}] in the SR lumen at 1mM during contraction (Hernandez et al. 2012). According to these evidences, Hernandez and colleagues (2012) showed that 7 days of NO_3^- supplementation increased contractile force at low stimulation frequencies in fast-twitch, but not in slow-twitch muscles. This result was accompanied by increased SR Ca^{2+} content and tetanic $[Ca^{2+}]_i$ increased, and expression of CASQ1 and DHPR. Hoon and colleagues (2015) demonstrated that dietary supplementation with NO_3^- -rich beetroot juice did not affect maximal force, submaximal contractile force output or the force–frequency relationship of the quadriceps muscles. Similarly, Fulford and colleagues demonstrated that dietary NO_3^- supplementation did not influence skeletal muscle force production during repeated isometric MVC (Fulford et al. 2013). Moreover, the effects of dietary NO_3^- supplementation seems to be different on evoked force production. Seven days of beetroot juice consumption increased peak force at low levels of electrically evoked contraction (1–20 Hz) and the rate of force development at 300 Hz (Haider & Folland 2014) as confirmed by Whitfield and colleagues (2017) and, in contrast to results in rodent muscle, these improvements were not explained by increased expression of proteins associated with intracellular calcium handling.

In conclusion the effects of increased NO bioavailability on muscle force generation are various and the precise mechanisms responsible for NO_3^--induced increase in human muscle contractility are still unknown, but changes in Ca^{2+} are likely to play a role (Coggan & Peterson 2018).

2.2.1.3 Glucose homeostasis

The concentration of blood glucose level is maintained constant by the action of two general opposing factors: the rate of glucose entrance to the blood and the rate of removal of blood glucose. Insulin and contractile activities regulate glucose utilization at skeletal muscle level

and they are influenced by NO (Stamler & Meissner 2001). Indeed, it has been demonstrated that NOS activity and, therefore, NO bioavailability increases glucose transport inside the skeletal muscle cells. This effect seems related to higher capillary recruitment and consequently increased blood flow in skeletal muscle after NO production by eNOS (Lundberg et al. 2009), whereas insulin-mediated GLUT-4 transport of glucose in isolated skeletal muscle is NO independent. Similarly, NO is able to improve insulin secretion by modulating blood flow in the gastrointestinal system and nutrients distribution in the peripheral tissues (Kobayashi 2015). Although this strong relationship between NO and glucose homeostasis, few studies have been conducted on this topic. In animal models, the seminal work by Carlström et al. (2012) demonstrated that eNOS-deficient mice with several of key features of diabetes benefitted from chronic NO_3^- supplementation. Restoring NO bioavailability resulted in improvements in glucose tolerance, glycosylated hemoglobin, fasting glucose, and circulating triglycerides. These findings have subsequently been reproduced and further investigated by several others (Khoo et al. 2014, Khalifi et al. 2015). However, to date, studies on humans have reported contrasting results and the efficacy of the utilization on increased NO bioavailability to manage glucose homeostasis and effectiveness in patients with diabetes (T2DM) has still to be determined.

2.2.1.4 Mitochondrial respiration and biogenesis

NO affects mitochondrial function at different levels. First of all by regulating blood flow to skeletal muscle fibers because of its vasodilatory effect, by facilitating the supply of respiratory substrates and O_2 to mitochondria (Stamler & Meissner 2001, Wolzt et al. 1999, Nisoli & Carruba 2006). NO also regulates hemoglobin binding and release of O_2 in red blood cells, thereby regulating O_2 delivery to tissues and O_2 supply to the mitochondria (Nisoli & Carruba 2006). Boveris and colleagues (2000) proposed a new concept of regulation of cellular respiration that depends on energy demands, which is controlled not only by the availability of ADP to F1-ATPase as classically accepted but also by O_2 and NO availability to cytochrome c oxidase.

NO can directly alter respiratory chain function through inhibition of cytochrome c oxidase activity, inhibition of electron transfer between cytochrome b and c, and inhibition of electron transfer and NADH-dehydrogenase function in Complex I (Boveris et al. 2000). In addition, NO directly interacts with complex IV in competition with O_2 to partially inhibit respiration. Another NO sensitive site in the respiratory chain is the electron transfer at complex III, ubiquinol-cytochrome c reductase, because NO can inhibit its activity (Boveris et al. 2000).

Following these evidences, recently some studies have investigated possible effects of increased NO bioavailability on mitochondrial function in human skeletal muscle. Respirometry of skeletal muscle biopsies from healthy subjects after $NaNO_3$ supplementation showed a significant increase in basal mitochondrial efficiency by an approximately 19 % increase of the amount of ATP produced per O_2 consumed (P/O ratio) (Larsen et al. 2011). At molecular level, these results were related to a downregulation of the expression of the adenine nucleotide transferase (ANT), which catalyzes the export and import of ATP and ADP from the mitochondrion respectively, and a decrease of the expression of uncoupling protein 3 (UCP3), both facilitating proton leak from the intermembrane space into the mitochondrial matrix (Larsen et al. 2011) (**Fig. 3**).

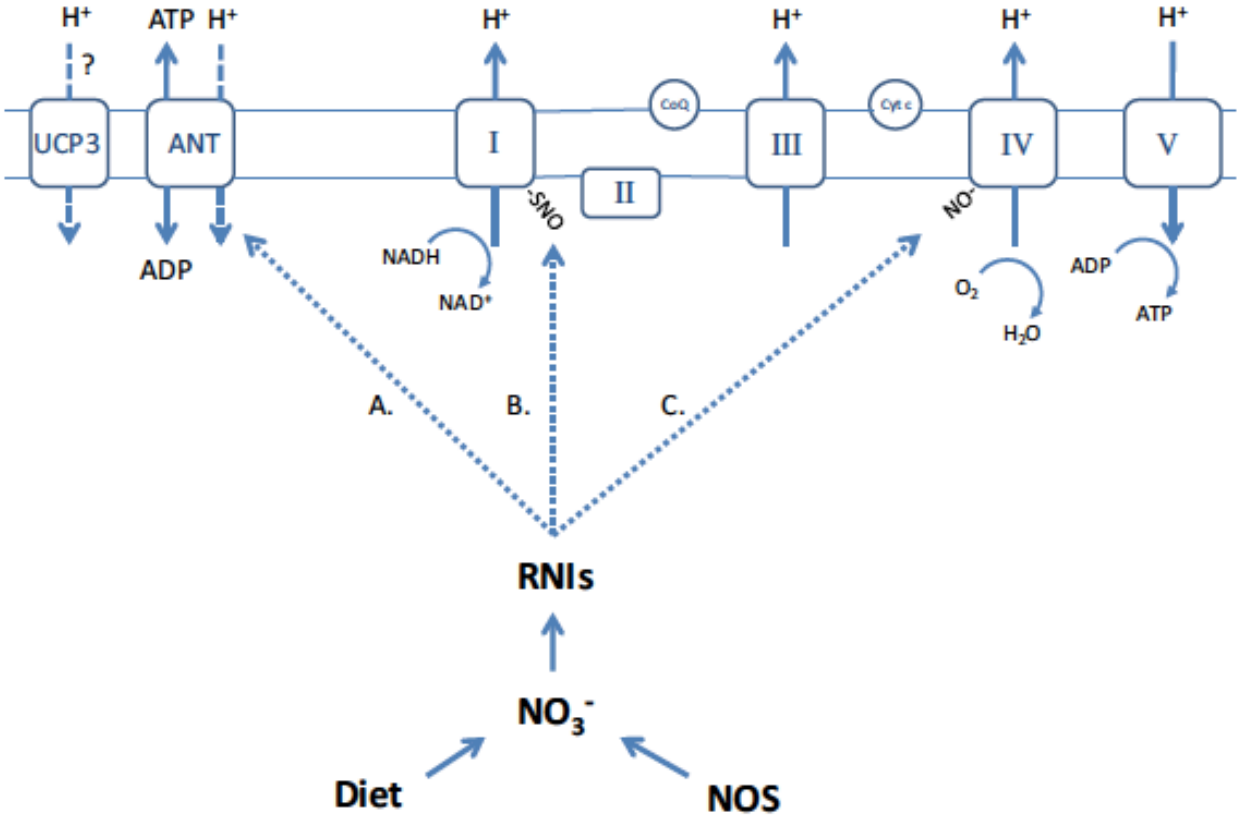

Figure 3 NO_3^- from NOS activity or dietary sources is metabolized in vivo to NO_2^-, NO, and other reactive nitrogen intermediates (RNIs). These RNIs can interact with mitochondria at different levels downregulates the expression of ANT (A). Other potential mitochondrial targets of RNIs include complex I (B) and complex IV (C). Previous studies have shown that nitrite can nitrosate (-SNO) complex I, which is associated with reduced ROS generation after ischemia reperfusion. In addition, NO directly interacts with complex IV in competition with O_2 to partly inhibit respiration. (from Larsen et al. 2011)

Downregulation of these proteins significantly decreased the protons leak and increased the number of protons pumped by the electron transport chain utilized for ATP production. As a consequence, the amount O_2 needed to produce a given amount of ATP decreased and the

mitochondrion was more efficient. The underlain mechanism by which nitrite modulates ANT and UCP3 protein expression need to be investigate.

Nevertheless, it should be noted that these effects of increased NO bioavailability on mitochondria have been recently challenged by Whitfield and colleagues (2016) who found that concentrated beetroot juice supplementation did not affect coupling P/O ratios and proton motive force. Moreover, UCP3 and ANT proteins were not reduced, and the apparent mitochondrial ADP respiratory sensitivity remained unaltered, suggesting a possible different effect of dietary NO_3^- vs $NaNO_3$ on mitochondrial function. Further future investigations should address this open question about the effects of increased NO bioavailability on mitochondrial efficiency.

NO plays also an important role in mitochondrial biogenesis of skeletal muscle (Nisoli and Carruba 2006). Several studies have shown that treatment of cells with NO donor increases mitochondrial markers and induces biosynthesis of functional mitochondria able to generate ATP via oxidative phosphorylation (Nisoli et al. 2004). The exact pathways involved in mitochondrial formation in response to NO signaling are not entirely understood, but in many tissues and cells, they have been associated to peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1α) (Nisoli et al. 2004). Unfortunately, only one study evaluated the effects of increased NO bioavailability on mitochondrial biogenesis: cultured myocytes were treated by concentrated beetroot juice and an increase in mitochondrial biogenesis in a PGC-1α-specific manner was observed (Vaughan et al. 2016).

2.2.2 Effects of increased NO bioavailability on exercise performance in normoxia

In the last years, several studies have been conducted to investigate the effects of increased NO bioavailability on exercise performance, according to the wide spectrum of actions of NO on skeletal muscle and related functions. The first seminal study in this field was conducted by Larsen and colleagues in 2007. After 3 days of supplementation with 0.1 mmol $NaNO_3 \cdot KgBM^{-1} \cdot day^{-1}$ or 0.1 mmol $NaCl \cdot KgBM^{-1} \cdot day^{-1}$, nine moderate active males ($\dot{V}O_2peak$ 55 $mL \cdot kg^{-1} \cdot min^{-1}$) performed five sub-maximal cycling bouts at work rates corresponding to 45, 60, 70, 80 and 85 % of $\dot{V}O_2peak$. Compared to NaCl, $NaNO_3$ increased plasma [NO_3^-] and [NO_2^-], significantly decreased $\dot{V}O_2$ during the first four sub-maximal stages (-5 %) but did not affect $\dot{V}O_2$ at 85 % or 100 % of $\dot{V}O_2peak$ (Larsen et al. 2007). Since one of the fundamentals of exercise physiology is the fixed amount of $\dot{V}O_2$ requested to carry out a specific workload regardless of sex, age and training status of the subject, this pioneering work opened a new line

of research aimed to investigate the effects of dietary NO_3^- supplementation on exercise economy and exercise tolerance. Few years later, Bailey and colleagues confirmed previous results demonstrating a reduction of $\dot{V}O_2$ at moderate intensity in recreationally active males after 6 days of beetroot juice supplementation (NO_3^- 5.6 mmol · day^{-1}). Moreover, they observed a reduction of $\dot{V}O_2$ slow component and an improvement in time to exhaustion (16 %) during cycling at severe intensity (70 % of the difference between the power output at the gas exchange threshold (GET) and $\dot{V}O_2peak$), highlighting the ability of increased NO bioavailability in improving exercise tolerance (Bailey et al. 2009).

Nowadays, it is largely demonstrated that in moderately trained subjects dietary NO_3^- supplementation reduces steady-state $\dot{V}O_2$ ($\sim$ 3-5 %) during moderate intensity exercise, reduces significantly ($\sim$ 20%) the amplitude of the $\dot{V}O_2$ slow component during high-intensity exercise, and increases time to exhaustion ($\sim$12-16%) (for a review see Pawlak-Chaouch et al. 2016, McMahon et al. 2017). The $\dot{V}O_2$ reduction following NO_3^- supplementation has consistently been demonstrated during different exercise modality: cycling (Lansley et al. 2011, Cermak et al. 2012, Muggeridge et al. 2014), running (Porcelli et al. 2015), kayaking (Muggeridge et al. 2013), and rowing (Hoon et al. 2014). It is important to note that ergogenic effects of increased NO bioavailabilty are influenced by several factors. First of all, exercise economy and exercise tolerance improvements after NO_3^- ingestion depend on plasma [NO_3^-] and [NO_2^-]. Wylie et al. (2013) demonstrated positive results on $\dot{V}O_2$ reduction 2.5 h only after the acute ingestion of 16.8 mmol of NO_3^- and no results after assumption of lower doses (8.4 and 4.2 mmol) of NO_3^-. Additionally, they reported an increase in time to exhaustion after ingestion of 8.4 mmol and 16.8 mmol of NO_3^- with no positive effects after the lowest dose. Secondarily, ergogenic effects of increased NO bioavailability are influenced by the duration of the supplementation. It has been demonstrated that the dose of NO_3^- exerting ergogenic effects can be reduced to about 6 mmol of NO_3^- per day if the supplementation period last more than 6 days (James et al. 2015, Wylie et al. 2016) (**Fig. 4**). Also, the conversion from NO_3^- to NO_2^- by oral commensal bacteria and the subsequent increase in plasma NO_2^- correlates with improvements in both exercise tolerance and performance. The importance of NO_3^- to NO_2^- conversion was shown in different studies that have eradicated or inhibited these bacteria by using antiseptic and antibacterial mouthwash treatments. The results showed a reduced salivary and plasma NO_2^- increase and lead to an increase in systemic blood pressure (Govoni et al. 2008, Woessner et al. 2016).

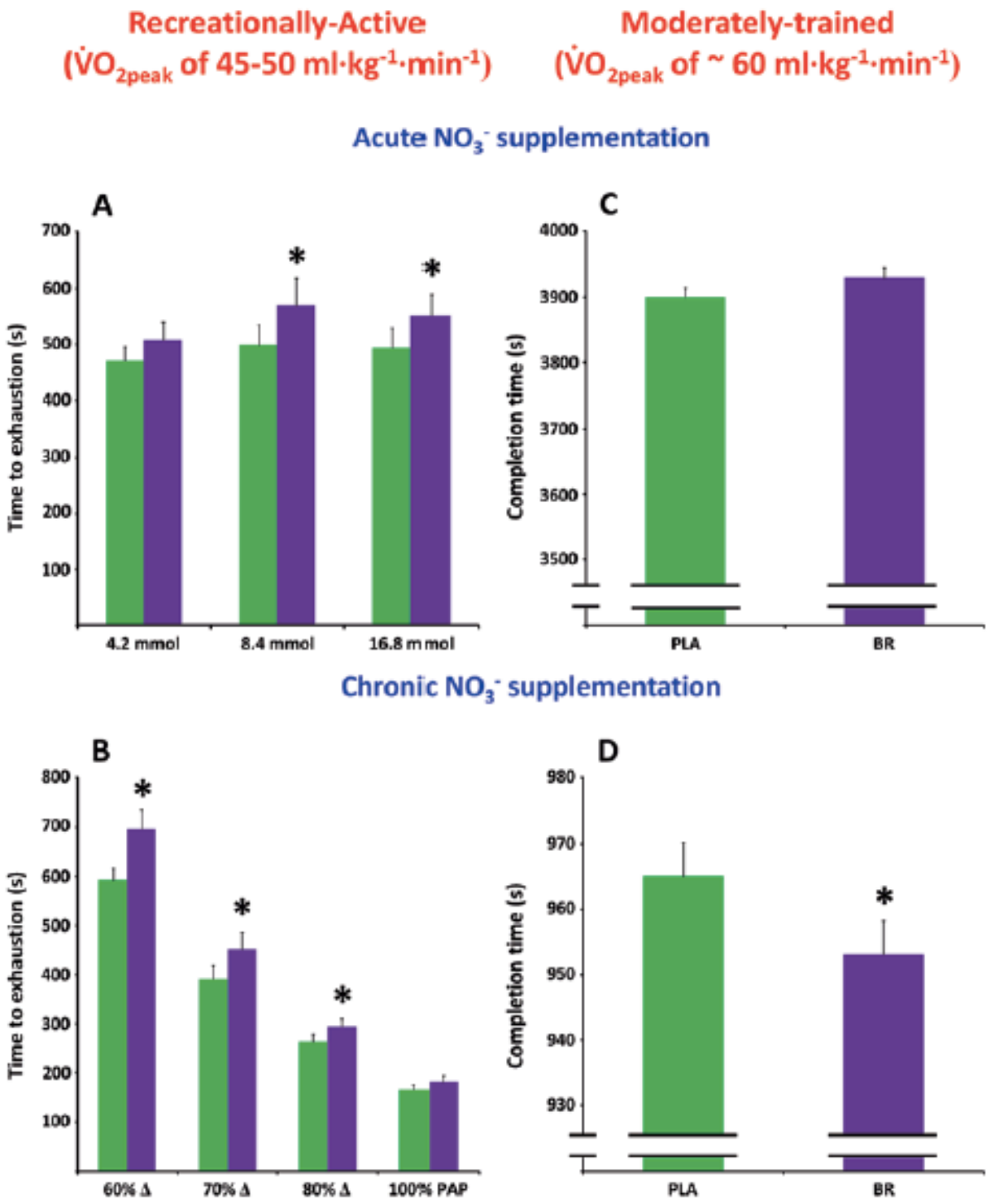

*Figure 4 Influence of acute and chronic NO_3^- rich beetroot juice supplementation (BR, purple bars), compared to placebo (PLA, green bars) in recreationally active and moderately trained subjects, after acute supplementation A and C and chronic supplementation B and D on severe-intensity exercise tolerance. A) Illustrate the effects with different concentration of NO_3^-. B) Illustrated the effects on exercise tolerance across a series of severe-intensity work rates. C and D illustrated the effects on time trial performance. * different from PLA (from Bailey "Nitrate and Nitrite in Human Health and Disease")*

Finally, the benefits deriving from increased NO bioavailability seems to be affected by the training status of the supplemented subjects. Indeed, some studies showed no improvements of exercise economy or performance after dietary NO_3^- supplementation in aerobically trained

subjects (Bescos et al. 2012, Boorsma et al. 2014, Porcelli et al. 2015) (**Fig. 5**).

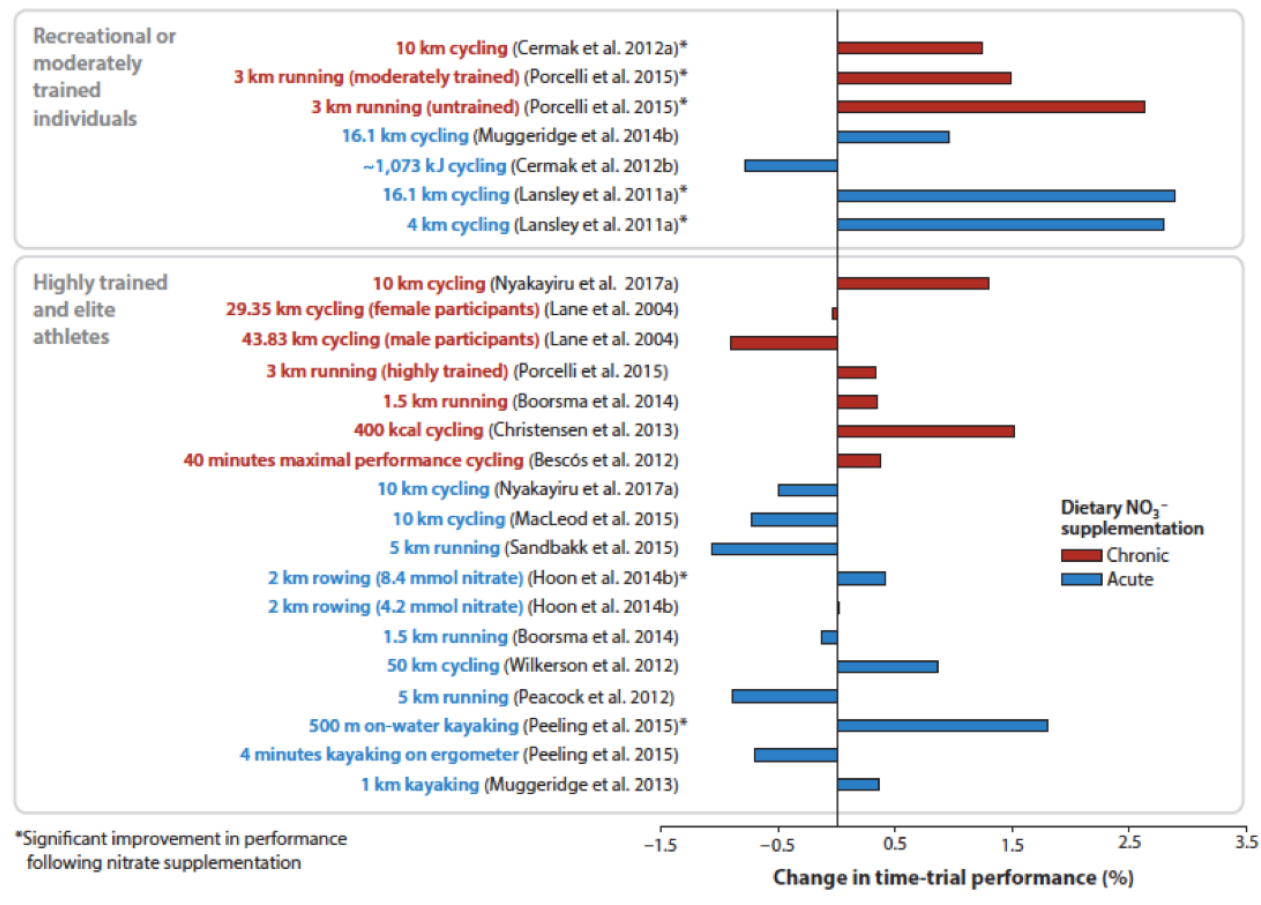

*Figure 5 Change in time-trial performance express as percentage following acute (blue bars) or chronic (red bars) NO_3^- supplementation in recreational and moderately trained individuals (top panel) and in highly trained and elite athletes (bottom panel). * significant improvement in performance following NO_3^- supplementation. (modified from Jones et al. 2018)*

The reasons for the lack of ergogenic effects from NO_3^- supplementation in these subjects are not completely understood but it has been hypothesized that aerobic training may lessen NO benefits through higher NOS enzymes expression (Green et al. 2004, McConnel et al. 2007), greater skeletal muscle capillarization (Jensen et al. 2004), increased content of skeletal muscle Ca^{2+}-handling proteins (Kinnunen & Mänttäri 2012), and a lower proportion of type 2 muscle fibers (Tesch & Karlsson 1985). Interestingly, there are evidences that NO_3^- supplementation may improve performance in highly trained subjects and elite endurance athletes when exercises involve upper body muscles. For instance, ergogenic effects have been observed in highly trained kayakers (Peeling et al. 2015) and in highly trained rowers (Hoon et al. 2014) (**Fig.6**).

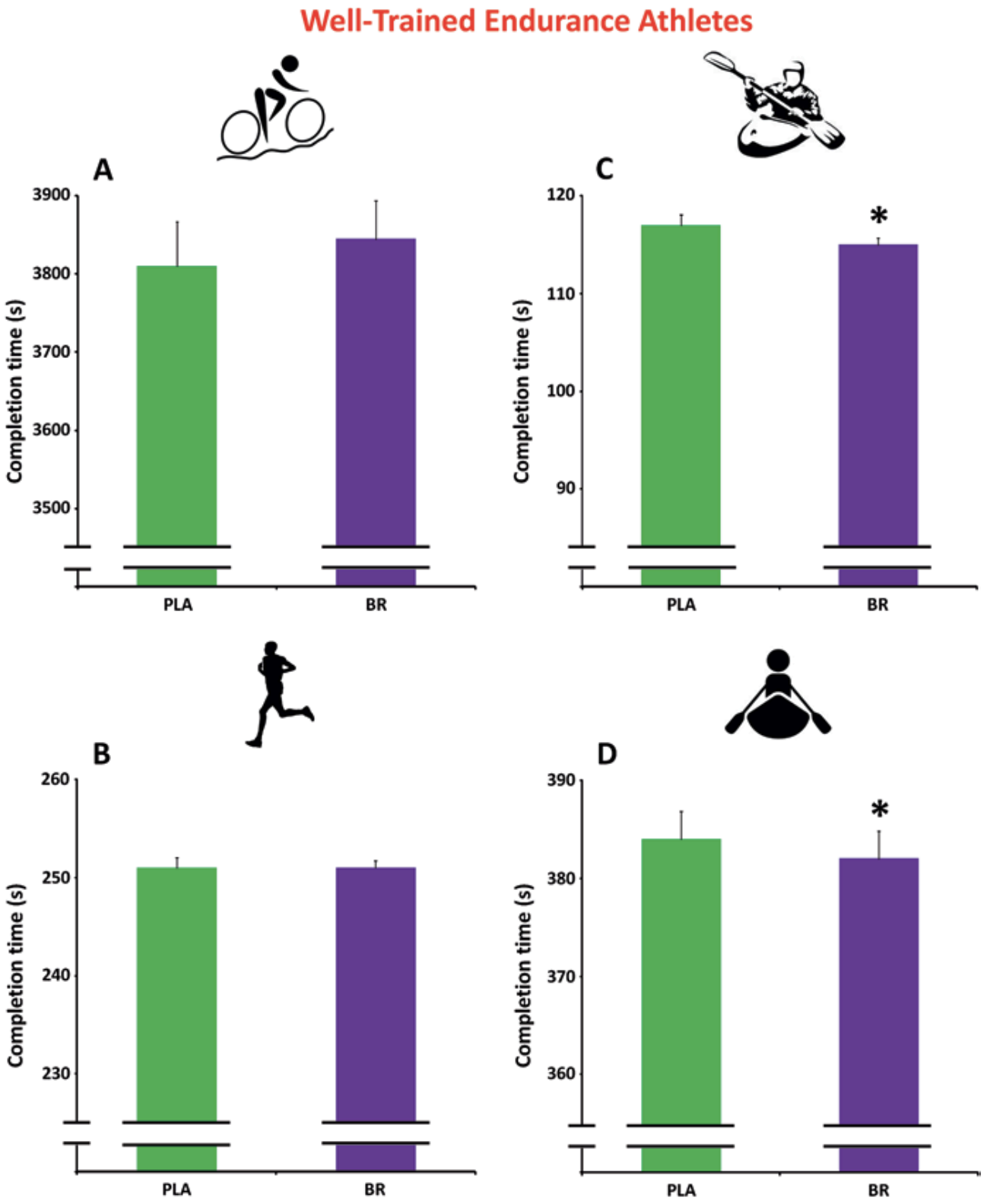

*Figure 6 Influence of exercise modality on the effectiveness of NO_3^- supplementation to improve endurance exercise performance in endurance-trained athlete on different exercise modalities. A) cycling B) running C) kayaking D) rowing * different from PLA (from Bailey cap 20 "Nitrate and Nitrite in Human Health and Disease" 2017)*

The reason for this discrepancy of results is not clear but one possible explanation of the positive effects of NO_3^- supplementation in highly trained individuals only when the exercise involves upper body muscle could be due to the greater proportion of type 2 fibers in these

muscles compared with lower limbs (Polgar et al. 1973). Indeed, it is known that NO_3^- supplementation has type 2 fiber specific effects, the relatively low O_2 tension present in type 2 fibers may promote the reduction of NO_2^- to NO, and lead to a better local perfusion, fatigue resistance, and fiber contractility (Jones et al. 2016).

2.2.3 Effects of increased NO bioavailability on exercise performance in hypoxia

As previous described, NO production coming from NOS enzymes requires the presence of O_2 and cofactors (i.e. BH4, FAD, FMN, NADPH), whereas the NO_3^- - NO_2^- - NO pathway is potentiated in situation of low O_2 availability and low pH, suggesting possible compensating contribution of these two pathways in order to maintain constant NO production (Umbrello et al. 2013). According to these characteristics, it has been hypothesized that potentiating the NO_3^- - NO_2^- - NO pathway through dietary NO_3^- supplementation in hypoxic condition such as normobaric hypoxia (simulated altitude) or high altitude may add further ergogenic power to the supplementation. This could be particularly useful considering the lower exercise tolerance in hypoxia due to the $\dot{V}O_2peak$ decrement and consequently the increase of relative percentage of $\dot{V}O_2peak$ to maintain a given sub-maximal work rate.

The first study evaluating the effects NO_3^- supplementation in hypoxia (14.5% O_2) was conducted by Vanhatalo and colleagues (2011). During high-intensity knee-extension exercise, they showed an increased time to exhaustion after beetroot juice supplementation (9.3 mmol of NO_3^-) compared with placebo and a faster PCr recovery kinetics after exercise (Vanhatalo et al. 2011). Interestingly, NO_3^- supplementation restored the PCr recovery kinetics to normoxic levels, suggesting that the increased NO bioavailability can attenuate muscle metabolic perturbation induced by acute hypoxia (Vanhatalo et al. 2011). In 2012, Masschelein and colleagues reported a 4% $\dot{V}O_2$ reduction during moderate intensity cycle ergometer exercise after NO_3^- supplementation in simulated hypoxia (11% O_2), confirming the magnitude of the effects previously found in normoxia. The authors also showed an increase (+4%) in tissue oxygenation index (ratio between absolute values of oxygenated and total hemoglobin plus myoglobin, TOI) evaluated by near infrared spectroscopy, suggesting positive effects of NO_3^- supplementation on O_2 delivery.

Following these studies, other investigations confirmed the positive ergogenic effects of NO_3^- supplementation in acute hypoxia. For example, Kelly and colleagues (2014) observed a reduction of $\dot{V}O_2$ during moderate intensity exercise (**Fig. 7A**) and increased time to exhaustion during severe intensity exercise (**Fig. 7B**).

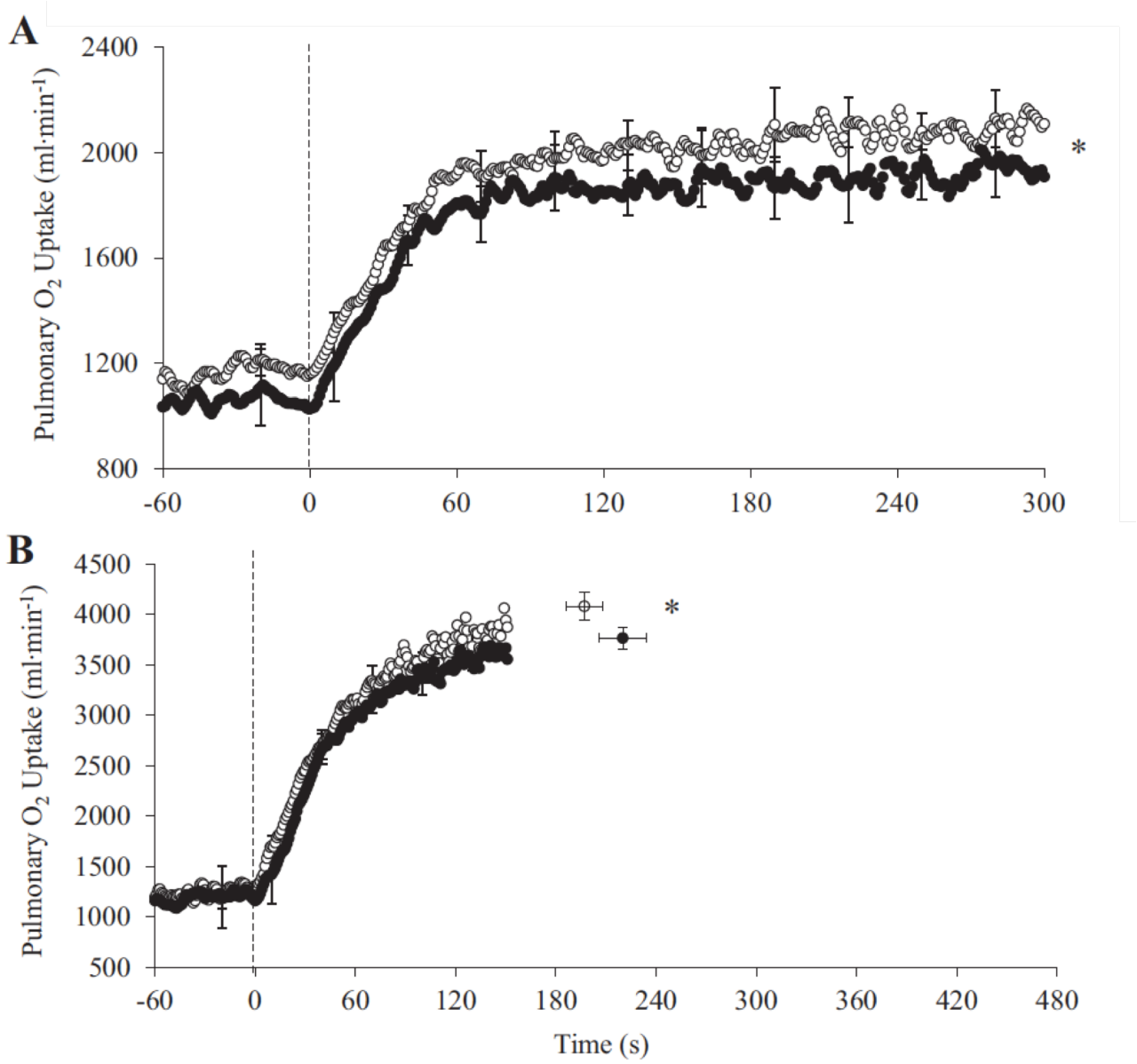

*Figure 7 Pulmonary O_2 uptake ($\dot{V}O_2$) responses during a step increment to a moderate-intensity (A) and severe intensity (B) work rate in hypoxia (13.2 % O_2). Responses following NO_3^- supplementation (BR) are represented as solid circles, with the placebo (PL) responses being shown as open circles. The dashed vertical line denotes the abrupt "step" transition from baseline. Error bars indicate the SE. A: group mean response to moderate-intensity exercise. *P < 0.05 compared with PL. B: group mean response to severe intensity exercise. *Time to exhaustion greater in BR compared with PL (P < 0.05; one-tailed t-test). (From Kelly et al. 2014)*

These studies involved subjects with different fitness level (untrained and trained), different exercise modalities (cycling and running), different levels of hypoxia (11-15,4% O_2) and different dose and duration of supplementation (for a review see Shannon et al. 2017). However not all studies conducted in hypoxic condition showed ergogenic effects after dietary NO_3^- supplementation. As reported in **Table 1** only 3 of 7 and only 2 of 5 studies showed positive effects after acute and chronic NO_3^- supplementation, respectively (Jones et al. 2018). Up to now, the effects of dietary NO_3^- supplementation in hypoxic condition has not been fully elucidated and the efficacy of supplementation may be modulated by factors such as acute or

chronic supplementation regime, training status, altitude level and evaluation tests (time trial, time to exhaustion during a constant-work-rate task or graded exercise test to determine maximum O_2 uptake). Further studies are needed to fully elucidate the possible effects of NO_3^- supplementation in hypoxic condition.

Acute or chronic?	Study	Subjects	NO_3^- intervention	Hypoxia intervention	Criterion exercise test	Egogenic?[b]
Acute	Shannon et al. 2016	Trained and untrained	15.2 mmol BR	~15.3% O_2 (2500 m)	1500 m treadmill TT	✓
	Gasier et al. 2017	Healthy	15 mmol $NaNO_3$	~12.3% O_2 (4300 m) hypobaric	Tlim handgrip exercise	×
	Nybäck et al. 2017	Trained skiers	13 mmol BR	~16.8% O_2 (1800 m)	1000 m roller-skiing TT	×
	Shannon et al. 2017	Healthy	12.5 mmol BR	~14% O_2 (3000 m) ~11.7% O_2 (4300 m)	3 km + 10 kg load treadmill TT	✓
	Arnold et al. 2015	Trained runners	7 mmol BR	~15.3% O_2 (2500 m) ~12.7% O_2 (4000 m)	10 km treadmill TT (2500 m) Tlim incremental test (4000 m)	×
	MacLeod et al. 2015	Trained cyclists	6 mmol BR	~15.3% O_2 (2500 m)	10 km cycle TT	×
	Muggeridge et al. 2014	Trained cyclists	~ 5mmol BR	~15.3% O_2 (2500 m)	16.1 km cycle TT	✓
Chronic	Vanhatalo et al. 2011	Moderately trained	1 day 9.3 mmol/24h	~14.5% O_2 (3000 m)	Tlim sigle-leg Knee extension	✓
	Buordillon et al. 2015	Trained cyclists	3 days ~7.4 mmol/day NaNo3	~11% O_2 (5200 m)	15 km cycle TT	×
	Kelly et al. 2014	Physically active	3 days ~8.4 mmol/day BR	~13.1% O_2 (3700 m)	Tlim at Δ75% cycle	×
	Masschelein et al. 2012	Healthy	6 days, ~5.1 mmol/day BR	~11% O_2 (5200 m)	Tlim incremental test cycle	✓
	Rossetti et al. 2017	Recreationally active	6 days ~6.4 mmol/day BR	~14.1% O_2 (3100 m)	Tlim at 80% hypoxic $\dot{V}O_2$max reserve + 15 kg load, treadmill	×

Table 1 The efficacy of supplementation may be modulated by factors such as acute or chronic supplementation regime and training status. b) A checkmark indicates a statistically significant positive effect on the criterion exercise test, and a cross indicates no significant effect. Abbreviations: BR, NO_3^--rich beetroot juice; $NaNO_3$, sodium nitrate; TT, time trial; Tlim, time to exhaustion during a constant-work-rate task; $\dot{V}O_2max$, maximum O_2 uptake; Δ75%, work rate occurring at 75% of the interval between the gas-exchange threshold and $\dot{V}O_2max$ (modified from Jones et al. 2018)

Up to date, the majority of the studies investigating the ergogenic effects of increased NO bioavailability in hypoxic conditions have exposed subjects to acute hypoxia and have been carried out in simulated altitude (normobaric hypoxia). Nevertheless, there are some evidences that normobaric hypoxia elicits different responses in term of NO bioavailability compared with hypobaric hypoxia. For example, Faiss et al. (2012) showed plasma NOx (i.e., NO_3^- and NO_3^-) concentration lower in hypobaric hypoxia than in normobaric hypoxia. They tested ten subjects during 24h in both hypobaric and normobaric hypoxia and they observed a significant reduction of NOx concentration (-37% at 24h) in the first condition while no differences in normobaric hypoxia compared with normoxic levels. Moreover, it has been reported that exposition to high altitude, from 1300 to 5050 m a.s.l., increases levels of serum, urinary, and salivary [NO_3^-] and [NO_2^-] with a peak the fifth day at 3440 m a.s.l. (Janocha et al. 2011). Similarly, Levett and colleagues demonstrated an increment in plasma [NO_3^-] and [NO_2^-] in caucasian subjects at 3500 m a.s.l. Thus, changes in NO bioavailability occurring during chronic exposure to hypobaric hypoxia seem to be different from those observed in subjects acutely exposed to normobaric hypoxia. According to these evidences, some studies tried to investigate the relationship between NO bioavailability and some physiological responses to hypoxia at altitude. In 2017, the research group of Extreme Everest showed a markedly enhanced levels of exhaled NO as well as higher NO_3^- and NO_2^- levels in saliva after dietary NO_3^- supplementation throughout the duration of the expedition, demonstrating that this supplementation successfully increases NO bioavailability at altitude (Cumpstey et al. 2017). Hennis and colleagues (2016) following a similar study design evaluated the effects of beetroot juice supplementation, 10 mmol of NO_3^- per day, on acute mountain sickness (AMS) and physiological responses to exercise in 40 adolescents trekking to mount Everest base camp. Exercise tests were performed at different altitudes: Kathmandou (1300 m, day 1), Monjo (2835 m, day 2), Namche Bazaar (3500 m, days 3, 4, 5), Deboche (3770 m, day 6), Pheriche (4250 m, days 7, 8, 9), Lobuche (4940 m, day 10) and Gorak Shep (5300 m, day 11). NO_3^- supplementation was taken between day 2 and day 9. Heart rate, Ventilatory rate and peripheral O_2 saturation were acquired immediately before, and in the last 10 s of a standardized 2-min stepping exercise protocol consisting in repeatedly stepping on and off a 20 cm step using alternate feet at 1 step (up or down) per second. No significant effects were

found on resting and exercising peripheral O_2 saturation, heart rate, and ventilation rate in NO_3^- supplementation compared with placebo. Finally, Bakker and colleagues demonstrated positive effects of NO_3^- supplementation on endothelium-dependent vasodilation at altitude. Following a double-blinded randomized controlled crossover design, flow mediated dilation (FMD), used to indirectly assess endothelium function, was measured 3 h after drinking beetroot juice (5.0 mmol of NO_3^-) and placebo supplementation at 3700 m a.s.l. in healthy young subjects (Bakker et al. 2015). Dietary NO_3^- supplementation restored FMD values of the brachial artery observed at pre-trek (1370 m), abolished the classical negative effect exerted by hypobaric hypoxia on endothelial function (Bakker et al. 2015).

2.3 Nitric oxide bioavailability and exercise tolerance in disease populations

A decrease in the production and bioavailability of NO is a hallmark of several cardiovascular and metabolic diseases and often lead to exercise intolerance and a poor quality of life. The idea of increasing NO bioavailability in the treatment of some pathologies is not a novelty. Several of the most commonly used pharmaceutical agents to prevent or treat cardiovascular diseases enhance NO signaling trough formation of NO, inhibition of NO breakdown or direct stimulation of downstream signal. For example, the administration of organic nitrate (e.g. nitroglycerin) has been used for more than a century to induce rapid vasodilation at coronary arteries and reduce angina symptoms via release of NO_2^- and NO (Omar et al. 2015). Nevertheless, although their efficacy in counteracting NO deficit, these drugs show some side effects like development of tolerance, headache, hypotension and in chronic use may lead to endothelial disfunction (Omar et al. 2015).

Conversely, inorganic nitrate has been demonstrated effective in increase NO bioavailability through NO_3^- - NO_2^- - NO pathway without major side effects. In light of these findings, several countries have started to recommend dietary interventions rich in inorganic NO_3^- for patients with cardiovascular diseases. The Dietary Approaches to Stop Hypertension (DASH) is the most striking example (Sacks et al. 2001). This lifelong approach to healthy eating was designed to help treat or prevent high blood pressure (hypertension) and it encourages subjects to reduce the sodium in their diet and eat a variety of foods rich in nutrients that help lower blood pressure, such as potassium, calcium and magnesium. However, recently it has been noticed that the consumption of fruits, vegetables, and low-fat dairy foods such as whole grains, poultry, fish, and nuts can also potentially increase inorganic NO_3^- assumption, suggesting the positive effects of DASH diet on blood pressure may reside also on the increased NO bioavailability.

According to the several physiological benefits associated to an increased NO bioavailability, including reduction in blood pressure, modification of platelet aggregation, and improvement of limb blood flow, several investigations have been conducted to evaluate the effects of dietary NO_3^- supplementation on health status and exercise tolerance in clinical population, especially in patients with limited O_2 availability at peripheral tissues (for a review see Woessner et al. 2018). In the following paragraphs, the main results of NO_3^- supplementation in different clinical populations will be described.

2.3.1 Chronic Heart Failure

Chronic heart failure (CHF) is characterized by an impairment of cardiac output, which is unable to meet the body's metabolic demands. This condition is exacerbated during physical activity and lead patients to exercise intolerance due to structural and functional defects in the O_2 transport pathway (Poole et al. 2012). In the past, the inability to augment cardiac output during exercise was considered the main contributor to the exercise intolerance in CHF patients. Nowadays, several factors at skeletal muscle level, such as capillary density rarefaction, decreased mitochondrial function and a preferential loss of type 1 oxidative fibers, have been demonstrated to be key determinants of impaired exercise capacity in these patients (Piepoli & Coats 2013). Thus, quality of life and exercise tolerance are limited by both central and peripheral factors. On the other hand, in CHF patients several evidences suggest that NO bioavailability is compromised by a combination of the reduction in BH4, SOD, catalase, and glutathione peroxidase protein expression and activity, as well as increased NADPH oxidase protein expression and activity (Poole et al. 2012). Starting from these concepts, in the last five years several studies evaluated the effects of increased NO bioavailability in CHF patients. In 2015, Zamani et al. evaluated the effects of acute beetroot juice supplementation (12.9 mmol of NO_3^-) on seventeen CHF patients with preserved ejection fraction (HFpEF). NO_3^- supplementation significantly improved $\dot{V}O_2peak$ and time to exhaustion during a maximal exercise test but did not improve exercise efficiency during a moderate intensity exercise. These changes were accompanied by a significant reduction in systemic vascular resistance and a significant increase in cardiac output at peak exercise, which provided greater O_2 delivery to exercising muscles thanks to the beneficial effects of increased NO bioavailability. In the same cohort of patients (CHF, HFpEF), Eggebeen and colleagues (2016) found no effects on exercise tolerance after acute supplementation but a greater increase in time to exhaustion after 7 days of supplementation (6.1 mmol NO_3^- per day). A more recent study from Zamani and colleagues (2017) confirmed the positive effects of chronic NO_3^- supplementation (6 mmol per day for 1

week, increasing to 18 mmol per day for the second week) in patients with similar characteristics. Taking together these results suggest that increased NO bioavailability can be considered a valid aid to improve exercise tolerance in CHF, HFpEF. However, contrasting results have been reported about the effects of beetroot juice supplementation on exercise tolerance in CHF patients with reduced ejection fraction (HFrEF). Hirai and colleagues demonstrated that 9 days of beetroot juice supplementation (12.1 mmol ·day^{-1}) did not improve exercise tolerance parameters as time to exhaustion or $\dot{V}O_2peak$. They also showed no significant changes in central hemodynamics, skeletal muscle oxygenation, or the O_2 cost of exercise (Hirai et al. 2017). The authors suggested that their negative findings could be due to the relatively normal peripheral oxygen extraction in HFrEF compared with HFpEF. Thus, the potential therapeutic application of dietary NO_3^- supplementation in CHF patients seems to depend from the etiology of the disease (HFrEF vs HFpEF) and future studies are needed to define the real application spectrum of inorganic NO_3^- supplementation in these patients.

2.3.2 Peripheral Arterial Disease

Peripheral arterial disease (PAD) is caused by atherosclerotic plaque formation in the large arteries of the lower limbs that results in reduced blood flow to the peripheral tissues, in particular during exercise for the increased O_2 demand, and leads to intermittent claudication (IC). In this disease the best way to increase exercise tolerance in patients seems to be a reduction of resistance in arteries, arterioles, and capillaries that serve the skeletal muscle tissue distal to the site of stenosis. Accordingly, increased NO bioavailability could be a beneficial intervention due to its potential vasodilatatory effect and the positive effects on muscle performance. The first pioneering work on this topic was performed by Kenjale and colleagues (2011). They supplemented patients with PAD by beetroot juice and they demonstrated an increase in time to claudication onset pain (18%) and a longer peak walking time (17%) compared with placebo (Kenjale et al. 2011). These findings were related to increase NO-signaling and consequent higher peripheral blood flow toward hypoxic tissues (Allen et al. 2012). This effect was confirmed by NIRS data that indicate a relative increase in blood volume to the region and therefore, vasodilation and perfusion following BR (Kenjale et al. 2011). In the last year, three more articles investigated the effects of chronic NO_3^- supplementation in PAD patients. They reported an enhancement of brachial artery blood flow and a reduction of the exaggerated pressor responses during moderate-intensity forearm exercise (Kruse et al. 2018), a better response to physical activity (Woessner et al. 2018) and on blood flow and vasodilator function (Bock et al. 2018) after NO_3^- supplementation. In

summary, these evidences suggest that NO_3^- supplementation could be useful per se on different aspects of the pathology and in combination to exercise training can maximize the beneficial peripheral vascular and skeletal muscle adaptations.

2.3.3 Diabetes Mellitus

Diabetes mellitus (DM) is a group of metabolic disorders characterized by high blood sugar levels over a prolonged period that can cause many acute and chronic complications. Diabetic ketoacidosis, hyperosmolar hyperglycemic state, or death can be ascribed in the first group. Serious long-term complications include cardiovascular disease, stroke, chronic kidney disease, foot ulcers, and damage to the eyes (Brownlee 2001). Several evidences show that impaired NO bioavailability is present in patients with type 2 diabetes mellitus (T2DM) and it contributes to decline in cardiovascular health in these patients. Moreover, the lack of NO production during maximal graded exercise test and a significant correlation between the increase in plasma NO_2^- during exercise and exercise capacity have been demonstrated, suggesting a strong relation between exercise intolerance and NO production defects in these patients (Allen et al. 2009). Similarly, studies on animal models of T2DM demonstrated that NO bioavailability influences several aspects of insulin signaling and glucose homeostasis, including regulation of insulin secretion, glucose uptake, mitochondrial function, and blood flow (see above *2.2.1.4 Glucose homeostasis*). Following these evidences, it has been hypothesized that NO_3^- supplementation can be a valid aid to counteract several aspects of the pathology. Some important beneficial properties in animal model, including regulation of glucose homeostasis and insulin signaling pathway, improvement of insulin resistance and vascular function, hypotensive, hypolipidemic as well as anti-inflammatory and anti-oxidative effects have been observed following administration of inorganic NO_3^-/ NO_2^- confirming this hypothesis (for a review see Bahadoran et al. 2015). However, similar studies conducted in T2DM patients, despite a significant increase in plasma [NO_2^-], did not find encouraging results. Cermak and colleagues (2015) showed no differences in oral glucose tolerance test after acute supplementation with 10.5 mmol of $NaNO_3$. Shepherd and colleagues (2015) failed to observe changes in exercise efficiency or exercise tolerance after 4 days of beetroot juice supplementation (6.43 mmol/day) in T2DM patients. Finally, no effects were found on endothelial function or insulin sensitivity in these patients after 2 weeks of NO_3^- supplementation (7.5 mmol/day) (Gilchrist et al. 2013). This discrepancy of results is not clear but the lack of physiological changes after NO_3^- supplementation in T2DM patients have been attributed to several factors: defects in NO transport at peripheral tissue for a preferential binding of NO to glycosylated red blood cells

and a consequent decreased NO bioavailability; longer duration of the disease in human patients compared to animal models; and the possible interference effects of diabetic medication. For instance, metformin, one of the principal drugs for diabetics used to lower blood glucose, may interfere with beneficial effects of dietary NO_3^- on aspects related to exercise tolerance (Woessner et al. 2018). In summary, contrasting results and few proper investigations are present about the utilization of NO_3^- in T2DM patients and future studies investigating the effects of longer-term supplementation on exercise capacity variables are needed.

2.3.4 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a progressive lung disease characterized by persistent airflow obstruction resulting in a systemic syndrome that includes inflammation, oxidative stress, insulin resistance, sympathetic activation, and hypoxemia and is typically develops over many years of smoke inhalation. Furthermore, patients with COPD have lower exercise efficiency and therefore exercise tolerance compared with healthy controls, possibly due to an increased oxygen cost of breathing and impaired muscle mitochondrial metabolism (Arne et al. 2009). NO_3^- supplementation could improve both exercise efficiency and cardiovascular health in COPD. Different studies investigated the effects of both acute or chronic dietary NO_3^- supplementation in COPD patients. Positive results were found in term of reduction of blood pressure at rest (Leong et al. 2015, Kerley et al. 2015, Berry et al. 2015) and during exercise after 3-8 days of dietary NO_3^- supplementation (Berry et al. 2015). Conversely, the beneficial effects of increased NO bioavailability on exercise efficiency and exercise tolerance in patients with COPD are more controversial. Beetroot juice ingestion have been reported to lower the O_2 cost of submaximal cycling (Curtis et al. 2015), increase in performance during incremental shuttle walk distance (Kerley et al. 2015) and extend time to exhaustion during submaximal constant-work-rate cycle exercise (Berry et al. 2015). On the other hand, other studies did not find significant improvement during walking (Leong et al. 2015, Shepherd 2015) or cycling exercise (Beijers et al. 2017, Friis et al. 2017). Thus, the effects of NO_3^- supplementation on exercise performance in COPD are not clear and further investigation are needed.

3. AIMS

The overall objective of this thesis is to investigate the effects of enhanced NO bioavailability on exercise tolerance in different conditions. More specific the projects carried out during these years aimed to:

- ⇒ gain insights into the mechanisms of action by which NO reduces skeletal muscle O_2 consumption during steady state exercise. To do so, the effects of $NaNO_2$ infusion on the metabolic response to exercise were evaluated in canine gastrocnemius model by investigating in vivo and ex vivo skeletal muscle oxidative metabolism. (Study I)

- ⇒ broaden the knowledge about the ergogenic effects of dietary NO_3^- on healthy subjects in hypoxia investigating the effects of beetroot juice supplementation on cycling and arm-cranking during a prolonged exposure to high altitude. (Study II)

- ⇒ evaluate possible beneficial effects of NO_3^- supplementation on other clinical population characterized by exercise intolerance. This object was pursued investigating the effects of a short-term dietary NO_3^- supplementation on physiological parameters associated with exercise tolerance in a group of obese adolescents. (Study III)

4. MANUSCRIPTS

4.1 Increased nitric oxide bioavailability does not alter isolated canine muscle oxidative metabolism in hypoxia

This study was carried out in the "Muscle Physiology Lab" of the Auburn University (Alabama, USA). I would like to thank the courtesy of Professor Bruce Gladden and his staff for letting me the unique opportunity to get access to their lab and sharing their knowledge with me.

Abstract

Previous studies have shown a decreased muscle oxygen consumption ($\dot{V}O_2$) during moderate intensity exercise in hypoxia after augmented NO bioavailability, which suggests an increased efficiency of oxidative metabolism. It is not clear if this effect is related to a NO-induced increase in convective O_2 delivery and/or to an enhanced mitochondrial coupling efficiency. Aim of this study was to examine skeletal muscle contraction economy during hypoxic exercise following nitrite infusion with unchanged convective O_2 delivery.

Canine gastrocnemius muscles (n=8) were surgically isolated and electrically stimulated via the sciatic nerve generating 1 contraction every 3 seconds for 3 minutes in hypoxic conditions. During contractions, sodium nitrite (NITRITE) or sodium chloride (SALINE) was infused in the popliteal artery. Muscle blood flow was kept constant by a perfusion pump. $\dot{V}O_2$ was calculated from the Fick principle. Muscle biopsies were obtained before and after NITRITE and mitochondrial respiration rates were recorded by high-resolution respirometry.

$\dot{V}O_2$was not significantly different between NITRITE (60.6±18.0 mL · Kg^{-1} · min^{-1}) and SALINE (61.7 ± 18.3 mL · Kg^{-1} · min^{-1}). No differences were found for ADP-stimulated mitochondrial respiration (both for complex I and complex II), leak respiration and oxidative phosphorylation coupling.

In hypoxic conditions, but in the presence of constant and normal convective O_2 delivery, nitrite infusion did not affect canine skeletal muscle oxidative metabolism. These results suggest that the effects of increased NO availability on muscle contraction efficiency in hypoxia, if present, are likely not attributable to changes in mitochondrial respiratory efficiency.

Keywords

Nitric oxide; Mechanism; Mitochondrial efficiency; Oxidative metabolism

Introduction

Nitric oxide (NO) is a gaseous signaling molecule which is involved in the regulation of many physiological processes, including skeletal muscle excitation–contraction coupling (Andrade et al. 1998), force production (Folland et al. 2000), microvascular blood flow (as reviewed by Tschakovsky & Joyner, 2008), and mitochondrial bioenergetics (Clerc et al. 2007; Boushel et al. 2012). NO can be produced from oxidation of the aminoacid L-arginine by a family of enzymes called NO synthases (NOS), before being rapidly oxidized to nitrate (NO_3^-) and nitrite (NO_2^-) (Moncada & Higgs, 1993). As alternative, NO_3^- and NO_2^- can be sequentially reduced to NO in a process dependent on anaerobes bacteria in the oral cavity (Govoni et al. 2008, Woessner et al. 2016). It is now acknowledged that the alternative pathway NO_3^- - NO_2^- - NO is a complementary system for NO synthesis and it is more effective in condition of low pH and reduced partial pressure of oxygen (PO_2) (Lundberg et al. 2009).

In these last years, the possibility to increase NO bioavailability by increasing NO_3^- and NO_2^- plasma concentration has become the focus of several investigations aimed to ameliorate exercise tolerance and improve performance (Jones 2014). The ingestion of foods rich in NO_3^- (e.g. beetroot juice) as well as the supplementation with sodium nitrate ($NaNO_3$) has been demonstrated effective in reducing oxygen cost of submaximal exercise and increasing time to exhaustion during severe intensity exercise (Jones 2014). The physiological basis explaining these observations has not been clearly defined and it involves several mechanisms. Previous data obtained in animals and humans showed that dietary NO_3^- supplementation improved excitation-contraction coupling resulting in an enhanced force production (Hernandez et al. 2012, Haider & Folland 2014). Bailey et al. (2010) reported a reduced ATP turnover rate during exercise after beetroot juice supplementation suggesting that supplementation may reduce ATP cost of force production by modulating actomyosin-ATPase sensitivity and/or Ca^{2+} handling. In addition, there are recent evidences that NO_3^- supplementation may enhance muscle O_2 delivery and improve O_2 distribution across and within the active muscle fibers (Ferguson et al. 2013, 2015). Finally, some authors have also hypothesized the reduction of O_2 cost of exercise after nitrate supplementation may be modulated by a different mitochondrial efficiency. Larsen et al. (2011) evaluated the effects of 3 days of sodium nitrate supplementation on whole body O_2 consumption and oxidative phosphorylation efficiency in healthy subjects. They observed a reduction of whole body O_2 consumption during moderate intensity constant work rate exercise and an improvement in mitochondrial bioenergetics through modifications in coupling efficiency (Larsen et al. 2011). However, some doubts have

been raised about the possible effects of improved NO bioavailability on mitochondrial function. Whitfield and colleagues (2016) reported no improvement of mitochondrial coupling and efficiency in human skeletal muscles after 7 days of supplementation of beetroot juice, although a reduced whole body oxygen consumption was observed. As such, the exact physiological mechanisms underlying the ergogenic effects of enhanced NO bioavailability are only partially understood and further study are needed.

NOS activity (which is O_2 dependent) is reduced in conditions of low PO_2, whereas NO_2^- reduction to NO is potentiated in hypoxic and acidic environments (Modin et al. 2001). Moreover, NO plays a key role in the physiological response and adaptation to hypoxia because it restores O_2 supply when arterial and intracellular PO_2 are reduced (Umbrello et al. 2014). Thus, physiological adaptations induced by dietary NO_3^- supplementation could be particularly beneficial when normal O_2 availability is limited and functional capacity reduced. Recent data have shown that dietary NO_3^- supplementation in hypoxia reduces muscle metabolic perturbations during knee-extension exercise (Vanhatalo et al. 2011), speeds up $\dot{V}O_2$ kinetics during moderate-intensity cycle exercise (Kelly et al. 2014) and improves severe-intensity exercise performance (Muggeridge et al. 2014), partially restoring exercise tolerance towards values observed in normoxia. However, the physiological mechanisms responsible for the beneficial effects of NO_3^- supplementation on oxidative function and exercise tolerance in hypoxia are not completely clear, and may be attributed to: i) NO_3^- - NO_2^- - NO mediated enhancement of O_2 delivery and improved distribution within the active muscle, and/or ii) an enhanced mitochondrial efficiency.

Aim of this study was to gain insights into the effects of increased NO bioavailability on the metabolic response to exercise by investigating in vivo and ex vivo skeletal muscle oxidative metabolism. More specifically, our purpose was to confirm, in the isolated dog gastrocnemius model, the positive effects of increased NO bioavailability, as obtained by sodium nitrite ($NaNO_2$) infusion, on skeletal muscle oxidative metabolism in hypoxia, and to discriminate between enhanced O_2 delivery/intramuscular blood flow distribution vs. enhanced mitochondrial function as the mechanism responsible for the positive effects.

The study was conducted in isolated, perfused canine gastrocnemius muscle model which allows to control several factors affecting physiological response to exercise. The isolated gastrocnemius preparation in situ allows pump-perfusion of the muscle with constantly elevated blood flows, preventing NO effects on O_2 delivery and leaving effect on mitochondrial respiration be fully manifest. Moreover, this experimental model permit to precisely control muscle force production by recruiting simultaneously all motor unit required for a given target

of force. Since there are some evidences that the effects of NO may be of a higher magnitude in fast type 2 fibers as opposed to slow type 1 fibers (Ferguson et al. 2014, Hernández et al. 2012), this model adds an additional element of control and insight, avoiding confounding effects of variable motor unit recruitment. Finally, the experiments were conducted at reduced partial pressure of inspired O_2 because there are evidences that the effects of NO might be revealed most readily in low O_2 conditions (Feelisch et al. 2008).

Materials and methods

Animals

The study was conducted with approval of the Institutional Animal Care and Use Committee of Auburn University (Auburn, Alabama) where the experiments were performed. Eight adult mongrel hound of either sex (4 F and 4 M; body mass 20.8 ± 2.6 kg, mean±SD) were anaesthetized with an initial bolus of sodium pentobarbital (30 mg · Kg^{-1}) intravenously via a prominent cephalic vein of a forelimb. Maintenance doses were given as required to maintain a deep plane of surgical anesthesia as indicated by a complete absence of pedal, palpebral and corneal reflexes. The dogs were intubated with an endotracheal tube and ventilated with a respirator (Model 613, Harvard, Holliston, MA, USA). Ventilation and gas (O_2 and N_2) concentration in the breathed air were adjust in order to ensure blood PO_2 values requested from the protocol (see below for further details). The dogs were warmed with a heating pad located under surgical plane of anesthesia. Blood coagulation was prevented by three divided doses of heparin at 2 h intervals to a total of 3000 U · Kg^{-1}. At the end of the experiments the dogs were euthanized with an overdose of pentobarbital along with saturated potassium chloride.

Surgical preparation

The gastrocnemius muscle complex (gastrocnemius + superficial digital flexor), for convenience referred to as 'gastrocnemius', was isolated as described previously (Grassi et al. 2011). Briefly, a medial incision was made through the skin of the left hindlimb from mid-thigh to the ankle. The insertion tendons of the sartorius, gracilis, semitendinosus and semimembranosus muscles were cut to allow these muscles to be folded back to expose the gastrocnemius. Popliteal artery guaranteed arterial supply to the gastrocnemius whereas venous outflow from the muscle was isolated to popliteal vein after ligation of all the other vessels draining from the gastrocnemius. The popliteal vein was cannulated, and flow ($\dot{Q}$) was measured with a flow-through-type transit-time ultrasonic flow probe (6NRB440, Transonic

Systems, Ithaca, NY). Venous outflow was returned to the animal via a reservoir attached to a cannula in the left jugular vein. The right femoral artery was also isolated and cannulated. Blood from this artery was passed through tubing to a peristaltic pump (Gilson Minipuls 3) and then through another cannula into the contralateral isolated popliteal artery supplying the gastrocnemius, allowing experimental control of muscle perfusion. Y-connectors positioned before and after the pump allowed either spontaneous perfusion of the gastrocnemius, at the animal's own blood pressure, or controlled flow at any desired level by adjusting the pump setting. A transducer (Model RP-1500, Narco Biosystems, Austin, TX) was inserted to measure perfusion pressure to the muscle. The distal tendons from the gastrocnemius, attached to a portion of the calcaneus, were cut away and clamped around a metal rod for connection to an isometric myograph via a load cell (Interface SM-250, Scottsdale, AZ). Two bone nails were used to fix the femur and tibia to the base of the myograph. The sciatic nerve, which innervates the gastrocnemius, was isolated and cut into two segments, and the distal nerve stump, ~1.5–3.0 cm in length, was pulled into a small tubular electrode for stimulation. The muscle was covered with saline-soaked gauze to prevent drying and cooling.

Experimental protocol

The experiments consisted of a preliminary trial and one experimental trial. Before each contraction protocol, the GS was set at optimal length in order to develop peak tension (total tension minus resting tension). To evoke muscle contractions, the nerve was stimulated by supramaximal square pulses of 6.0–8.0 V amplitude and 0.2 ms duration (Grass S48 stimulator). For the preliminary trial, isometric tetanic contractions were triggered by nerve stimulation with trains of stimuli (6–8 V, 200 ms duration, 50Hz frequency) at a rate of one contraction per second for a 3 min period. Based on studies of $\dot{V}O_2peak$ in this model (Ameredes et al. 1998), this stimulation pattern elicited about 100% of peak metabolic rate for this muscle in the current experiments. This trial was performed with spontaneous adjustment of self-perfused cardiac output ($\dot{Q}$). Ventilation and gas (O_2 and N_2) concentration in the breathed air were adjusted to a level that established and maintained normal arterial PO_2, PCO_2, pH; initial settings were 20 ml · Kg^{-1}BW for tidal volume and a frequency of 15-20 breath · min^{-1}.

As for the experimental trial, isometric tetanic contractions were triggered by nerve stimulation with trains of stimuli (6–8 V, 200 ms duration, 50Hz frequency) at a rate of one contraction every three seconds for a 9 min period. The first three minutes of exercise were performed to attain a steady-state level of the investigated variables, avoiding confounding effects of the transition phase. During the subsequent 3 minutes (SALINE), physiological saline

solution (0.9%) was infused into the right femoral artery at an infusion rate of ≈0.013 mL · min^{-1} infusate per 1.0 mL · min^{-1} blood flow. In the last 3 minutes (NITRITE), sodium nitrite was infused at same rate as previous saline infusion. No resting phase was interposed between SALINE and NITRITE. This exercise was performed with a pump-perfused constant $\dot{Q}$, adjusted 15-30 s before the start of the contractions at a level corresponding to about 50% of $\dot{Q}peak$ value determined in the preliminary trial in order to maintain a reasonably high perfusing pressure. Ventilation and gas (O_2 and N_2) concentration in the breathed air were adjusted to ensure desired values of hypoxia (FiO_2≈12%, PaO_2 ≈43 mmHg).

Measurements

Sample of arterial and venous blood were drawn before and after pre-trial. In trial 1 and 2, blood samples were collected before contractions start, after 3 minutes of exercise and at the end of each trial. In these samples, PO_2, PCO_2, plasma lactate concentration and pH were determined with a blood gas analyzer (GEM PREMIER 3000, Instrumentation Laboratory, Lexinton, MA). The difference between venous and arterial whole blood lactate concentration (ΔLa^-) was multiplied by muscle blood flow in order to obtain blood lactate efflux from muscle (Laefflux). Total haemoglobin (Hb) concentration and Hb oxygen saturation (SO_2) were measured by CO-Oximeter (IL682, Instrumentation Laboratory, Lexinton, MA). The arterial oxygen concentration (CaO_2) was calculated as follows:

$$CaO_2 = \{[(1.39mL\ O_2 \cdot g\ Hb^{-1}) \cdot (gHb \cdot dL\ arterious\ blood^{-1})] \cdot SaO_2\%\} + \{[(0.003mL\ O_2 \cdot dL\ blood^{-1}) \cdot (mmHgPO_2)^{-1}] \cdot PaO2\}$$

where PaO_2 is the partial pressure of oxygen in the arterial blood. Dissolved O_2 was also considered for the calculation of blood O_2 concentration. The venous oxygen concentration (CvO_2) was calculated as follows:

$$CvO2 = \{[(1.39mL\ O2\ O_2 \cdot g\ Hb^{-1}) \cdot (gHb \cdot dL\ venous\ blood^{-1})] \cdot SaO_2\%\} + \{[(0.003mL\ O_2 \cdot dL\ blood^{-1}) \cdot (mmHgPO2)^{-1}] \cdot PvO_2\}$$

where PvO2 is the partial pressure of oxygen in the venous blood. Blood from venous samples was also separated into EDTA vacutainer (BD Vacutainer) and centrifuged at 4000 rpm and 4°C for 10 min immediately after collection. The plasma was then separated, frozen at -80°C and analyzed within 6 months from initial collection for determination of [NO_3^-] and [NO_2^-]. Measurements of [NO_3^-] and [NO_2^-] were made using chemiluminescence as previously described (Lanzarone et al. 2010).

Output from the pressure transducer was recorded on a strip chart recorder while outputs from the load cell and flowmeter (T206, Transonic Systems) were fed through strain gauge and transducer couplers, respectively, into a computerized (PowerComputing PowerBase 240 Macintosh clone) data acquisition system (GW Instruments Inc., SuperScope II and InstruNet Model 100B D/A input/output system). The load cell reached 90% of full response within 1ms while the flowmeter was set to its highest pulsatile cut-off frequency of 100 Hz; both signals were sampled at a rate of 100 Hz by the computerized data acquisition system. The load cell was calibrated with known weights prior to each experiment. The flowmeter was calibrated with a graduated cylinder and clock during and after each experiment. Peak force and force–time integral (FTI) were calculated for each contraction and averaged across last five contractions evoked at the end of each 3-minute exercise phase. To calculate FTI, the pre-stimulation baseline was set as zero and the FTI was taken as the integral of force from the onset of force development in one contraction to the data sample before the beginning of force development in the next contraction. Any increase in baseline tension during contractions was accounted for by subtracting the area described by the straight line joining these two points and the pre-stimulation baseline. All these analyses were performed with commercial software (Prism 6.0, Graphpad, CA, USA). Vascular resistance was calculated as muscle perfusion pressure (BPm) divided by $\dot{Q}$. $\dot{V}O_2$ of the gastrocnemius was calculated by Fick's principle as $\dot{V}O_2 = \dot{Q} \cdot C(a\text{-}v)O_2$, where $C(a\text{-}v)O_2$ is the difference in O_2 concentration between arterial blood (CaO_2) and venous blood (CvO_2). $\dot{V}O_2$ was calculated at discrete time intervals corresponding to the timing of the blood samples.

A continuous-wave near infrared spectroscopy (NIRS) system (Oxymon Mk III, Artinis Medical Systems BV) was used to assess relative changes in muscle oxygenation. Two fiber-optic bundles were used to emit and receive light at two different wavelengths (760 and 850 nm). The optodes were placed over the belly of the medial head of the left GS and held in place with an elastic band. The muscle was covered with a dark plastic sheet throughout the experiment to block extraneous light. The two optodes were held 25 mm apart, resulting in a penetration depth of approximately 12 mm. Relative changes in oxygenated hemoglobin and myoglobin (Δ[oxy(Hb+Mb)]), deoxygenated hemoglobin and myoglobin (Δ[deoxy(Hb+Mb)]), and total hemoglobin and myoglobin (Δ[tot(Hb+Mb)]) were output from the system. The NIRS signal was biased to zero before the start of each trial. For analysis of the NIRS results, raw data were 5-s averaged to obtain Δ[oxy(Hb+Mb)], Δ[deoxy(Hb+Mb)] and Δ[tot(Hb+Mb)] values at the end of each trial.

Muscle biopsies were obtained by superficial excision of muscle pieces with a scalpel, at rest and during the last 10 s of the contraction period of each trial. Biopsy samples were immediately frozen in liquid nitrogen. Subsequently, samples were freeze-dried and dissected from visible connective tissue and blood. The skeletal muscle samples were minced and then homogenized with a VITRUS polytron and trypsin was added to allow the release of mitochondria from the myofibrils. The resulting homogenate was centrifuged and the supernatant was then decanted. The mitochondria pellet obtained was washed and resuspended in a mannitol–sucrose solution and this extract was utilized to determined polarigraphically mitochondrial respiration (Oxytherm, Hansatech Instruments, Pentney, UK) following procedures previously described (Mowry et al. 2017). Respiration was measured using 2 mmol · L^{-1} pyruvate, 2 mmol · L^{-1} malate and 2 mmol · L^{-1} glutamate as a substrate. Maximal respiration (state 3) was defined as the rate of respiration in the presence of ADP and was initiated by adding 0.25 mmol · L^{-1} ADP to the chamber containing respiratory substrates and buffered mitochondria. State 4 respiration was measured after the phosphorylation of ADP was complete. The state 3 and 4 respirations were normalized to mitochondrial citrate synthase. Respiratory control ratio (RCR) was calculated as the ratio between state 3 respiration and state 4 respiration.

Calculations of mean capillary PO_2 and O_2 diffusional conductance. During exercise , mean O_2 partial pressure at capillary level ($P_{\bar{c}}O_2$) and O_2 diffusional conductance (DmO_2) were calculated based on a Bohr integration technique in which, by a forward integration procedure, a lumped parameter tissue diffusing capacity-to-muscle blood flow ratio ($DmO_2/\dot{Q}$), is selected to produce the measured venous PO_2, given the actual arterial PO_2. The calculation is based on Fick's first law of diffusion, which states that, at any point along the capillary in a small element of blood, instantaneous $\dot{V}O_2$ (t) is given by:

$$\dot{V}O_2 = \frac{VC}{100} \cdot \frac{dC_{O_2}(t)}{dt} = DmO_2\,[PO_2(t) - Pmit_{O_2}(t)] \qquad \text{Eq.1}$$

The middle portion of Eq. 1 represents the rate of removal of O_2 from blood whereas the right-hand portion reflects the rate of tissue diffusion of O_2 that is utilized by mitochondrial oxidative phosphorylation. DmO_2 is the tissue diffusing capacity, VC is capillary volume, $dC_{O_2}(t)$ and $PO_2(t)$ are instantaneous capillary O_2 content and partial pressure, respectively, and $Pmit_{O_2}(t)$ is mitochondrial PO_2, here assumed to be zero. If transit time is taken to be $VC/\dot{Q}$, where $\dot{Q}$ is muscle blood flow, Eq. 1 can be evaluated numerically in small-time steps (0.01 s) requiring only an estimate of DmO_2 from which effluent venous PO_2 is calculated for a given arterial PO_2. Briefly, the value of $DmO_2/\dot{Q}$ is varied iteratively until the calculated end-capillary PO_2 and

PCO_2 correspond to the measured venous PO_2 and PCO_2. Mean capillary PO_2 is then a time-weighted averaged PO_2 along the capillary, easily determined from the capillary PO_2 values calculated at each equal time step along the capillary.

Statistical analysis

Values were expressed as means ± SD. Normal distribution of values was assessed by Shapiro-Wilk normality test. To determine the statistical significance of differences between two means, a Student's paired t test (two-tailed) was performed. The level of significance was set at $P < 0.05$. Data fitting and statistical analyses were carried out by using a commercially available software package (Prism 6.0, Graphpad, CA, USA).

Results

The weight of gastrocnemius muscle was 93.0 ± 11.3 g.

Preliminary trial

Resting and peak values of the main variables pertinent to O_2 transport and utilization, acid-base status and hemodynamics are shown in **Table 1**. In this normoxic self-perfused trial, $\dot{V}O_2peak$ was 183.1 ± 43.6 mL · Kg^{-1} · min^{-1} and $\Delta[La^-]_b$ corresponded to 1.44 ± 0.73mM.

Table 1. Values at rest and at end of the preliminary trial for the main variables pertinent to O_2 transport and utilization, acid-base status and haemodynamic.

	Rest	Maximal
pH_a	7.40 ± 0.02	7.39 ± 0.02
P_aO_2 (Torr)	101 ± 12	94 ± 10
P_aCO_2 (Torr)	33 ± 3	33 ± 3
$[Hb]_a$ (g · 100mL^{-1})	15.4 ± 1.3	15.5 ± 1.5
S_aO_2 (%)	95.0 ± 0.9	94.3 ± 1.3
C_aO_2 (mL·100mL^{-1})	20.7 ± 1.7	20.6 ± 1.9
$\dot{Q}_m$ (mL · 100g^{-1} · min^{-1})	17.0 ± 4.5	112.1 ± 26,6
$\dot{Q}_m \cdot C_aO_2$ (mL · 100g^{-1} · min^{-1})	3.6 ± 1.2	23.1 ± 5.6
$m\dot{V}O_2$ (mL · 100g^{-1} · min^{-1})	0.3 ± 0.1	18.3 ± 4.4
$m\dot{V}O_2$ (mL · kg^{-1} · min^{-1})	3.4 ± 1.2	183.1 ± 43.6
$C_{(a-v)}O_2$ (mL · 100ml^{-1})	2.1 ± 0.8	16.4 ± 1.1
O_2 extraction (%)	10.4 ± 4.6	79.6 ± 5.1

Table 1 pH_a, arterial pH; P_aO_2 , oxygen partial pressure in arterial blood; P_aCO_2 , carbon dioxide partial pressure in arterial blood; $[Hb]_a$, arterial haemoglobin concentration; S_aO_2, haemoglobin saturation in arterial blood; C_aO_2 , arterial oxygen concentration; Q_m, muscle blood flow; $Q_m \cdot C_aO_2$, muscle O_2 delivery; mVO_2, muscle O_2 consumption; $C(a-v)O_2$, artero-venous oxygen concentration difference; O_2 extraction, percentage of O_2 extraction. Values are means ± SD; n = 8.

Experimental Trial

Resting and steady state values obtained after three minutes of exercise of the main variables pertinent to O_2 transport and utilization, acid-base status and hemodynamics are shown in **Table 2**. According to the reduced PO_2 in the inspired air, P_aO_2, C_aO_2 and S_aO_2 resting values were significantly lower than values observed during preliminary trial. $[Hb]_a$ concentration was slightly higher than in preliminary trial due to a possible reduction in plasma volume. At steady state, $m\dot{V}O_2$ was about 30% of $\dot{V}O_2peak$ whereas muscle blood flow was kept higher (at 45% of $\dot{Q}$peak) than that requested by the relative exercise intensity.

Table 2. Values at rest and after 3 minutes of constant work rate exercise for the main variables pertinent to O_2 transport and utilization, acid-base status and haemodynamic.

	Rest	Steady-state
pH_a	7.38 ± 0.03	7.39 ± 0.04
P_aO_2 (Torr)	43 ± 3	43 ± 3
P_aCO_2 (Torr)	30 ± 3	29 ± 3
$[Hb]_a$ ($g \cdot 100mL^{-1}$)	16.1 ± 1.1	16.2 ± 1.1
S_aO_2 (%)	71.9 ± 3.1	70.6 ± 4.0
C_aO_2 ($mL \cdot 100mL^{-1}$)	16.2 ± 1.3	16.0 ± 1.8
$\dot{Q}_m$ ($mL \cdot 100g^{-1} \cdot min^{-1}$)	13.4 ± 5.6	47.7 ± 16.7
$\dot{Q}_m \cdot C_aO_2$ ($mL \cdot 100g^{-1} \cdot min^{-1}$)	2.2 ± 1.0	7.7 ± 2.8
$m\dot{V}O_2$ ($mL \cdot 100g^{-1} \cdot min^{-1}$)	0.1 ± 0.1	5.9 ± 2.4
$m\dot{V}O_2$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	1.3 ± 0.9	58.9 ± 23.6
$C_{(a-v)}O_2$ ($mL \cdot 100ml^{-1}$)	1.1 ± 0.9	12.2 ± 1.7
O_2 extraction (%)	6.5 ± 5.1	76.6 ± 10.1

Table 2 pH_a, arterial pH; P_aO_2 , oxygen partial pressure in arterial blood; P_aCO_2 , carbon dioxide partial pressure in arterial blood; $[Hb]_a$, arterial haemoglobin concentration; S_aO_2, haemoglobin saturation in arterial blood; C_aO_2 , arterial oxygen concentration; Q_m, muscle blood flow; $Q_m \cdot C_aO_2$, muscle O_2 delivery; mVO_2, muscle O_2 consumption; $C(a-v)O_2$, artero-venous oxygen concentration difference; O_2 extraction, percentage of O_2 extraction. Values are means ± SD; n = 8.

NO_3^- and NO_2^- plasma concentrations after infusion interventions are shown in **Figure 1**. In NITRITE, sodium nitrite infusion increased significantly both NO_3^- and NO_2^- concentration in plasma. In SALINE, sodium chloride did not affect the plasma concentration of these metabolites.

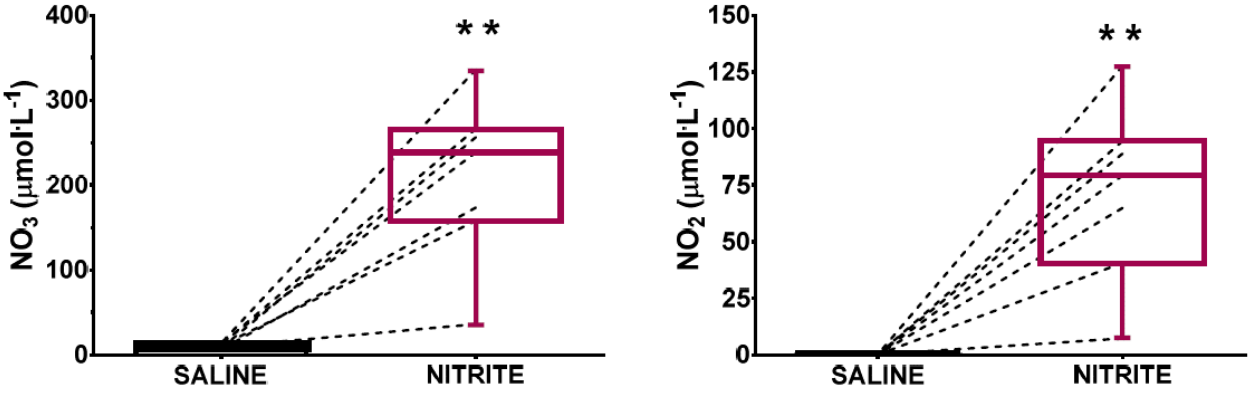

*Figure 1 Box and Whisker plots representing 10-90% percentile for nitrate (upper panel) and nitrite (lower panel). Each single observation in SALINE and NITRITE is connected by a dotted line. ** p<0.01*

Steady state values of the main variables pertinent to O_2 transport and utilization, acid-base status and hemodynamics for the two conditions are shown in **Table 3**.

Table 3. Values for the main variables pertinent to O_2 transport and utilization, acid-base status and haemodynamic during constant work rate exercise in SALINE and NITRITE.

	SALINE	NITRITE
pH_a	7.38 ± 0.04	7.37 ± 0.04
P_aO_2 (Torr)	43 ± 5	42 ± 3
P_aCO_2 (Torr)	29 ± 2	30 ± 3
$[Hb]_a$ (g · 100mL^{-1})	16.1 ± 1.3	16.2 ± 1.2
S_aO_2 (%)	69.0 ± 7.5	68.2 ± 5.5
C_aO_2 (mL·100mL^{-1})	15.6 ± 2.6	15.5 ± 2.2
$\dot{Q}_m$ (mL · 100g^{-1} · min^{-1})	52.9 ± 15.2	53.5 ± 13.2
$\dot{Q}_m \cdot C_aO_2$ (mL · 100g^{-1} · min^{-1})	8.2 ± 2.3	8.3 ± 2.2
$m\dot{V}O_2$ (mL · 100g^{-1} · min^{-1})	6.2 ± 1.8	6.1 ± 1.8
$m\dot{V}O_2$ (mL · kg^{-1} · min^{-1})	61.7 ± 18.3	60.6 ± 18.0
$C_{(a-v)}O_2$ (mL · 100ml^{-1})	11.6 ± 1.5	11.4 ± 1.9
O_2 extraction (%)	75.6 ± 11.2	74.1 ± 13.0

Table 3 pH_a, arterial pH; P_aO_2 , oxygen partial pressure in arterial blood; P_aCO_2 , carbon dioxide partial pressure in arterial blood; $[Hb]_a$, arterial haemoglobin concentration; S_aO_2, haemoglobin saturation in arterial blood; C_aO_2 , arterial oxygen concentration; Q_m, muscle blood flow; $Q_m \cdot C_aO_2$, muscle O_2 delivery; mVO_2, muscle O_2 consumption; $C(a-v)O_2$, artero-venous oxygen concentration difference; O_2 extraction, percentage of O_2 extraction. Values are means ± SD; n = 8.

Muscle blood flow was 52.9 ± 15.2 mL · 100g^{-1} · min^{-1} and 53.5 ± 13.2 mL · 100g^{-1} · min^{-1}, in SALINE and NITRITE respectively. The high blood flow ensured an identical O_2 delivery to GS muscles in the two conditions (52.9 ± 15.2 and 53.5 ± 13.2 mL · 100g^{-1} · min^{-1}, in SALINE and NITRITE respectively). $m\dot{V}O_2$ was not significantly different between SALINE and NITRITE (6.2 ± 1.8 vs 6.2 ± 1.8 mL · 100g^{-1} · min^{-1}, respectively). Accordingly, calculated mean capillary O_2 partial pressure did not change between SALINE and NITRITE (**Figure 2**).

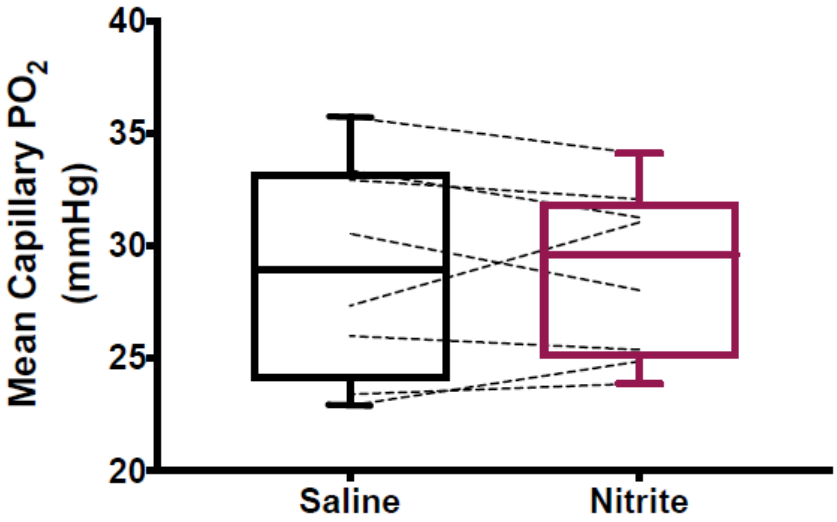

*Figure 2 Box and Whisker plots representing 10-90% percentile for mean capillary O_2 partial pressure calculated from Eq 1 (see methods for further details). Each single observation in SALINE and NITRITE is connected by a dotted line. * P<0.05*

Although muscle oxygen delivery was kept constant, microvascular blood volume (as detected by NIRS) was different between NITRATE and SALINE. Indeed, [oxy(Hb+Mb)] and [tot(Hb+Mb)] were significantly increased in NITRITE (vs. SALINE) whereas no differences were observed for [deoxy(Hb+Mb)] (**Figure 3**).

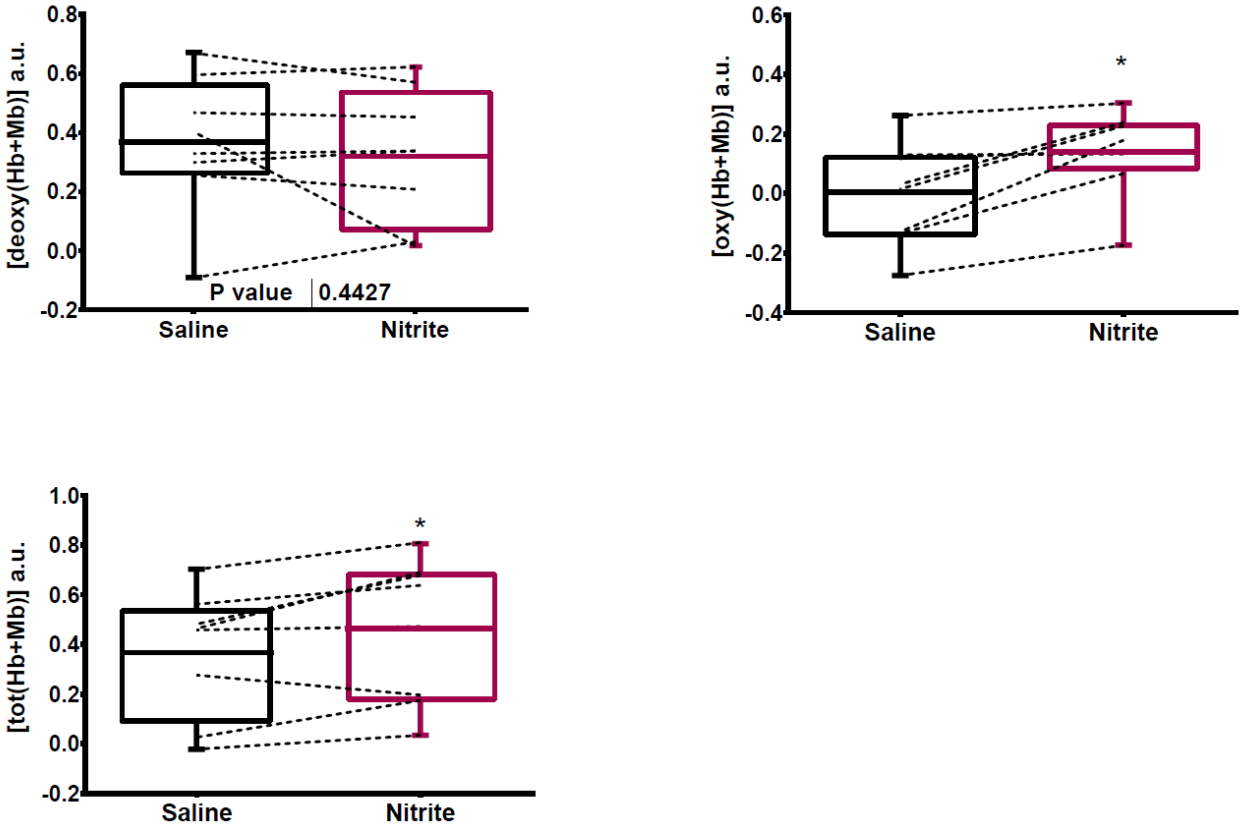

*Figure 3 Box and Whisker plots representing 10-90% percentile for [deoxy(Hb+Mb)], [oxy(Hb+Mb)] and [tot(Hb+Mb)] variables obtained by NIRS. Each single observation in SALINE and NITRITE is connected by a dotted line. * P<0.05*

Figure 4 shows force data recorded during SALINE and NITRITE. Force Time Integral (see methods for further details) was not significantly different in NITRITE (231.8 ± 36.8 N · min · g^{-1}) vs. SALINE (237.4 ± 40.6 N · min · g^{-1}), corresponding to a peak force of about 35 kp · 100g^{-1} (left panel). Fatigue index, calculated as the ratio between final peak force and initial peak force, was 97 ± 3 % (right panel), suggesting that the high blood flow utilized in the present study presumably minimized muscle fatigue in both conditions. $m\dot{V}O_2$ data, normalized for force production, were not significantly different between NITRITE and SALINE (0.18 ± 0.004 vs 0.17 ± 0.04 $mLO_2 \cdot min^{-1} \cdot kp^{-1}$, respectively).

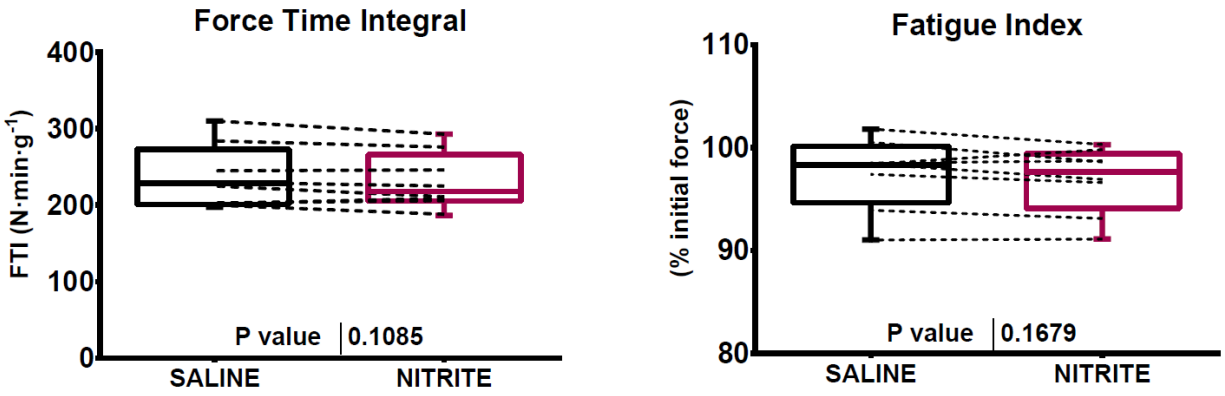

*Figure 4 Box and Whisker plots representing 10-90% percentile for peak force (left side) and Force Time Integral (right side). Each single observation in SALINE and NITRITE is connected by a dotted line. * P<0.05*

No differences were found for ADP-stimulated mitochondrial respiration (both for complex I and complex II), leak respiration and oxidative phosphorylation coupling, suggesting that mitochondrial efficiency was not influenced by nitrite infusion (**Figure 5**).

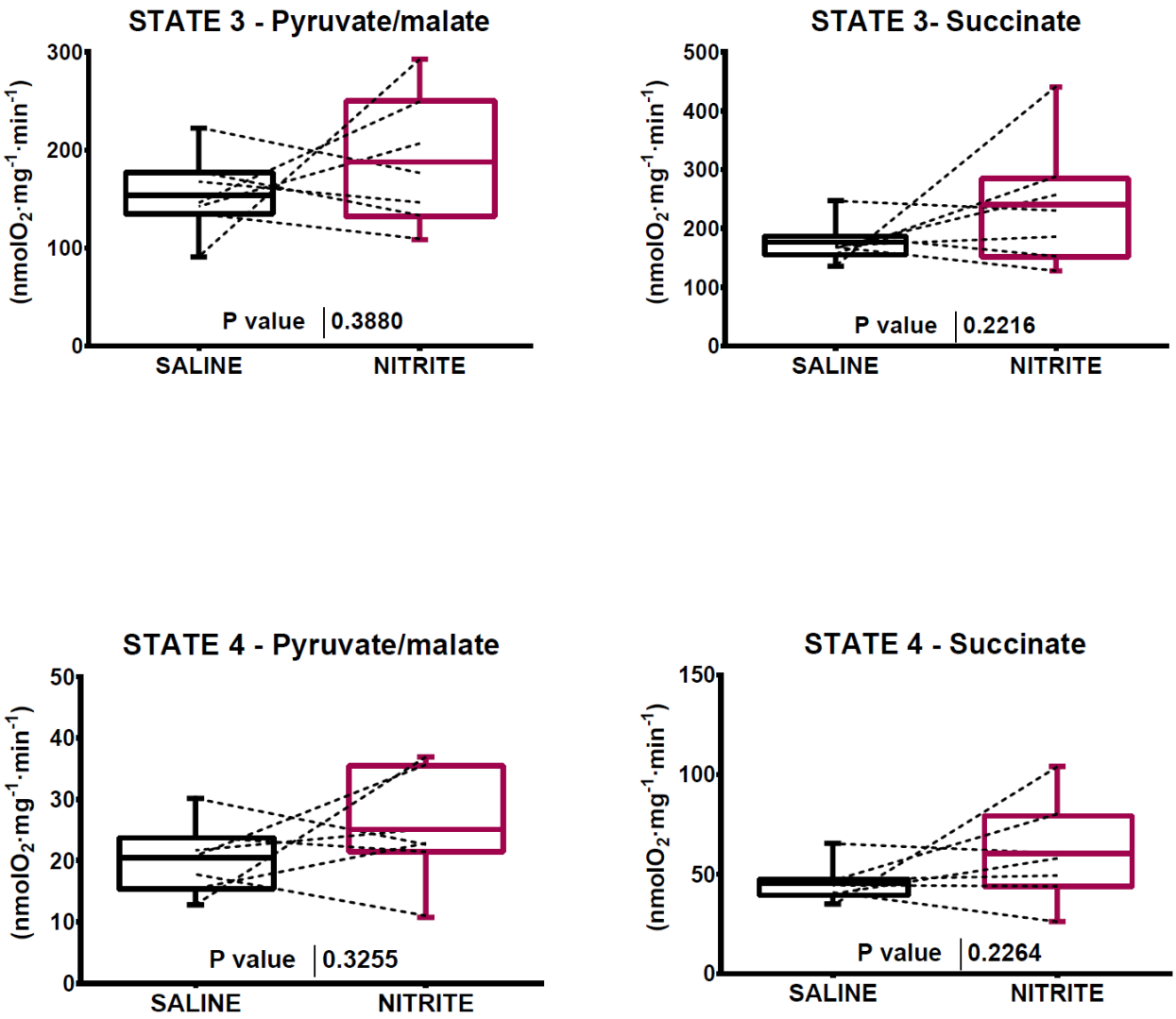

Figure 5 Box and Whisker plots representing 10-90% percentile for variables related to mitochondrial function. Each single observation in SALINE and NITRITE is connected by a dotted line. Data using succinate as the substrate are shown. Maximal ADP-stimulated mitochondrial respiration (state 3) and "leak respiration" (state 4) were not different in NITRITE vs. SALINE. RCR, which estimates the degree of coupling of oxidative phosphorylation, was similar in the two conditions.

Discussion

This study aimed to evaluate the effects of increased NO bioavailability on gastrocnemius canine muscle oxidative metabolism and force production during moderate intensity exercise in hypoxia. Keeping O_2 delivery high in order to avoid effects of elevated NO on this parameter, nitrite infusion did not affect muscle force neither muscle oxygen consumption. Moreover, we did not observe any differences in mitochondrial respiration between SALINE and NITRITE infusion. Thus, in hypoxic muscle increased NO bioavailability, in the presence of normal convective O_2 delivery, does not affect muscle function.

In recent years, evidence has accumulated that increased NO bioavailability enhances skeletal muscle function and improve exercise performance in humans (see Jones et al. 2014 for

review). For example, the O_2 cost of submaximal cycling has been reported to be lower by 3-5% following sodium nitrate supplementation (Larsen et al. 2007) as well as a 5% decrease in $\dot{V}O_2$ at low-intensity exercise and a 16% improvement in performance of fatiguing high-intensity exercise has been described after dietary NO_3^- supplementation (Bailey et al. 2009). It has also been demonstrated the ergogenic effects of NO_3^- are affected by several factors such as dose and duration of NO_3^- ingestion (Wylie et al. 2016), individual fitness level (Porcelli et al. 2015) and exercise intensity (Jones et al. 2016). However, the exact physiological mechanisms underlying the ergogenic effects of nitrate supplementation are only partially understood. Bailey et al. (2010) demonstrated a reduced PCr utilization during knee-extension exercise after NO_3^- supplementation, suggesting an increased ATP turnover rate as mechanistic basis of the ergogenic benefits. Similar findings were reported by Karolinska research group evaluating the effects of 3 days of $NaNO_3$ supplementation on whole body O_2 consumption and oxidative phosphorylation efficiency in healthy subjects. They observed a reduction of O_2 consumption during moderate intensity constant work rate exercise and an improvement in mitochondrial bioenergetics through modifications in coupling efficiency (Larsen et al. 2011). Other researchers have shown an increased production of muscle force after dietary NO_3^- assumption and an improved excitation-contraction coupling (Hernandez et al. 2012, Haider & Folland 2014). Finally, Ferguson et al. (2013, 2015) showed better muscle O_2 delivery after NO_3^- supplementation in rat skeletal muscle, particularly when type 2 fibers percentage was high. Thus, there is still debate about the mechanistic bases of the ergogenic effects of NO_3^- supplementation and further studies investigating these aspects are needed. In our study we manipulated NO bioavailability by NO_2^- infusion and we assessed in vivo contraction economy and force production directly in a contracting canine skeletal muscle. During experiments, dog inspired a reduced partial pressure of O_2 in order to increase the reduction of NO_2^- to NO and ensure elevated level of this gaseous molecule at tissue level. We used in vivo Gastrocnemius muscle model because it is a well-established method to study whole muscle function and exercise metabolism including the physiological effects of blood flow manipulation and metabolic stimulators/inhibitors (Grassi et al. 2005). Plasma $[NO_3^-]$ and $[NO_2^-]$ after NO_2^- infusion significantly increased compared with saline infusion and values were higher than those usually reached after dietary NO_3^-supplementation. This is probably due to the different type of administration, NO_2^- infusion ($\approx$0.013 mL $\cdot$ min^{-1} $NaNO_2$ per 1.0 mL $\cdot$ min^{-1} blood flow) instead of NO_3^- add in drinking water ($\approx$100 mg $\cdot$ L^{-1} $NaNO_3$) usually utilized in murine studies. The infusion modality used in the present study could affect the normal NO_3^- - NO_2^- - NO pathway, indeed the reduction of NO_3^- to NO_2^- in the oral cavity that normally occurred in

dietary NO_3^- supplementation has been avoided, leading to a different metabolization of NO_2^-. It is important also to note that the surgical preparation and the experimental session has brought to a partial hemolysis affecting plasma [NO_3^-] and [NO_2^-] levels.

Although muscle oxygen delivery was kept constant through the entire experiment, microvascular blood volume detected by NIRS was higher after NO_2^- infusion. In particular, [oxy(Hb+Mb)] and [tot(Hb+Mb)] were significantly increased, whereas no differences were observed in [deoxy(Hb+Mb)]. These data suggest a vasodilatory effect at microvascular level despite the constant blood flow imposed, and partially confirm previous data obtained on human subjects during exercise. Indeed, Breese and colleagues (2017) after NO_3^- supplementation found higher end-exercise deoxy [Hb + Mb] and total [Hb + Mb] levels, suggesting an increased capacity for muscle diffusive O_2 transport across active muscle sites and no differences in deoxy [Hb + Mb] responses. Although the NIRS utilized by Breese and colleagues was a time-resolved NIRS, it is important to note that the continuous-wave NIRS utilized in the present study was placed directly on muscle mass avoiding skin and subsequent superficial light scattering. These findings contribute to understand the mechanisms by which NO bioavailability can improve efficiency during submaximal exercise (Breese et al. 2017). Although a significant increase of plasma NO_3^- and NO_2^- concentrations were observed, NO_2^- infusion did not modify muscle force production neither muscle fatigue during moderate-intensity exercise Indeed, force time integral and fatigue index values were not significantly different from data recorded during control (SALINE) condition. These results do not confirm previous findings suggesting enhanced contractile properties of skeletal muscle following prolonged beetroot juice supplementation, particularly during low frequency of contraction stimulation (Hernandez et al. 2012). However, similar results have been reported after acute NO_3^- supplementation (Fulford et al. 2013). Thus, the short period of NO "exposure" may have not been sufficient to increase expression of calcium-handling proteins and thus may have not enhance excitation-contraction coupling.

As for muscle oxidative metabolism, we directly measured muscle oxygen consumption by Fick method analyzing arterial and venous blood across gastrocnemius muscle. We did not observe differences between NITRITE and SALINE conditions on muscle O_2 consumption even when we normalized data for force production, suggesting no effects of increased NO bioavailability on contraction economy. Some previous finding demonstrated a reduction of whole body $\dot{V}O_2$ after NO_3^- supplementation but other authors failed to find a positive effect of NO_3^- supplementation on O_2 cost of exercise (for a review see Hoon et al. 2013). The reasons of this discrepancy in the results may come from different aspects. First of all, we should consider the duration of the

supplementation. It has been demonstrated that longer durations of nitrate supplementation result in a significant $\dot{V}O_2$ decrease during moderate intensity exercise whereas shorter supplementations do not affect contraction economy (Pawlak-Chaouch et al. 2016). In our study we increased nitric oxide bioavailability by infusing directly NO_2^- in the venous blood. Thus, the brief period of exposure to high concentrations of NO may have not been sufficient to induce changes in contraction efficiency. These results are confirmed by the absence of effects of nitrite infusion on muscle mitochondrial activity observed in our experiments (fig. 5). It is important to notice these results are partially in accordance with data presented by Whitfield and colleagues (Whitfield et al. 2016) who demonstrated that beetroot juice supplementation does not alter mitochondrial bioenergetics. They measured mitochondrial respiration in permeabilized muscle fibres and showed no change in leak respiration, the content of proteins associated with uncoupling (UCP3, ANT1, ANT2), maximal substrate-supported respiration, or ADP sensitivity (apparent Km). In addition, P/O ratio, respiratory control ratios and membrane potential were unaltered, suggesting no effect of beetroot juice supplementation on mitochondrial efficiency.

Another possible explanation of the lack of effects of nitrite infusion on muscle function can be found in the fiber composition of the muscle activated. Gastrocnemius canine muscle is an aerobic muscle, with a very high percentage of type 1 fibers. Maximal O_2 consumption was 18.3 ± 4.4 mL · $100g^{-1}$ · min^{-1}, corresponding to more than 100 mL · kg^{-1} · min^{-1}. Nevertheless, it has been reported nitrate supplementation has preferential effects on type 2 muscle fibers in terms of blood flow distribution (Ferguson et al. 2013), reliance on fractional O_2 extraction (McDonough et al. 2005) and impact on skeletal muscle calcium-handling proteins (Hernandez et al. 2012). Thus, it is plausible to think the muscle characteristics of our canine muscle had a negative effect on the potential benefits coming from increased NO bioavailability. Finally, there is also some evidence that the effects of NO may be of a higher magnitude in fast type II fibers as opposed to slow type I fibers (Ferguson et al. 2014, Hernández et al. 2012), rendering the response variable with motor unit recruitment. In the gastrocnemius muscle model contractions are generated from electrical nerve stimulations and all motor units are recruited simultaneously. Thus, the stimulation frequency of contraction may have influenced our results.

Conclusions

Altogether, in the current study we provide evidence that in hypoxic conditions, but in the presence of constant and normal convective O_2 delivery, increased NO bioavailability obtained

by arterial nitrite infusion did not affect canine skeletal muscle oxidative metabolism and muscle force production. These results suggest that the effects of increased NO availability on muscle contraction efficiency in hypoxia, if present, are likely not attributable to changes in mitochondrial respiratory efficiency.

4.2 "BEet On Alps": ergogenic effects of dietary nitrate supplementation on cycling and arm-cranking during a prolonged exposure to high altitude

Abstract

Several studies have shown dietary nitrate supplementation increases nitric oxide bioavailability through nitrate-nitrite-nitric oxide pathway (NO_3^--NO_2^--NO). Since NO is involved in several physiological processes related to muscle function, NO_3^- supplementation has been utilized to improve sea level exercise performance, especially when type 2 muscle fibers are recruited. Ergogenic effects of NO_3^- supplementation have also been investigated in acute hypoxia due to enhanced reduction of NO_3^- to NO_2^- by reduced level of PO_2. No studies have been carried out in chronic hypoxia, although prolonged permanence at altitude changes NO bioavailability and impairs physical performance. This study aimed to investigate the ergogenic effects of dietary NO_3^- supplementation on cycling and arm-cranking during a prolonged exposure to high altitude. Fourteen subjects (28 ± 6 yrs) participated in a double-blind randomized crossover study carried out at Casati hut (3269 m a.s.l). Following 5 days of acclimatization, an incremental exercise test was carried out in order to assess $\dot{V}O_2peak$ and gas exchange threshold (GET) by cycle- and arm-ergometers. Then, each subject was supplemented for 3 days either with beetroot juice (2x70mL · day^{-1}, 8.4mmol NO_3^- · day^{-1} [BR]) and placebo [PLA]. At the end of each supplementation period, subjects exercised at two different intensities: an 8 min moderate-intensity (80% of GET) constant work rate (MOD) and a severe-intensity (50% of the difference between $\dot{V}O_2peak$ and GET) constant work rate exercise up to exhaustion (SEVERE) were performed. These exercises were conducted by both cycle- and arm-ergometers. Plasma [NO_3^-] and [NO_2^-] were measured by chemiluminescence at discrete intervals during acclimatization as well as before and after supplementation periods. Before supplementation period, plasma [NO_3^-] and [NO_2^-] increased from sea level values and they reached a peak value at day 3 (11.8±7.0 vs. 36.0±13.2 μM, $p<0.05$ and 325.0±69.8 vs. 744.7±324.6 nM, $p<0.05$). After BR, [NO_3^-] and [NO_2^-] were significantly higher compared with PLA (309.9±153.7 vs. 25.3±11.9.7 μM, $p<0.001$ and 966.5±386.4 vs. 490.0±150.3 nM, $p<0.01$). In MOD, $\dot{V}O_2$ was significantly reduced in BR vs. PLA in both cycling ($p<0.05$) and arm cranking ($p<0.05$). Interestingly, the oxygen cost of exercise was not affected by supplementation in the subjects with higher aerobic fitness only in cycling. In SEVERE, time to exhaustion in BR (vs. PLA), significantly improved during cycling and arm cranking. This study shows that exposure to hypoxia influence nitric oxide metabolites per se. Moreover, dietary nitrate supplementation reduces oxygen cost during moderate-intensity exercise and improves severe-intensity

exercise tolerance during prolonged exposure to hypobaric hypoxia, independently from the muscle mass involved (cycling and arm-cranking).

Keywords

Nitric oxide; Hypobaric hypoxia; Adaptation; Exercise tolerance; Oxidative metabolism

Introduction

Nitric oxide (NO) is a gaseous signaling molecule produced endogenously starting from the aminoacid L-arginine in an oxygen dependent pathway catalyzed by NOS enzymes (Moncada & Higgs 1993). Its effects are very rapid and, if not immediately involved in redox-related signaling events, it is oxidized to nitrite (NO_2^-) and nitrate (NO_3^-). Several years ago, these metabolites were considered inert compounds but recently it has been demonstrated that NO_3^- and NO_2^- have intrinsic effects and they can be reverted to NO. Indeed, NO_3^- can be converted in NO_2^- in the oral cavity thanks to anaerobic bacterial present on tongue surface and then NO_2^- are reduced to NO in the peripheral tissue. This alternative NO_3^- - NO_2^- - NO pathway has been demonstrated to be more effective in condition of low partial pressure of oxygen as well as low pH and it can be potentiated by the ingestion of food rich in NO_3^-, like beetroots or green leafy vegetables.

NO is involved in several physiological processes within our body (Culotta & Koshland 1992) and, from an exercise physiological prospective, it can alter muscle activity and functions by inducing vasodilation, adjusting muscular glucose uptake, regulating calcium handling at sarcoplasmic reticulum level, and affecting mitochondria efficiency (Stamler & Meissner 1992). Several studies investigated the ergogenic effects of dietary NO_3^- supplementation and increased NO bioavailability through this intervention and it has been demonstrated to be effective in improving exercise efficiency and exercise tolerance in normoxia (for a review see Jones et al. 2018). In the last years, some studies have also been conducted to investigate the effects of dietary NO_3^- supplementation on exercise capacity in hypoxia, since NO_3^- - NO_2^- - NO pathway is more effective in hypoxic condition (for a review see Shannon et al. 2017). These studies suggested that NO_3^- supplementation lessens muscle metabolic perturbation during high-intensity exercise and considerably improves exercise tolerance in moderate hypoxia by enhancing muscle oxygenation and partially restoring muscle oxidative function (Vanhatalo et al. 2011, Kelly et al. 2014).

Up to date, most of the studies have investigated the effects of acute nitrate supplementation on physiological responses to exercise in normobaric hypoxia. Carriker et al. (2016) were the only ones who investigated the ergogenic effects of nitrate supplementation at altitude. They

supplemented ten well-trained males ($\dot{V}O_2peak$ at 1600 m a.s.l. 61.0 ± 7.4 mL · Kg^{-1} · min^{-1}) with ~12.8 mmol NO_3^- rich beverage or placebo 2.5 hours prior four 5-min cycling bouts (40, 50, 60, 70% of $\dot{V}O_2peak$) at 3500 m and they did not observe any improvements (Carriker et al. 2016). No data have been reported after prolonged exposure to hypobaric hypoxia. Nevertheless, population living at high altitude from generations and well adapted to hypoxia (i.e. Tibetans) show 10-fold-higher plasma [NO_3^-] and [NO_2^-] than lowlanders and this difference in NO metabolism has been linked to their better forearm blood flow (Erzurum et al. 2007). Moreover, $\dot{V}O_2$ during steady-state submaximal exercise has been reported to be significantly lower in Tibetans than in Nepali lowlanders (Marconi et al. 2005) and this higher exercise efficiency has been hypothesized to be related to enhanced NO bioavailability (Martin et al. 2013).

Some evidences suggest that plasma level of NO metabolites is differently affected by hypobaric hypoxia vs normobaric hypoxia. After 24 h of exposure, NOx concentrations were decreased in hypobaric hypoxia compared with normoxic levels (-37% at 24h) whereas no differences were observed in normobaric hypoxia (Faiss et al. 2013). Moreover, it has been reported that altitude changes during a trekking expedition (from 1300 to 5050 m a.s.l.) modify levels of serum, urinary, and salivary [NO_3^-] and [NO_2^-] with peak values at about 3500 m a.s.l. (Janocha et al. 2011, Levett et al. 2011).

The primary aim of this study was to investigate the effects of dietary NO_3^- supplementation on physiological responses to exercise during a prolonged period at altitude. We hypothesized that NO_3^- supplementation positive influence exercise performance by reducing $\dot{V}O_2$ during moderate intensity exercise and by increasing time to exhaustion during severe intensity exercise. A secondary aim was to evaluate in lowlanders plasma NO_3^- and NO_2^- concentrations during a period of well controlled acclimatization.

Materials and methods

Subjects

Fifteen subjects were initially recruited for this study. One subject dropped out for personal reasons, and fourteen subjects completed the entire protocol (11 males, 3 females). The subjects' anthropometrical characteristics are shown in table 1. Each subject performed an incremental exercise until exhaustion on a cycle-ergometer at sea level (Fig. 1). $\dot{V}O_2peak$ resulted of 46.2 ± 8.7 mL · Kg^{-1} · min^{-1}.

Table 1. Subjects anthropometrical characteristics. (n=14)

Age (years)	Body Mass (Kg)	Height (m)	BMI ($Kg \cdot m^{-2}$)
28±6	70.8±11.8	1.76±0.09	22.7±2.4

Tabella 1 Mean (± SD) values of Age; Body Mass; Height; BMI, Body Mass Index.

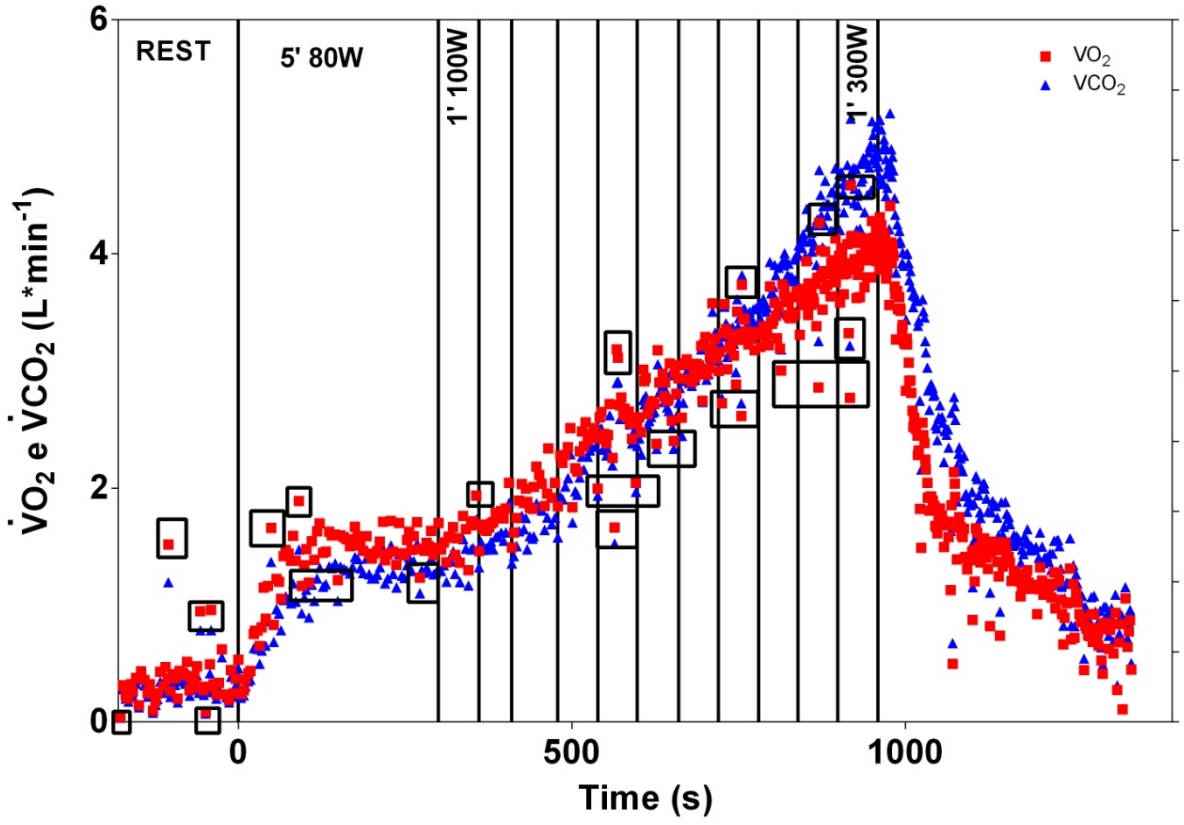

Figure 1 Typical example of breath by breath oxygen consumption ($\dot{V}O_2$, red squares) and carbon dioxide production ($\dot{V}CO_2$, blue triangles) during incremental exercise carried out until voluntary exhaustion. Exercise start at time 0 and after 5 min at 80 W (warm-up) power was increased by 20 W every minute. breaths inside black rectangles were identified as errant breaths (caused by coughing, swallowing, sighing, etc., and excluded from the analyses.

All participants were not assuming any drugs and were not expose to altitude (> 500 m a.s.l.) in the last 6 months before the experimental sessions. They had no signs or symptoms related to cardiovascular, pulmonary, or orthopedic diseases contraindicating the possibility to perform exercise and did not experience symptoms related to acute mountain sickness in their past altitude experiences. The subjects were fully informed of the procedures and possible risks associated with the experiments before giving their written consent to participate to the study. The protocol was approved by the local ethics committee (Verbale 27, Stralcio 8; 09/03/2016).

All procedures were in accordance with the Declaration of Helsinki (2000) of the World Medical Association.

Study design

The experiments were performed at Casati hut (3300 m a.s.l.) on the Alps. The study followed a randomized crossover double-blind placebo-controlled trial design. After an acclimatization period lasted 5 days, the subjects were divided into two groups and they were supplemented for 3 days with beetroot juice [BR – 140 mL containing 8.4 mmol of NO_3^- (70 mL in the morning + 70 mL in the afternoon) (Beet It, James White Drinks, Ipswich, UK)] or placebo [PLA - 140 mL with negligible content of NO_3^- (70 mL in the morning + 70 mL in the afternoon) (Beet It)]. After a wash out period lasting 4 days, the two groups were inverted, and the second 3-day period of supplementation was carried out (Fig. 2). Each subject was tested at the end of the two supplementation periods (PLA and BR).

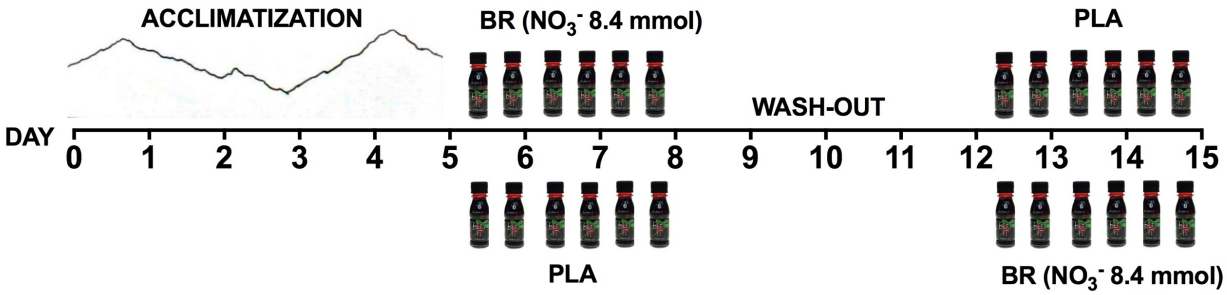

Figure 2 Experimental protocol.

Exercise testing

At altitude, after 5 days of acclimatization, subjects performed two incremental exercises until voluntary exhaustion: one on the arm-ergometer (Monark Ergomedic 891E, Vansbro, Sweden) and one on the cycle-ergometer (Corival; Lode BV). In the arm-cranking exercise, a warm-up period of 5 min at 30 W was followed by incremental steps of 15 W (male subjects)/10 W (female subjects) every minute. In the cycling exercise, the warm-up (5 min) started at 80 W (males)/ 60 W (females) and then the work-rate was increased by 10 W every minute. For both exercises, exhaustion was defined when at least three of the following criteria were reached: 1) inability to maintain the pedaling or cranking frequency (60 and 80 revolutions/min in arm-cranking and cycling exercise, respectively) despite vigorous verbal encouragement by the operators; 2) maximal levels (higher than 15) of self-perceived exertion, using the Borg's CR20 scale (Borg 1973); 3) heart rate (HR) values higher than 85% of the age-predicted maximum; 4) gas exchange ratio (R) value equal or above 1.1; and 5) Blood lactate concentration during recovery at the end of exercise higher than 8 mM. No validation test for the determination of

maximal oxygen consumption (Poole & Jones 2017) was carried out in the recovery following the incremental test. Data collected during these incremental tests were utilized to identify individual gas exchange threshold (GET) and peak oxygen consumption ($\dot{V}O_{2\text{peak}}$), and to set the intensity of the constant work-rate (CWR) exercises to be carried out at the end of each supplementation period. In both BR and PLA, 2.5 h after the last supplementation dose, each subject performed an 8-min moderate-intensity CWR exercise (approximately at 80% of GET) and a severe-intensity CWR exercise (at a work rate representing GET plus 50% of the difference between the work rate at GET and at $\dot{V}O_2$peak) until voluntary exhaustion. These exercises were separated by 10 min of passive recovery. Arm-cranking exercise were performed in the morning whereas cycling exercise were performed in the afternoon.

Measurements

Acute Mountain Sickness (AMS) score. Each participant also recorded medications taken and their symptoms according to the Lake Louise score as validated score for diagnosing and grading AMS.

Blood samples. Resting blood samples were collected to determine plasma levels of [NO_3^-] and [NO_2^-] at different time points during the acclimatization phase and at the end of each supplementation period, approximately 2.5 h after the ingestion of the last NO_3^- rich or NO_3^- depleted drink. Venous blood was drawn from the antecubital vein into a 5-ml EDTA vacutainer tube (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). The plasma was then separated, frozen at -80°C and analyzed within 6 months from initial collection. Measurements of [NO_3^-] and [NO_2^-] were made using chemiluminescence as previously described (Lanzarone et al. 2010).

Physiological response to exercise. Tidal volume, breathing frequency, pulmonary ventilation ($\dot{V}E$), $\dot{V}O_2$, and carbon dioxide output ($\dot{V}CO_2$) were measured breath-by-breath by a computerized metabolic cart (Vyntus CPX; CareFusion, Hoechberg, Germany). The gas exchange ratio (R) was calculated as $\dot{V}O_2/\dot{V}CO_2$. GET was determined by standard methods (Beaver et al. 1986), and peak values of the main cardiovascular, respiratory, and metabolic parameters were taken as the highest 20-s mean values reached during the incremental test. During CWR, breath-by-breath data were initially examined to exclude errant breaths caused by coughing, swallowing, sighing, etc., and those values lying more than 4 SDs from the local mean were removed. Data from the last 30 s of exercise were utilized to determine steady-state values of all variables. HR was determined continuously by a chest band (Polar Electro; Oulu, Finland). At 1, 3, and 5 min of recovery, 20 μL of capillary blood was obtained from a preheated

earlobe for the determination of blood lactate concentration ($[La^-]_b$) by an enzymatic method (Biosen C-line; EKF). Ratings of perceived exertion were obtained at the end of exercise using the Borg's CR20 scale (Borg 1973).

Statistical analysis

Data are expressed as mean values ± SD. Normal distribution of values was assessed with the Kolmogorov-Smirnov normality test. O_2 cost of exercise (the ratio between $\dot{V}O_2$ and mechanical power output) was considered the primary outcome of this study for power analysis. Considering the mean difference in this parameter previously observed in normoxia in subjects supplemented with BR, a sample size of twelve was requested to achieve a power of 80% with a significance level of 0.05. To check the statistical significance of differences between BR and PLA, paired Student's t-tests were performed. Values obtained during acclimatization period were analyzed using a one-way ANOVA for repeated measures and post hoc analysis was completed using Dunnett's multiple comparisons. Linear regression and correlation analyses were carried out by the least-squared residual method. The significant level was set at $P < 0.05$. Statistical analysis was performed using a software package (Prism 6.0, GraphPad Software, La Jolla, CA).

Results

Lake Louis Score

Lake Louise Score (LLS) were recorded at sea level in Segrate (115 m a.s.l.), and at different time points (day 1, 2, 3, and 4) at Casati hut (3300 m a.s.l.). Although, a significant increase in LLS in the first day at altitude no subject reached a score of 6 indicating severe ACM and needed to descend (Fig. 3).

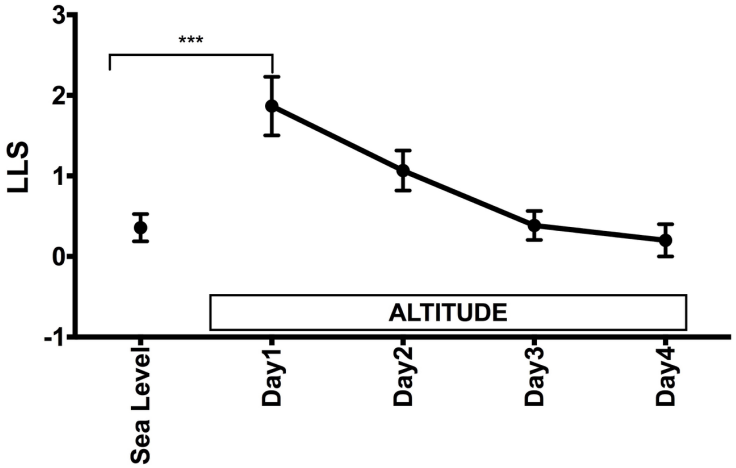

Figure 3 Lake Louise Score (LLS) for acute mountain sickness at sea level and at different time points at altitude (Day 1, 2, 3, 4). Asterisks indicate that P<0.001 for the comparison with baseline values by Dunnet's post hoc

Plasma nitrate and nitrite levels

During acclimatization period (day 1, 2, 3, and 4), plasma [NO_3^-] and [NO_2^-] increased significantly from sea level values and reached a peak value at day 3 (11.8±7.0 vs. 36.0±13.2 µM, $p<0.05$ and 325.0±69.8 vs. 744.7±324.6 nM, $p<0.05$). Then, plasma [NO_3^-] and [NO_2^-] were reduced, keeping significantly higher values than sea level. In order to take in account only the effects of the altitude exposure and not the possible effects of the supplementation, subjects supplemented by beetroot juice during the first period were excluded from this analysis and only the subjects supplemented with placebo were considered for day 7 and day 12. Thus, data at day 7 and day 12 correspond to n=7 subjects (Fig. 4).

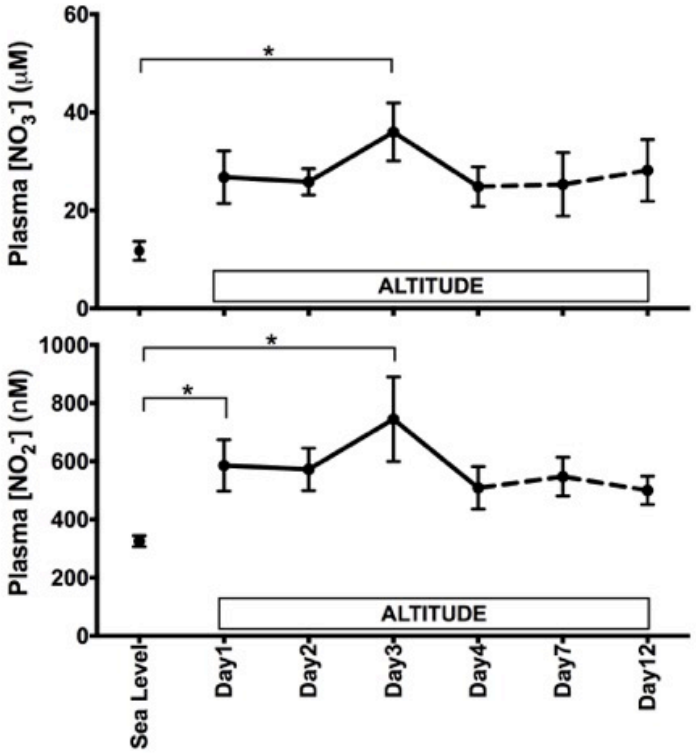

Figure 4 Mean values (± SEM) of plasma nitrate (NO_3^-, upper panel) and plasma nitrite (NO_2^-, lower panel) concentration at sea level and at different time points at altitude (Day 1, 2, 3, 4 ,7, 12). Dotted lines show the decrease in sample size (n=7, see text for further details). Asterisks indicate that P<0.05 for the comparison with baseline values by Dunnet's post hoc.

After BR, [NO_3^-] and [NO_2^-] were significantly higher compared with PLA (309.9±153.7 vs. 25.3±11.9.7 μM, p<0.001 and 966.5±386.4 vs. 668.3±150.3 nM, p<0.01) (Fig. 3). In BR condition, plasma [NO_3^-] and [NO_2^-] reached values previously observed by our group (Porcelli et al. 2015) and others (Jones et al. 2018) at sea level and they approached levels previously found in Tibetans. As a reference, values obtained by Erzurum et al. (2007) in Tibetan highlanders (TIBETANS) are also shown in the figure (Fig. 5).

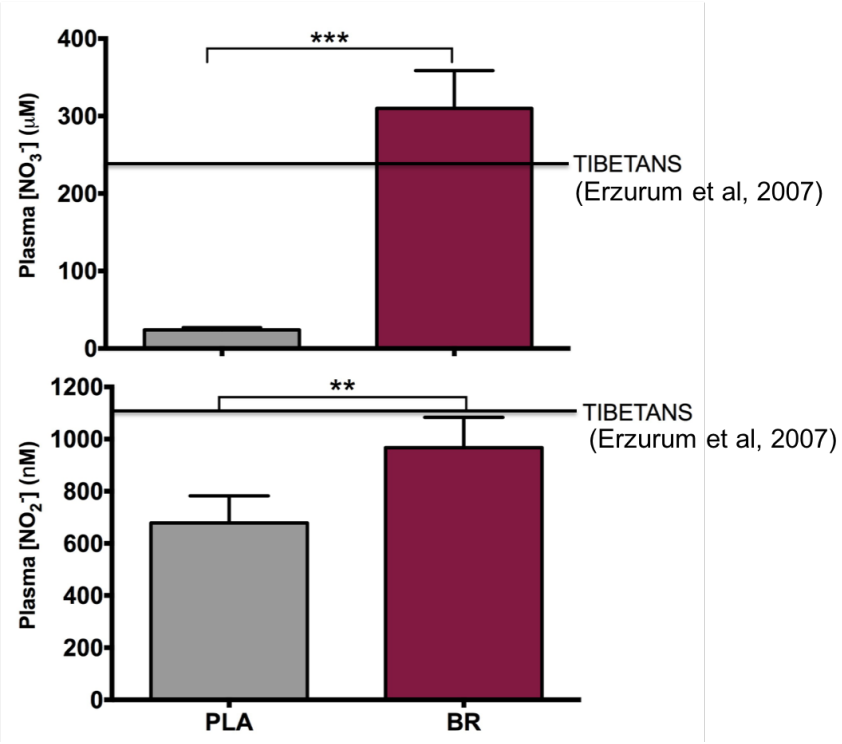

*Figure 5 Mean values (± SEM) of plasma nitrate (NO_3^-, upper panel) and plasma nitrite (NO_2^-, lower panel) concentration after placebo (PLA) and beetroot juice (BR) supplementation (light gray and purple columns, respectively). Dotted lines show individual changes. P value of paired Student's t-test is also shown. **P < 0.01 ***P < 0.001, significantly different from PLA.*

Incremental exercises

Cycle-ergometer

Peak values of the main respiratory, metabolic and cardiovascular variables obtained during cycle-ergometer incremental exercise are shown in Table 2. HR values reached ~95% of maximal predicted HR. GET occurred at a $\dot{V}O_2$ value of 2.177 ± 0.364 L · min^{-1} and a work-rate of 145±25 W, corresponding to 68 ± 4% of maximal work rate and 75 ± 4% of $\dot{V}O_{2peak}$.

Table 2. Peak and GET values of the main respiratory, metabolic and cardiovascular variables determined during cycle-ergometer incremental exercise.

$\dot{V}O_2$ (mL·min^{-1}·kg^{-1})	$\dot{V}O_2$ (L·min^{-1})	$\dot{V}CO_2$ (L·min^{-1})	$\dot{V}E$ (L·min^{-1})	R	Work rate (W)	HR (b·min^{-1})	$[La^-]_b$ (Mm)	RPE (6-20)
41.3 ± 6.7	2.889 ± 0.435	3.328 ± 0.481	155.5 ± 29.9	1.15 ± 0.08	214 ± 34	180 ± 12	9.71 ± 1,00	18 ± 1

Values are mean (± SD). $\dot{V}O_2$, oxygen uptake; R, gas exchange ratio; HR, heart rate; GET, gas exchange threshold;$[La^-]b$, blood lactate concentration; RPE,rating of perceived exertion..

Arm-ergometer

Peak values of the main respiratory, metabolic and cardiovascular variables obtained during cycle-ergometer incremental exercise are shown in Table 3. HR values reached ~90% of

maximal predicted HR. GET occurred at a $\dot{V}O_2$ value of 1.209 ± 0.266 L · min^{-1} and a work-rate of 80 ± 18 W, corresponding to 60 ± 6% of maximal work rate and 59 ± 7% of $\dot{V}O_{2peak}$.

Table 3. Peak and GET values of the main respiratory, metabolic and cardiovascular variables determined during arm-ergometer incremental exercise.

$\dot{V}O_2$ (mL·min^{-1}·kg^{-1})	$\dot{V}O_2$ (L·min^{-1})	$\dot{V}CO_2$ (L·min^{-1})	$\dot{V}E$ (L·min^{-1})	R	Work rate (W)	HR (b·min^{-1})	$[La^-]_b$ (Mm)	RPE (6-20)
29.6 ± 6.1	2.077 ± 0.427	2.472 ± 0.536	124.2 ± 28.2	1.19 ± 0.12	129 ± 26	169 ± 13	9.30 ± 1.77	17 ± 2

Values are mean (± SD). $\dot{V}O_2$, oxygen uptake; R, gas exchange ratio; HR, heart rate; GET, gas exchange threshold;[La$^-$]b, blood lactate concentration; RPE,rating of perceived exertion;.

Moderate intensity CWR exercises

Cycle-ergometer

All subjects completed the imposed 8 min of exercise. Work rate was set to be identical in each subject in the two conditions, and the value corresponded to ~50% of peak work rate. Figure 4 shows a typical example of the time courses of $\dot{V}O_2$ during the moderate-intensity CWR exercise in both PLA and BR (Fig. 6A). In the transition from rest to exercise, $\dot{V}O_2$ increased from resting values following a mono-exponential function and reached a steady state after about 3 min. A significant difference ($p < 0.05$) was found in $\dot{V}O_2$ at steady state between BR (1.811 ± 0.332 L · min^{-1}) and PLA (1.885 ± 0.304 L · min^{-1}), corresponding to a reduction of about ~4% (Fig. 6B). No differences were observed for other pulmonary and metabolic parameters.

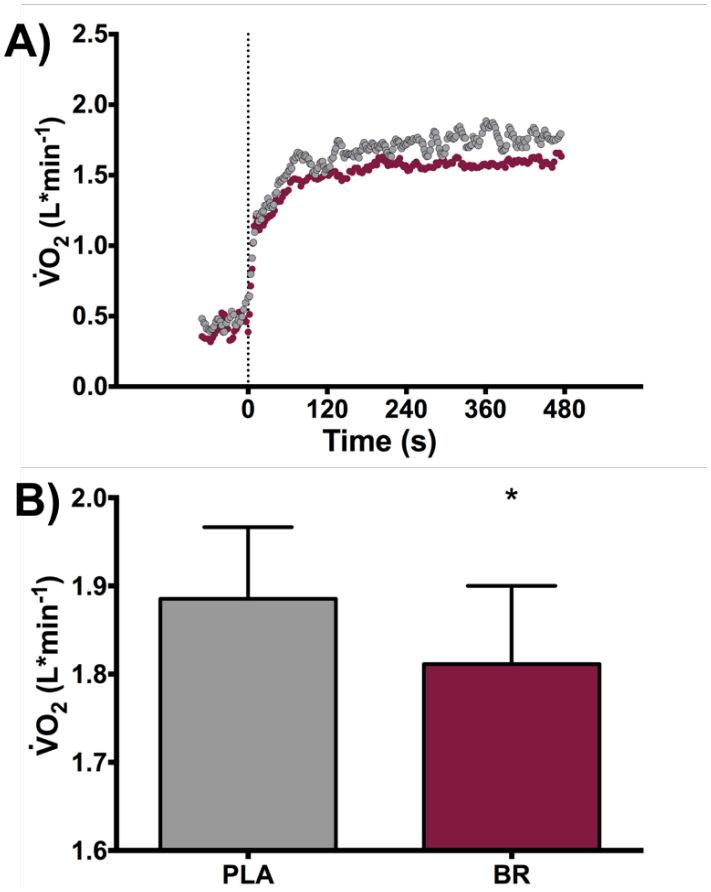

*Figure 6 - A) Typical example of oxygen consumption ($\dot{V}O_2$) during moderate-intensity exercise following placebo (PLA, grey circles) and beetroot juice (BR, purple circles) supplementation. Vertical line: exercise started at time 0. B) Mean values (± SD) of $\dot{V}O_2$ calculated in the last 30 s of exercise in both PLA (grey column) and BR (purple column). P value of paired Student's t-test is also shown. *P < 0.05, significantly different from PLA.*

HR, expressed as percentage of maximal HR reached during the incremental exercise, was 78 ± 11 % and 78 ± 9 % in PLA and BR, respectively (no significant difference).

A significant linear relationship was observed between steady-state $\dot{V}O_2$ reduction, expressed as percentage of PLA values, and subjects' aerobic fitness level represented by $\dot{V}O_2peak$ values collected at altitude. Lower-fit subjects showed a greater reduction of O_2 cost of exercise whereas higher fit subjects showed less or no effects on O_2 consumption following BR supplementation (Fig. 7).

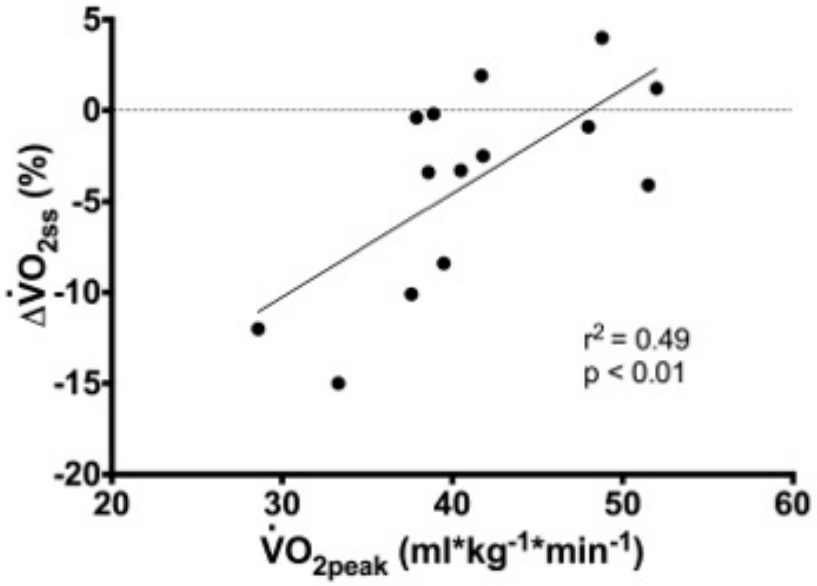

Figure 7 Percentage reduction of oxygen consumption during moderate constant load exercise after NITR vs individual peak oxygen consumption values.

Arm-ergometer

All subjects completed the imposed 8 min of exercise. Work rate was set to be identical in each subject in the two conditions, and the value corresponded to ~45% of peak work rate. In the transition from rest to exercise, $\dot{V}O_2$ increased from resting values following a mono-exponential function and reached a steady state after about 3 min. $\dot{V}O_2$ at steady state was significantly ($p< 0.05$) lower in BR (0.989 ± 0.224 L·min^{-1}) compared with PLA (1.023 ± 0.236 L·min^{-1}), corresponding to a reduction of about ~5% (Fig. 8). No differences were observed for other pulmonary and metabolic parameters. HR, expressed as percentage of maximal HR reached during the incremental exercise, was 72 ± 15 % and 69 ± 12 % in PLA and BR, respectively (no significant difference).

Severe intensity CWR exercises

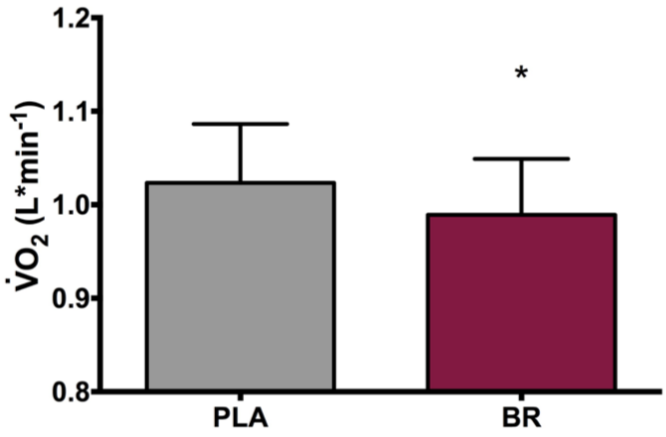

*Figure 8 - Mean values (± SD) of $\dot{V}O_2$ calculated in the last 30 s of exercise in both PLA (grey column) and BR (purple column). P value of paired Student's t-test is also shown. *P < 0.05, significantly different from PLA.*

No significant linear relationship was observed between steady-state $\dot{V}O_2$ reduction, expressed as percentage of PLA values, and subjects' aerobic fitness level represented as $\dot{V}O_2peak$ values collected at altitude (Fig. 9).

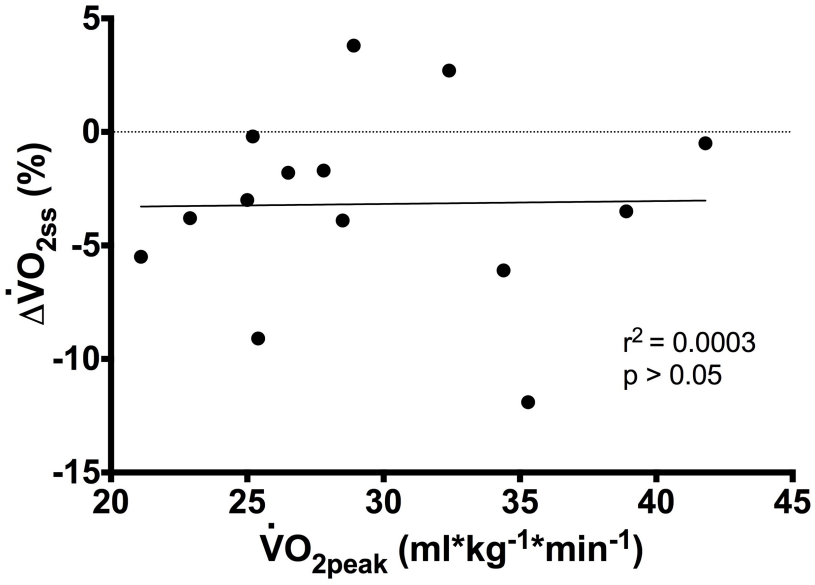

Figure 9 Percentage reduction of oxygen consumption during moderate constant load exercise after NITR vs individual peak oxygen consumption values.

Cycle-ergometer

Each subject cycled at the same work-rate in the two conditions, corresponding to 83 ± 16% of peak work rate. Figure 10 (A) shows a typical example of the time courses of $\dot{V}O_2$ during the severe-intensity CWR exercise in both PLA and BR. In the transition from rest to exercise, $\dot{V}O_2$ increased from resting values following a mono-exponential function and then increased linearly reaching ~95% of $\dot{V}O_2peak$ values.

Mean exercise time was 785 ± 180 s and 715 ± 235 s, in BR and PLA respectively. Thus, time to exhaustion was significantly increased (by 11%) by NO_3^- supplementation (Fig. 10B). At the end of exercise, no significant differences were found in $\dot{V}O_2$ between BR vs PLA (2.728 ± 0.449 L·min^{-1} vs 2.763 ± 0.467 L·min^{-1}, P = 0.379). However, lower $\dot{V}O_2$ values were observed after 6 minutes of exercise in BR vs PLA (2.588 ± 0.425 L · min^{-1} vs 2.686 ± 0.438 L · min^{-1}, P < 0.01). No differences were observed for other pulmonary and metabolic parameters. HR was 98 ± 7% of peak HR in PLA and 97 ± 7% of peak HR in BR.

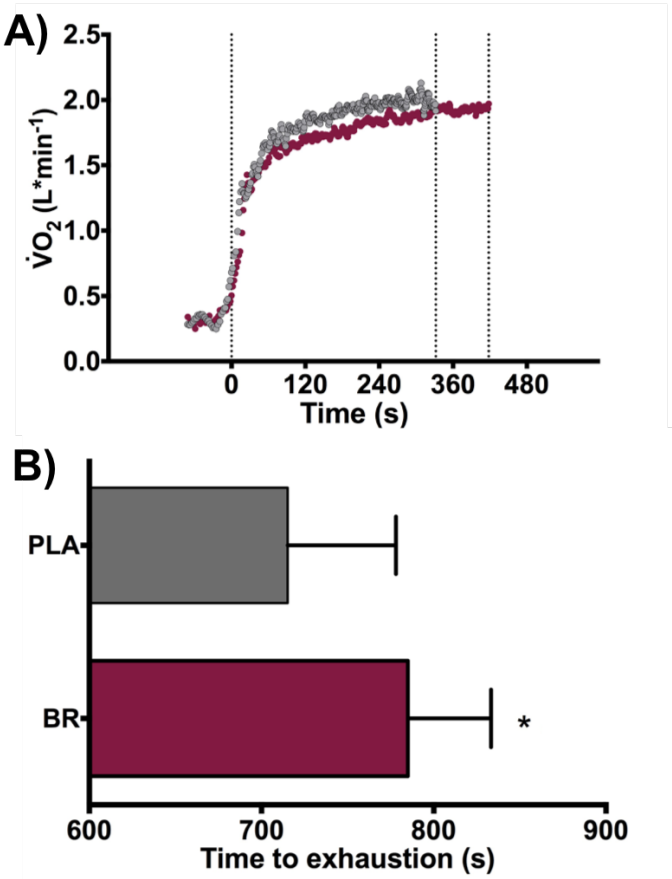

*Figure 10 - A) Typical example of oxygen consumption ($\dot{V}O_2$) during severe-intensity exercise following placebo (PLA, grey circles) and beetroot juice (BR, purple circles) supplementation. Vertical lines indicate start and end of exercises e. B) Mean values (± SD) of time to exhaustion in both PLA (grey column) and BR (purple column). P value of paired Student's t-test is also shown. *P < 0.05, significantly different from PLA.*

Arm-ergometer

Each subject performed the exercises at the same work-rate in the two conditions, corresponding to 75 ± 15% of peak work rate. Figure 11 (A) shows a typical example of the time courses of $\dot{V}O_2$ during the severe-intensity CWR exercise in both PLA and BR. In the transition from rest to exercise, $\dot{V}O_2$ increased from resting values following a mono-exponential function and then increased linearly reaching ~105% of. $\dot{V}O_2peak$ values.

Mean exercise time was 796 ± 152 s and 673 ± 243 s, in BR and PLA respectively. Thus, time to exhaustion was significantly increased (by 15%) by NO_3^- supplementation (Fig. 11 B). Moreover, lower $\dot{V}O_2$ values were observed after 6 minutes of exercise in BR vs PLA (1.905 ± 0.418 L·min^{-1} vs 1.989 ± 0.414 L·min^{-1}, P < 0.05). No differences were observed for other pulmonary and metabolic parameters. HR was 103 ± 6% of peak HR in PLA and 103 ± 6% of peak HR in BR.

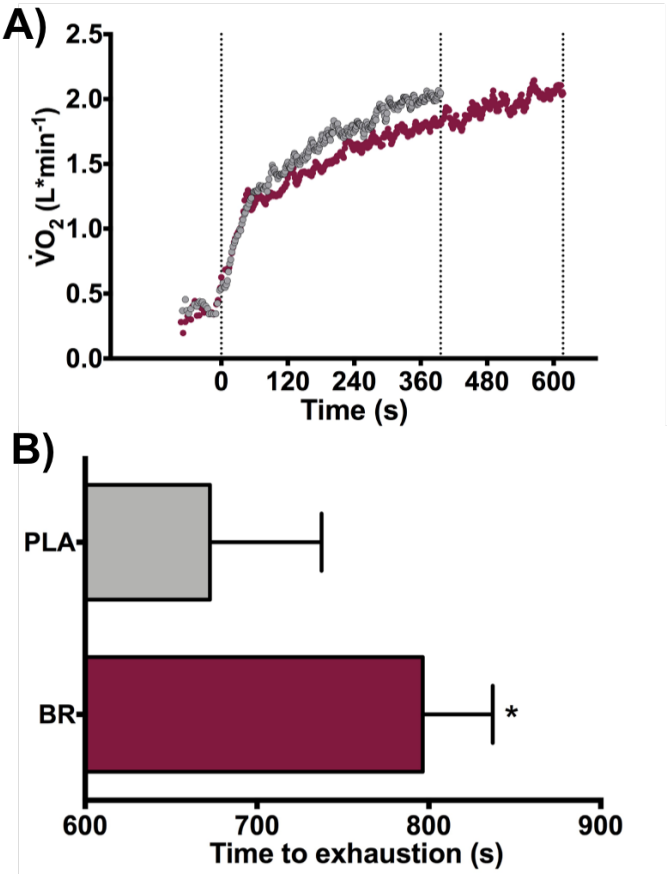

*Figure 11 - A) Typical example of oxygen consumption ($\dot{V}O_2$) during severe-intensity exercise following placebo (PLA, grey circles) and beetroot juice (BR, purple circles) supplementation. Vertical lines indicate start and end of exercises e. B) Mean values (± SD) of time to exhaustion in both PLA (grey column) and BR (purple column). P value of paired Student's t-test is also shown. *P < 0.05, significantly different from PLA.*

Discussion

This study demonstrates NO_3^- supplementation has ergogenic effects in cycling and arm-cranking exercises following several days of exposure to hypobaric hypoxia. Indeed, after several days of permanence at 3300 m a.s.l., three days of supplementation with beetroot juice (8.4 mmol of $NO_3^- \cdot day^{-1}$) were able to decrease O_2 cost of exercise during moderate and severe intensity exercises and increase time to exhaustion during severe intensity exercises in activities involving different muscle mass (cycling and arm-cranking). Thus, this study confirms previous results obtained after acute NO_3^- supplementation in normobaric hypoxia and suggests NO_3^- supplementation as a possible effective intervention to counteract exercise intolerance at altitude.

Hypobaric hypoxia, the decrease in partial pressure of O_2 due to increment in terrestrial altitude, is associated to an impairment of oxidative metabolism according to a reduction of the partial pressure of O_2 along all the so called O_2 cascade in our body, starting from the lungs to the peripheral tissue. The impairment in oxidative metabolism induces a decrease in maximal

O_2 consumption and, consequently, higher muscle metabolic perturbations for a given work-rate (Cerretelli et al. 2009). Similarly, altitude affects also exercise performance as shown by shorter time to exhaustion and worst time-trial performance (Cerretelli et al. 2009). Several studies have demonstrated that dietary NO_3^- supplementation can positively affect exercise performance in normoxia, by lowering O_2 cost of exercise including an improved mitochondrial efficiency (Larsen et al. 2011) and/or reductions in the ATP cost of muscle contraction (Bailey et al. 2010), which may be linked to enhanced Ca^{2+}-related muscle contractility (Hernández et al. 2012). Similarly, enhanced NO bioavailability has been demonstrated to be effective in lessening muscle metabolic perturbation during knee-extension exercise as well as improving time to exhaustion in acute normobaric hypoxia (Vanhatalo et al. 2011). In literature no data are available about the effects of NO_3^- supplementation on physiological responses to exercise during prolonged exposure to hypobaric hypoxia. In the present study dietary NO_3^- supplementation was effective in reducing O_2 consumption during moderate intensity exercises during cycling. The reduction in O_2 cost of exercise was ~4%, in accordance with data reported by previous studies in moderately trained subjects in normoxia (for a review see Jones et al. 2018). Moreover, this improvement in exercise efficiency is consistent with data obtained in nomobaric hypoxia (for a review see Shannon et al. 2017). Kelly et al. (2014) reported a 7% reduction in steady-state $\dot{V}O_2$ in hypobaric hypoxia (corresponding to 3800 m altitude) during cycling at 80% of GET. Masschelein et al. (2012) reported a 4% reduction in steady-state $\dot{V}O_2$ in simulated altitude (corresponding to 5000 m altitude) during cycling at 45% of $\dot{V}O_2peak$. Muggeridge et al. (2014) reported a ~6–8% reduction in steady-state $\dot{V}O_2$ in hypobaric hypoxia (corresponding to 2800 m altitude) during cycling at 60% of maximum work rate following BR supplementation. Interestingly, the effects of dietary NO_3^- supplementation on O_2 cost of exercise were not similar in all the subjects and a positive correlation between aerobic fitness level and percentage reduction in steady-state $\dot{V}O_2$ was found in the present study: beetroot juice reduced at a greater extent steady state $\dot{V}O_2$ in less fitness subjects than in highly fit subjects and it had no effects in the three subjects with the highest fitness level. These results confirm previous findings obtained by our group in normoxia (Porcelli et al. 2015) but are in contrast with results reported by Shannon et al. (2016). They tested twelve healthy males with different aerobic fitness level ($\dot{V}O_2peak$ ranging from 47.1 - 76.8 mL · Kg^{-1} · min^{-1}) on treadmill running performance in moderate normobaric hypoxia (equivalent to 2500 m altitude). After a high nitrate dose (~ 15.2 mmol NO_3^-) in the form of beetroot juice, all subjects improved running performance without no apparent relationship to individual aerobic fitness. The

differences may be due to the dose of NO_3^- administered as well as to the different duration to hypoxic exposure. However, further research is required to address the physiological bases for responders and non-responders to dietary NO_3^- supplementation in both normoxic and hypoxic conditions.

In this study, dietary nitrate supplementation was also effective in improving exercise efficiency during moderate-intensity arm-cranking. The reduction in O_2 consumption was similar (~5%) to that observed for cycling. Although no data have been reported about the effects dietary NO_3^- supplementation on arm exercises in hypoxia yet, the decrease in O_2 cost of exercise is comparable to that observed (~3%) in kayaking during exercise in the same intensity domain (Muggeridge et al. 2013). Thus, during chronic exposure to hypoxia dietary NO_3^- supplementation can be a valid intervention to improve exercise efficiency in moderate-intensity exercise performed with both upper and lower limbs.

In the last years, several studies have been conducted to understand if dietary NO_3^- supplementation has ergogenic effects during severe intensity domain exercise. It has been demonstrated a significant ~ 20 % reduction in the amplitude of the $\dot{V}O_2$ slow component, which is considered to reflect a progressive loss of muscle efficiency as high-intensity exercise proceeds (Jones 2014), and a significant increase in time to exhaustion in open-ended test (Campos et al. 2018). In some cases, the same amount of dietary NO_3^- supplementation positively affected exercise tolerance to high-intensity exercise without modifying physiological responses to moderate intensity exercise. For example, Breese and colleagues showed an improvement in the tolerable duration of severe intensity cycle exercise initiated from a moderate-intensity baseline work rate (by 22% on average) was not associated with a reduced steady-state $\dot{V}O_2$ during unloading to moderate intensity exercise (Breese et al. 2013). The reasons of the different effects in relation to the intensity domain are not clear but it has been hypothesized that during these types of exercise, the conditions of low pH and PO_2 can guarantee a higher nitric oxide bioavailability from nitrate and nitrite. Moreover, several evidences suggest that beetroot juice supplementation is more effective in type 2 compared with type 1 fibers (Jones et al. 2016) in term of blood flow elevation (Ferguson et al. 2013). In our study, the positive effects observed in the moderate-intensity domain were associated to an improved exercise efficiency after beetroot juice supplementation during severe-intensity exercise in both cycling and arm-cranking. Indeed, after 6 min of exercise $\dot{V}O_2$ was significantly lower in BR, compared with PLA. These results are in accordance with data reported in literature in healthy subjects in both normoxia and normobaric hypoxia (Bailey et al. 2009, Kelly et al. 2014). Moreover, time to exhaustion was significantly increased after cycling

(+11%) and, to a greater extent, after arm-cranking (+15%), although no differences were observed in $\dot{V}O_2$ at the end of exercise. These ergogenic effects of dietary NO_3^- supplementation are comparable to previous findings reported by Peeling et al. (2015) in 500 m on-water kayak time-trial and by Hoon et al. (2014) 2000-m rowing performance. However, this is the first time the effects of dietary NO_3^- supplementation are investigated within the same subject in different exercise modalities. Even if we do not have any mechanistic evidences to explain the greater improvement in arm vs leg exercise, our hypothesis is that during severe intensity the greater ergogenic effects in arm-cranking compared with cycling could be related to different characteristics in term of muscle fibers composition and recruitment pattern between these two muscle groups. Direct comparisons of arm and leg muscles indicate that arm muscles are less oxidative and less capable of extracting oxygen from the circulation, with a greater percentage of type 2 fibers and a greater variability in blood flow during exercise (Van Hall et al. 2003, Calbet et al. 2005). Thus, the same relative intensity in exercise performed with upper limbs may cause greater muscle perturbation than with lower limbs. Since dietary NO_3^- supplementation causes reduction in muscle metabolic perturbation [i.e., slower rates of change of muscle pH and phosphocreatine and inorganic phosphate concentrations] during severe-intensity in hypoxia (Vanhatalo et al. 2011), it is plausible that during arm-ergometer exercise the enhanced intramuscular NO bioavailability better improved matching between O_2 delivery and O_2 uptake, reducing metabolic perturbation, delaying fatigue, and consequently enhancing exercise tolerance (Grassi et al. 2011). Finally, we should also take into consideration that greater recruitment of type 2 fibers in arm-cranking vs cycling may cause lower pH value and guarantee higher NO bioavailability (Jones et al. 2016).

The secondary aim of our study was to evaluate the effects of altitude on plasma NO_3^- and NO_2^- concentrations. Exposure to altitude elicits several physiological responses aimed to counteract the decrease in partial pressure of O_2. The acclimatization mechanisms involve acute and chronic adaptations, depending on the time of exposure to hypoxia, and are prerogative of different organs, although all changes try to increase O_2 delivery to peripheral tissues. Among the others (e.g., erythropoiesis and hypoxic ventilatory response), NO bioavailability has been demonstrated to be a fundamental component in altitude acclimatization. Indeed, NO plays a crucial role in achieving cardiocirculatory homeostasis during acute hypoxia through both vasodilation and metabolic suppression (Umbrello et al. 2013) and defect in NO synthesis may contribute to high-altitude pulmonary edema, clear sign of high-altitude maladaptation (Scherrer et al. 1996). Similarly, the inhalation of NO has been demonstrated effective in improving arterial oxygenation in high-altitude pulmonary edema (Scherrer et al. 1996). Up to

now, only one study evaluated the effects of NO_3^- supplementation on AMS. Cumpstey and colleagues (2017) demonstrated that 3 days of 0.10–0.18 mmol/kg/day of NO_3^- rich juice is well tolerated at altitude and significantly increases pulmonary NO availability and both salivary and exhaled breath condensate NO metabolite concentrations. Surprisingly, this was not associated with changes in hemodynamics, oxygen saturation or AMS development.

It is important also to consider that plasma NO metabolites changes in altitude have been implicated in hypoxia induced vasodilation, a physiological response to elicit appropriate matching between O_2 delivery and O_2 uptake of muscle and cerebral tissue when O_2 availability is low (Umbrello et al. 2013). In the present study plasma NO_3 and NO_2 levels, which represent valid indexes of NO bioavailability, were significantly higher than sea level values during all the permanence at altitude, reaching a peak value the third day of exposure to hypobaric hypoxia. These data are similar to previous results obtained in Caucasian in terms of magnitude of increase and time-course kinetics (Janocha et al. 2011, Levett et al. 2011). Indeed, Janocha et al. (2011) showed from 1300 to 5050 m a.s.l., increased levels of serum, urinary, and salivary [NO_3^-] and [NO_2^-] with a peak the fifth day at 3440 m a.s.l. and Levett et al. (2011) demonstrated an increment in plasma [NO_3^-] and [NO_2^-] at 3500 m a.s.l. both studies in investigated Caucasian people during trekking. However, our plasma NO_3^- and NO_2^- concentrations were evaluated at a fixed altitude and no confounding factors such exercising for several hours in a day, altitude changes, etc. were present. Thus, altitude influences per se NO bioavailability and should be considered an important mechanism activated by our body to cope with the reduced O_2 availability at peripheral tissues. Unfortunately, at the moment we have not analyzed data about SaO_2 and arterial pressure yet and it is not possible to rule out the importance of NO metabolites' changes in terms of physiological adaptations to altitude. It is to note that the amount of increase plasma NO_3^- and NO_2^- concentrations is far from plasma NO metabolites levels found in Tibetans highlander, although at altitude were significantly higher than at sea level. Indeed, Tibetans chronic exposed to hypobaric hypoxia shows NO_3^- and NO_2^- levels >10-fold-higher than lowlanders (Erzurum et al. 2007). Thus, several days of exposure to hypobaric hypoxia are not sufficient to activate mechanisms that, at least in part, explain the peculiar adaptations to hypoxia observed in high altitude populations. Interestingly, after nitrate supplementation our Caucasian subjects showed level of plasma [NO_3^-] and [NO_2^-] not only similar to those obtained in previous study (Kelly et al. 2014) but also close to those observed in Tibetan highlanders (Fig. 5). As previously discussed, these changes in NO_3^- and NO_2^- were associated to an increase of exercise efficiency in different intensity domains in both arm-cranking and cycling. At the same time, it is well known Tibetans have an extraordinary exercise

economy during walking and running at different slopes ($\dot{V}O_2$ < 10%; Marconi et al. 2005). Thus, increased NO bioavailability can be considered a possible reason of the difference in exercise economy between Caucasian and Tibetan, although studies addressing this specific issue should be performed.

Conclusion

In conclusion, dietary NO_3^- supplementation affects physiological responses to moderate and severe intensity exercises at altitude by reducing O_2 cost of exercise in both leg and arm exercises. Interestingly, only during cycling exercise this positive effect was related to aerobic fitness level. Additionally, after several days of exposure to hypobaric hypoxia dietary NO_3^- supplementation increased time to exhaustion in both cycle- and arm-ergometer exercises, suggesting that this intervention can be considered a valid aid to counteract the negative effect hypoxia has on exercise tolerance.

4.3 Ergogenic effects of beetroot juice supplementation during severe-intensity exercise in obese adolescents

This article has been published in the 'AMERICAN JOURNAL of PHYSIOLOGY – Regulatory, Integrative and Comparative Physiology' 315: R453–R460 (2018) as 'Ergogenic effects of beetroot juice supplementation during severe-intensity exercise in obese adolescents' by Rasica L, Porcelli S, Marzorati M, Salvadego D, Vezzoli A, Agosti F, De Col A, Tringali G, Jones AM, Sartorio A, Grassi B.

Abstract

Previous studies showed a higher O_2 cost of exercise, and therefore a reduced exercise tolerance, in obese patients during constant work rate (CWR) exercise compared to healthy subjects. Among the ergogenic effects of dietary nitrate (NO_3^-) supplementation in sedentary healthy subjects, a reduced O_2 cost and enhanced exercise tolerance have often been demonstrated. The aim of this study was to evaluate the effects of beetroot juice supplementation, rich in NO_3^-, on physiological variables associated with exercise tolerance in obese adolescents. In a double-blind, randomized, crossover study, ten obese adolescents (8F, 2M; age=16±1 yr; BMI=35.2±5.0 kg · m^{-2}) were tested after 6 days of supplementation with beetroot juice (5 mmol NO_3^- per day) (BR) or placebo (PLA). Following each supplementation period, patients carried out two repetitions of 6-min moderate-intensity CWR exercise and one severe-intensity CWR exercise until exhaustion. Plasma NO_3^- concentration was significantly higher in BR vs. PLA (108±37 vs. 15±5 μM, P<0.0001). The O_2 cost of moderate-intensity exercise was not different in BR vs. PLA (13.3±1.7 vs. 12.9±1.1 mL · min^{-1} · W^{-1}, P=0.517). During severe-intensity exercise, signs of a reduced amplitude of the O2 uptake slow component were observed in BR, in association with a significantly longer time to exhaustion (561±198 s in BR vs. 457±101 s in PLA, P=0.0143). In obese adolescents, short-term dietary NO_3^- supplementation is effective in improving exercise tolerance during severe-intensity exercise. This may prove to be useful in contrasting early fatigue and reduced physical activity in this at-risk population.

Keywords

Nitric oxide; Oxidative metabolism; Endurance exercise; Obesity; O_2 uptake kinetics.

Introduction

Obesity is one of the main public health challenges in industrialized countries and the number of people affected by this condition is growing from year to year. Taking into consideration children and adolescents (2-19 years), the percentage of obese in United States is an alarming 46.9%. Obesity during childhood is associated with an excess in body mass in adulthood and among adults, obesity is a significant risk factor for several chronic diseases and, conditions, including cardiovascular diseases, diabetes and cancer (Health 2015).

Obese patients show a significant exercise intolerance and an impairment in physical activities of daily living, due to a generalized sense of fatigue, which negatively affects their quality of life and may lead to severe deconditioning. The reduced exercise tolerance is associated with a higher O_2 cost for submaximal exercise (Wasserman & Whipp 1975, Salvadori et al. 1999, Salvadego et al. 2010) a lower gas exchange threshold, slower adjustment of the fundamental component of $\dot{V}O_2$ kinetics (Salvadego et al. 2010), and a more pronounced amplitude of the slow component of $\dot{V}O_2$ kinetics during heavy intensity exercise (Salvadego et al. 2010). These impairments are attributable to cardiovascular (Salvadori et al. 1999), respiratory (Salvadego et al. 2015, Salvadego et al. 2017) and skeletal muscle factors (Salvadego et al. 2010, Lazzer et al. 2013). In a vicious circle, the impaired exercise tolerance and the associated reduced level of physical activity worsen obesity and interfere with interventions (such as exercise prescription) aimed at its control.

Obese patients show an impaired endogenous nitric oxide (NO) synthesis, in particular in the presence of the metabolic syndrome (Siervo et al. 2011). The mechanisms seem to be related to an increased generation of reactive oxygen species, an impaired function of NO synthase (NOS) and to a decreased insulin signaling (Siervo et al. 2011). More specifically, the inhibition NOS enzymes activity in obesity is prevalent in the endothelium (Kraus et al. 2012) but it can be also found in skeletal muscle (Tengan et al. 2012), suggesting a possible shortage of NO bioavailability at the skeletal muscle level.

NO is produced endogenously from the oxidation of L-arginine in a reaction catalyzed by a family of NOS enzymes (L-arginine - NOS - NO pathway) (Kobayashi et al. 2015). An alternative pathway of NO synthesis is however present. The ingestion of foods rich in nitrate (NO_3^-), like green leafy vegetables and beetroot, can increase NO bioavailability through the conversion of NO_3^- to nitrite (NO_2^-) in the entero-salivary circulation, and the subsequent conversion of NO_2^- to NO by peripheral enzymes or proteins (such as deoxygenated hemoglobin and myoglobin) after NO_2^- has entered the systemic circulation (Kobayashi et al. 2015).

Recent evidence suggests that an enhanced NO bioavailability can positively affect exercise tolerance through a variety of mechanisms (Jones 2014). For example, an increased NO bioavailability may improve the efficiency of mitochondrial respiration, as evaluated by the P/O ratio (Larsen et al. 2011); ameliorate muscle metabolic perturbations found during exercise, which may decrease the ATP cost of force production (Bailey et al. 2010); and increase skeletal muscle microvascular blood flow, at least towards type 2 fibers, thereby improving the intramuscular matching between O_2 delivery and O_2 utilization and increasing microvascular PO_2 and peripheral O_2 diffusion (Ferguson et al. 2013). Thus, an increased oxidative efficiency (i.e. a lower O_2 requirement leading to a lower pulmonary O_2 uptake [$\dot{V}O_2$] for the same work rate) could be of utmost interest in obese patients, who typically show an increased O_2 cost of exercise ($\Delta\ \dot{V}O_2/\Delta$ work rate) (Wasserman & Whipp 1975, Salvadori et al. 1999), which is partly attributable to an increased O_2 cost of breathing (Salvadego et al. 2015, Salvadego et al. 2017).

The aim of the present study was to evaluate the effects of a short-term (6 days) dietary NO_3^- supplementation on physiological parameters associated with exercise tolerance in a group of obese adolescents. We hypothesized that in this population NO_3^- supplementation would lead to an enhanced exercise tolerance by reducing the O_2 cost of exercise in different intensity domains and by increasing the time to exhaustion during severe-intensity constant work rate (CWR) exercise. These results would extend to the obese population the ergogenic effects of dietary NO_3^- supplementations previously demonstrated in healthy subjects (Bailey et al. 2009, Porcelli et al. 2015) and in patients with peripheral arterial disease (Kenjale et al. 2011), chronic obstructive pulmonary disease (Curtis et al. 2015) and chronic heart failure (Zamani et al. 2015, Zamani et al. 2017).

Materials and methods

Subjects

Ten obese adolescents, 2 males and 8 females (age, 16±1 years [mean ± SD]; body mass, 95.5±16.8 kg; height, 1.64±0.08 m), BMI 35.2±5.0 $kg \cdot m^{-2}$ (above the age-specific 97th percentile) (Cacciari et al. 2006) participated in the present study. The standard deviation score (SDS) of BMI, calculated by applying the LMS method (Cacciari et al. 2006) to Italian reference values for adolescents, was 2.8±0.5.

Bioelectric impedance analysis was used to assess fat-free mass. Whole-body resistance to an applied current (50 kHz, o.8 mA) was measured with a tetrapolar device (Human IM, Dietosystem, Italy). Fat-free mass (FFM), calculated with equations derived with a 2-

compartment model (Gray et al. 1989), was 60.4 ± 5.5 Kg. Fat mass (FM) was calculated as the difference between total body mass (BM) and FFM and resulted in 36.4 ± 6.6 Kg. Patients were recruited from those involved in a multidisciplinary body weight reduction program at the Division of Auxology, San Giuseppe Hospital, IRCSS Istituto Auxologico Italiano (Italian Institute for Auxology), Piancavallo (VB), Italy. All patients had no signs or symptoms related to cardiovascular, pulmonary or orthopedic diseases contraindicating the possibility to perform exercise. The patients and their parents were fully informed of the procedures and possible risks associated with the experiments before giving their written consent to participate to the study. The protocol was approved by the local ethics committee (reference code: 01C407-2014; acronym: SUPNITESEROB). All procedures were in accordance with the Declaration of Helsinki (2000) of the World Medical Association.

Study design

The patients were involved in a placebo-controlled, randomized, double-blind crossover study. Following a pre-evaluation test (see below for details), each patient completed two periods of 6 days of supplementation with beetroot juice (BR) (70 ml · day^{-1} containing 5 mmol of NO_3^-, [Beet It; James White Drinks, Ipswich, UK]) or nitrate-depleted placebo (PLA), matched in flavor, appearance and packaging (70 ml · day^{-1} with negligible content of NO_3^-, [Beet It; James White Drinks, Ipswich, UK]). A washout period lasting 7 days was interspersed between the two supplementation periods. A similar intervention in terms of dose and duration of supplementation has been demonstrated effective in improving exercise tolerance in healthy subjects (see e.g. 2). On day 6, at the end of each supplementation period, the patients performed three constant work rate (CWR) exercises at two different intensities (see below). Patients were required to abstain, during the study period, from using antibacterial mouthwashes and chewing gums, which are known to alter the oral bacteria responsible for the reduction of NO_3^- to NO_2^- (Woessner et al. 2016). All participants were hospitalized for a 3-week multidisciplinary body weight reduction program, entailing in constant and monitored physical activity, psychological counseling, nutritional education and moderate energy restriction (approx. 500 kcal lower than the measured resting energy expenditure), provided and daily monitored by expert nutritionists. The amount of ingested NO_3^- (about 150 mg·day^{-1}, 2.5 mmol · day^{-1}) was in accord with dietary guidelines, estimated considering the food intake and kept constant along the intervention period.

Exercise tests

At the beginning of the study each patient performed an incremental exercise on a cycle-ergometer (Monark Ergomedic 839E, Sweden) until voluntary exhaustion. The work rate started with 40 watt (W) for 2 minutes and then was increased by 20 W every minute. Exhaustion was defined when at least 3 of the following criteria were reached: 1) inability to maintain the pedaling frequency (60-80 revolutions.min-1) despite vigorous verbal encouragement by the operators; 2) maximal levels (higher than 7.5) of self-perceived exertion, using the Borg's modified CR10 scale (Borg 1973); 3) heart rate (HR) values higher than 85% of the age-predicted maximum; and 4) gas exchange ratio (R) value equal or above 1.1. No validation test for the determination of $\dot{V}O_2$max (see Poole & Jones 2017) was carried out in the recovery following the incremental test.

Data collected during the incremental test were utilized to identify individual gas exchange threshold (GET) and peak oxygen consumption ($\dot{V}O_2peak$), and, to set the intensity of the constant work rate exercises to be carried out at the end of each supplementation period.

Both in BR and in PLA, 3 hours after the last supplementation, each patient performed two repetitions of 6-min moderate-intensity CWR exercise (approximately at 80% of GET) separated by 10 min of passive recovery. Thereafter, a severe-intensity CWR exercise (at a work rate representing GET plus 65% of the difference between the work rate at GET and at $\dot{V}O_2peak$ about 80% of peak work rate) was performed until voluntary exhaustion, or until the maximal duration of 15 minutes was reached. CWR exercises were carried out at the same absolute work rate after the two supplementation periods.

Measurements

Blood samples

Resting blood samples were collected to determine plasma levels of NO_3^- before the incremental test and on day 6 of both supplementation periods; blood samples were taken, at least 2.5 hours after the last PLA or BR intake. Venous blood was drawn from the antecubital vein into a 5 mL EDTA vacutainer tube (Vacutainer, Becton Dickinson, USA). Plasma was immediately separated by centrifuge (5702R, Eppendorf, Germany) at 1000 g for 10 min at 4 °C. Plasma samples were then ultrafiltrate through a 10 kDa molecular weight cut-off (AmiconUltra; Millipore, EMD Millipore Corporation, Billerica, USA) utilizing an ultracentrifuge (4237R, ALC, Italy) at 14000 g for 60 min at 4 °C, in order to reduce background absorbance due to the presence of hemoglobin. The ultrafiltrate was recovered and used to measure NO_3^- concentration by the Griess method using a commercial kit (Cayman Chemical, USA). Samples were read by the addition of Griess reagents at 545 nm by a microplate reader

spectrophotometer (Infinite M200, Tecan, Austria). A linear calibration curve was computed from pure NO_3^- standard. All samples were determined in duplicate and the inter-assay coefficient of variation was in the range indicated by the manufacturer.

Physiological response to exercise. Tidal volume (VT), breathing frequency (fR), pulmonary ventilation ($\dot{V}E$), $\dot{V}O_2$ and carbon dioxide output ($\dot{V}CO_2$) were measured breath-by-breath by a computerized metabolic cart (Quark b2; Cosmed, Italy). The gas exchange ratio (R) was calculated as $\dot{V}CO_2/\dot{V}O_2$. GET was determined by standard methods (Beaver et al. 1986) and peak values of the main cardiovascular, respiratory and metabolic parameters were taken as the highest 20 s mean values reached during the incremental test. During moderate CWR, breath-by-breath $\dot{V}O_2$ data in the two repetitions were initially examined to exclude errant breaths caused by coughing, swallowing, sighing, etc., and those values lying more than 4 SDs from the local mean were removed. These data were time aligned and then superimposed for each subject. Oxygen cost (O_2cost) was calculated as $\Delta\dot{V}O_2$ ($\dot{V}O_2$ during last 30 s of constant work rate exercise minus resting $\dot{V}O_2$, defined as the mean $\dot{V}O_2$ measured over the final 60 s of resting period) divided by work rate. HR was determined continuously by a chest band (Polar Electro, Oulu, Finland); mean values every 5 s were calculated. Ratings of perceived exertion (RPE) were obtained at rest and every minute during exercise using the Borg's modified CR10 scale (Borg 1973). Considering the low number of exercise repetitions, a formal $\dot{V}O_2$ kinetics analysis was not performed (Lamarra et al. 1987).

Statistical analysis

Data are expressed as mean values ± standard deviation (x ± SD). Normal distribution of values was assessed with the Kolmogorov-Smirnov normality test. Time to exhaustion during severe-intensity CWR was considered the primary outcome of this study for power analysis. Considering the mean difference in this parameter previously observed in healthy subjects supplemented with beetroot juice, a sample size of 8 was requested to achieve a power of 80% with a significance level of 0.05.

To check the statistical significance of differences between BR and PLA paired Student's t-tests were performed. Linear regression and correlation analyses were carried out by the least-squared residual method. The $\dot{V}O_2$ values obtained during CWR at moderate intensity were analyzed using a two-way ANOVA for repeated measures (treatment × time). Post-hoc analysis was completed using Bonferroni multiple comparisons. The significant level was set at $P < 0.05$. Statistical analysis was performed using a software package (Prism 6.0; GraphPad Software, USA).

Results

Adherence and safety

All participants completed the intervention and follow-up testing. Adherence to BR supplementation, estimated by returned bottle count and consumption log, was 100%. There were no adverse events related to the supplementation, except that a temporarily red urine and red stools.

Plasma nitrate levels

Plasma [NO_3^-] was significantly higher in BR (108.4±37.2 μM) compared to PLA (15.4±5.0 μM) (P<0.0001) (Figure 1). Higher values in BR were observed in all patients. The increase in plasma [NO_3^-] was inversely related to the patients' body mass (r2 = 0.63; P=0.0072).

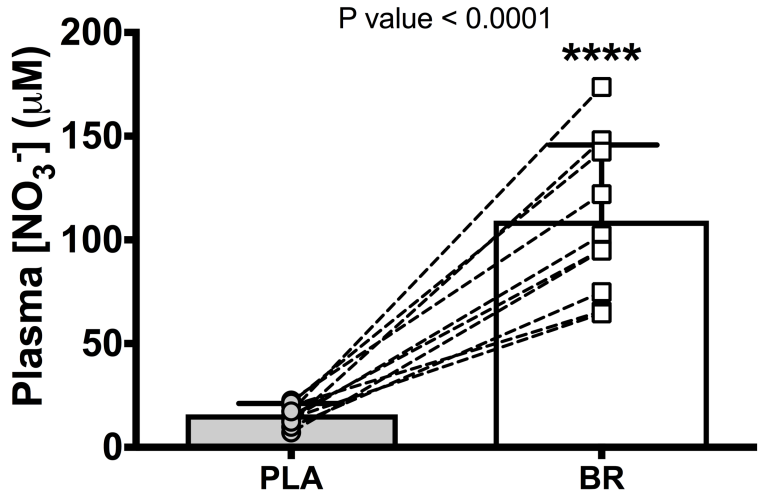

*Figure 1 Mean values (±SD) of plasma nitrate concentration (NO_3^-) after placebo (PLA) and beetroot juice (BR) supplementation (light grey and white columns, respectively). Dotted lines show individual changes. The P value of Paired Student's t-test is also shown. The symbol **** represents P < 0.0001, significantly different from PLA.*

Incremental exercise

Peak values of the main respiratory, cardiovascular and metabolic variables obtained at exhaustion during incremental exercise, are shown in Table 1. HR values reached about 85% of maximal predicted HR. GET occurred at 59±9% of maximal work rate and 63±9% of $\dot{V}O_2peak$.

Table 1. Peak values of the main respiratory, cardiovascular and metabolic variables determined at exhaustion during incremental exercise.

Work rate (W)	$\dot{V}O_2$ (L·min^{-1})	$\dot{V}O_2$ (mL·kg^{-1}·min^{-1})	$\dot{V}CO_2$ (L·min^{-1})	R	V_T (L)	fR (br·min^{-1})	$\dot{V}E$ (L·min^{-1})	$PETO_2$ (mmHg)	$PETCO_2$ (mmHg)	HR (bpm)	GET (W)	GET (L·min^{-1})	RPE (0-10)
174 ± 44	2.330 ± 0.676	24.4 ± 5.5	2.707 ± 0.806	1.16 ± 0.07	1.711 ± 0.412	39 ± 6	66.7 ± 17.0	103.1 ± 5.1	45.8 ± 5.2	174 ± 10	100 ± 21	1.455 ± 0.356	7.9 ± 2.4

Table 1 Mean (± SD) values of PO, power output; $\dot{V}O_2$, oxygen uptake; $\dot{V}CO_2$, CO2 output; RQ, gas exchange ratio; VT, tidal volume; fR, respiratory frequency; $\dot{V}E$, pulmonary ventilation; PET O_2, end tidal O_2 partial pressure; PET CO_2, end tidal CO_2 partial pressure; HR, heart rate; GET, gas exchange threshold; RPE, rating of perceived exertion

Moderate-intensity constant work rate exercise

All patients completed the imposed 6 minutes of exercise. Breath by breath values obtained in the two repetitions of the exercise were time aligned and then superimposed for each subject. Mean values of the main physiological variables determined in both BR and PLA during the last 30 s of moderate-intensity CWR exercises are presented in Table 2.

No significant differences were found for any of the variables between the two conditions. Work rate was set to be identical in each patient in the two conditions, and the value corresponded to about 45% of peak work rate. HR, expressed as percentage of maximal HR reached during the incremental exercise, was 73 ± 8% and 73 ± 9% in PLA and BR, respectively (no significant difference). Mean values of $\dot{V}O_2$ calculated at different time points (last 3 min and last 1 min) were not different between BR (1.35 ± 0.23 and 1.35 ± 0.22 L.min^{-1}, respectively) and PLA (1.32 ± 0.25 and 1.32 ± 0.25, L.min^{-1}, respectively), suggesting that steady state was reached. The O_2 cost of cycling was 13.3 ± 1.7 mL · min^{-1} · W^{-1} in BR and 12.9 ± 1.1 mL · min^{-1} · W^{-1}.

Figure 2 shows the time courses of $\dot{V}O_2$ obtained every 30 s during the moderate-intensity CWR exercise in both PLA and BR. The time-courses of the variable in the two conditions were comparable and no statistical differences were observed between the values obtained in the two groups at any given time.

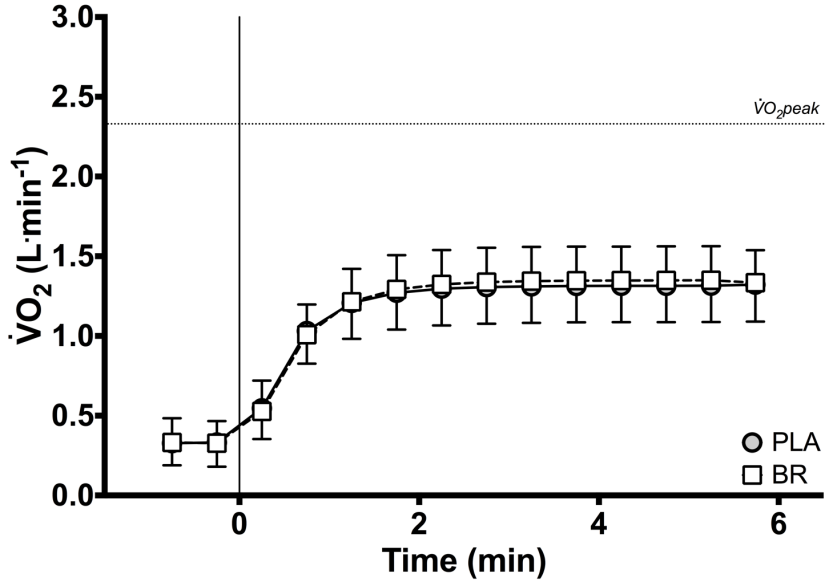

Figure 2 Group mean (±SD) of oxygen consumption ($\dot{V}O_2$) every 30 s during moderate-intensity exercise following placebo (PLA - light grey circles) and beetroot juice supplementation (BR – white squares). Horizontal dotted-line indicates mean peak $\dot{V}O_2$ values reached during the incremental exercise. Exercise started at time zero, vertical line. See text for further details.

Severe-intensity constant work rate exercise

Mean values of the main physiological variables determined in BR and PLA during the last 30 s of constant work rate severe-intensity CWR exercises are shown in Table 2. Work rate was designed to be the same in each patient in the two conditions and corresponded to 83 ± 4 % of peak work rate. HR was 96 ± 9% of peak HR in PLA and 103 ± 4% of peak HR in BR.

Table 2. Main respiratory, cardiovascular and metabolic variables determined at the end of the moderate and severe intensity constant work rate exercises after placebo (PLA) and beetroot juice supplementation (BR).

	Work rate (W)	$\dot{V}O_2$ (L·min^{-1})	$\dot{V}O_2$ (mL·kg^{-1}·min^{-1})	$\dot{V}O_2$ (%peak)	$\dot{V}CO_2$ (L·min^{-1})	R	$\dot{V}E$ (L·min^{-1})	HR (bpm)	HR (%peak)
MODERATE									
PLA	77 ± 13	1.318 ± 0.212	14.2 ± 1.3	59 ± 12	1.291 ± 0.172	0.98 ± 0.06	34.3 ± 6.2	124 ± 12	73 ± 8
BR	77	1.344	14.6	60	1.299	0.97	34.1	124	73

	±	±	±	±	±	±	±	±	±
	13	0.209	2.2	12	0.152	0.06	4.6	12	9
SEVERE									
	142	2.334	24.7	100	2.568	1.10	73.8	169	96
PLA	±	±	±	±	±	±	±	±	±
	38	0.535	4.8	24	0.563	0.07	18.0	16	9
	142	2.223	23.1	95	2.445	1.10	69.9	172	103
BR	±	±	±	±	±	±	±	±	±
	38	0.558	4.5	25	0.576	0.04	16.6	10	4

Table 2 Mean (± SD) values of PO, power output; $\dot{V}O_2$, oxygen uptake; $\dot{V}CO_2$, CO2 output; RQ, gas exchange ratio; $\dot{V}E$, pulmonary ventilation; HR, heart rate.

No patients in PLA and only one patient in BR completed the 15-minute exercise. Time to exhaustion was significantly increased (by 23%) by NO_3^- supplementation (561 ± 198 s and 457 ± 101 s, in BR and PLA respectively, P=0.0143) (Figure 3). In 3 of the patients, time to exhaustion did not increase following NO_3^- supplementation. No correlation was observed between the increase in plasma [NO_3^-] and time to exhaustion (r^2=0.13; P=0.30). The O_2 cost of cycling (14.1 ± 0.9 mL · min^{-1} · W^{-1} in BR vs. 14.9 ± 1.3 mL · min^{-1} · W^{-1} in PLA) was not different following NO_3^- administration (P=0.38). No correlation was observed between the increase in plasma [NO_3^-] and O_2 cost of cycling (r^2=0.38; P=0.10).

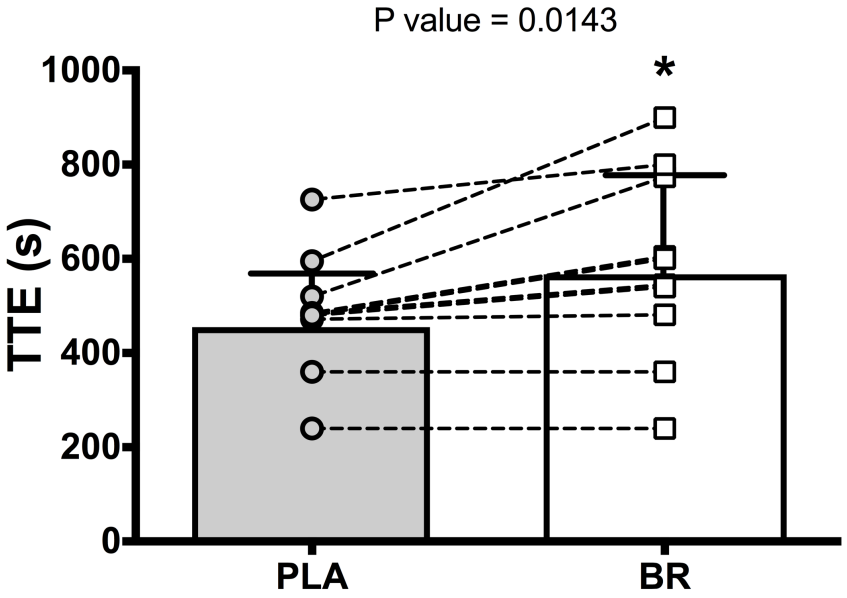

*Figure 3 Time to exhaustion (TTE) reached during severe-intensity exercise after placebo (PLA) and beetroot juice (BR) supplementation (light grey and white columns, respectively [mean±SD]). Dotted lines show individual changes. The P value of Paired Student's t-test is also shown. The symbol * represents P < 0.05, significantly different from PLA.*

Figure 4 (left panel) shows the time courses of $\dot{V}O_2$in the two conditions. To obtain this figure, individual $\dot{V}O_2$values were grouped for discrete time intervals, which were determined at discrete percentages of the individual total exercise time. Mean absolute individual values were calculated for the x and the y variables, for each time interval, and these data were utilized to obtain the mean group values shown in the Figure. Until 180 seconds of exercise, the exponential time courses of $\dot{V}O_2$ in the two conditions were substantially superimposed. After 180 seconds, the time courses of $\dot{V}O_2$ followed a linear increase without reaching a steady-state. The slopes of the regression lines, calculated from the 3rd minute to the end of exercise were significantly different from zero (p<0.0001). The rate of $\dot{V}O_2$increase, calculated as $\Delta\dot{V}O_2$ ($\dot{V}O_2$ at end-exercise minus $\dot{V}O_2$ at 3 minutes) divided by time (time at end-exercise minus 3 minutes) was significantly lower in BR vs. PLA (29.3 ± 18.1 vs. 60.9 ± 18.1 mL · min^{-2}, p=0.0002) (Figure 4, right panel).

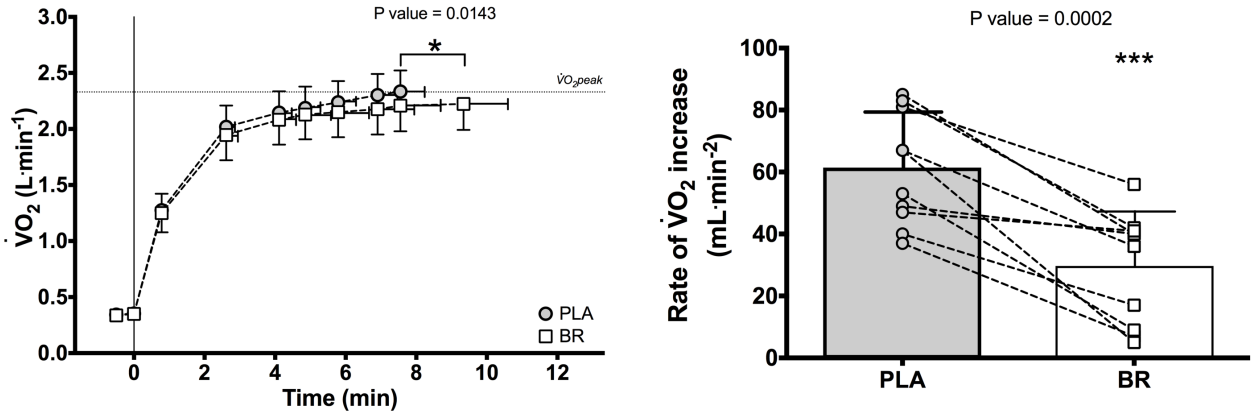

*Figure 4 Group mean values of oxygen consumption ($\dot{V}O_2$), grouped for discrete time intervals, during severe-intensity exercise following placebo (PLA, light grey circles) and beetroot juice (BR, white squares) supplementation (left panel). Exercise started at time zero, vertical line. Horizontal dot-point line indicates mean peak $\dot{V}O_2$ reached during incremental exercise. The P value of Paired Student's t-test is also shown. Time to exhaustion was significantly higher after BR compared to PLA (*P<0.05). In the right panel, mean values (±SD) of rate of $\dot{V}O_2$ increase calculated from the 3rd minute to the end of exercise in both PLA and BR (light grey and white columns, respectively). Dotted lines show individual changes. The P value of Paired Student's t-test is also shown. The symbol *** represents P < 0.001, significantly different from PLA. See text for further details.*

Discussion

The main result of the present study was that in obese adolescents short-term (6 days) dietary NO_3^- supplementation enhanced exercise tolerance by significantly increasing the time to exhaustion during severe-intensity CWR exercise. The increased exercise tolerance was associated with a less pronounced slow component of pulmonary $\dot{V}O_2$kinetics. On the other hand, NO_3^- supplementation had no effects on other physiological variables determined during moderate-intensity CWR exercise.

Obesity, also in adolescence, is usually associated with reduced cardiorespiratory fitness and exercise intolerance (Salvadori et al. 1999, Salvadego et al. 2010). Whereas $\dot{V}O_2peak$, expressed in absolute units (e.g. $L \cdot min^{-1}$), is usually normal, compared to normal-weight controls, when it is normalized per unit of body mass ($mL \cdot kg^{-1} \cdot min^{-1}$) values in obese patients are about 40% lower than in controls (Salvadego et al. 2010, Lazzer et al. 2013). Other factors contributing to the impaired exercise tolerance in obesity include a higher O_2 cost for submaximal exercise (Wasserman & Whipp 1975, Salvadori et al. 1999, Salvadego et al. 2010), which is at least in part attributable to an increased O_2 cost of breathing (Salvadego et al. 2015, Salvadego et al. 2017), a lower gas exchange threshold, slower adjustment of the fundamental component of $\dot{V}O_2$ kinetics (Salvadego et al. 2010), and a more pronounced amplitude of the slow component of $\dot{V}O_2$ kinetics during heavy intensity exercise (Salvadego et al. 2010). At least in part, these impairments can be overcome by exercise training (McNarry et al. 2015), including training specifically directed towards respiratory muscles (Salvadego et al. 2017).

In the present study, we evaluated the effects of a 6-day dietary NO_3^- supplementation on a group of obese adolescents and we observed an improved exercise tolerance after the intervention. More specifically, time to exhaustion during severe-intensity CWR exercise, which represents a classic parameter for the evaluation of exercise tolerance, was significantly increased (by about 25%) after dietary NO_3^- supplementation. Figure 4 shows that the linear increase in $\dot{V}O_2$ from 200 seconds to the end of the exercise was characterized by a significantly lower slope in BR than in PLA. This represents clear evidence that the "excess $\dot{V}O_2$" (Jones et al. 2011, Grassi et al. 2015), was reduced by NO_3^- supplementation. The term "excess $\dot{V}O_2$" is usually utilized to indicate the progressive increase in $\dot{V}O_2$ associated with the "slow component" of the $\dot{V}O_2$ kinetics, that is the $\dot{V}O_2$ "in excess" above that determined as the asymptote of the fundamental component. Since work rate is constant, this "excess $\dot{V}O_2$" indicates a reduced efficiency of muscle contraction. Considering the close interconnections between the loss of efficiency and fatigue, and ultimately exercise tolerance, during severe-intensity exercise (Jones et al. 2011, Grassi et al. 2015), it is not surprising that the lower slope of $\dot{V}O_2$ as a function of time in BR was associated with (or was responsible for) a longer time to fatigue and an enhanced exercise tolerance, as demonstrated in previous works on healthy subjects (Breese et al. 2013, Porcelli et al. 2015). Interestingly, in BR the time to exhaustion did not improve in 3 subjects out of 10, whereas it was remarkably higher in 5 subjects out of 10. This heterogeneity of results has been already reported by other authors both in healthy subjects (Porcelli et al. 2016) and in patients (Zamani et al. 2017), and it has been shown to be correlated with changes of plasma $[NO_3^-]$ or $[NO_2^-]$ after supplementation. In our subjects, we did not observe any correlation between time to exhaustion and plasma $[NO_3^-]$. On the other hand, in BR the rate of $\dot{V}O_2$ increase during severe-intensity exercise was reduced, suggesting less oxidative inefficiency, in all subjects.

The improved exercise tolerance of the present study confirms similar findings obtained in healthy subjects (Bailey et al. 2009, Porcelli et al. 2015) and in disease populations (Kenjale et al. 2011, Curtis et al. 2015, Zamani et al. 2015), such as patients with peripheral arterial disease (Kenjale et al. 2011,), patients with chronic obstructive pulmonary diseases (Curtis et al. 2015) and heart failure patients (Zamani et al. 2015). Similar results have also been shown by different interventions aimed to increase NO bioavailability. For example, Sperandio et al. (Sperandio et al. 2012) demonstrated that sildenafil consumption in chronic heart failure patients improved muscle oxygenation and increased exercise capacity.

In the present study dietary NO_3^- supplementation did not affect physiological responses to moderate-intensity CWR exercise. In this exercise domain the O_2 cost of cycling was not significantly different in BR (12.9 ± 1.1 mL · min^{-1} · W^{-1}) vs. PLA (13.3 ± 1.7 mL · min^{-1} · W^{-1}), being in both conditions substantially higher than the values usually observed in normal weight subjects (about 9-10 mL · min^{-1} · W^{-1}) (Wasserman & Whipp 1975). This confirms our previous data (Salvadego et al. 2010, Salvadego et al. 2015, Salvadego et al. 2017). Thus, in this exercise domain, NO_3^- did not have any effect on the $\dot{V}O_2$ kinetics. These results are in accordance to the results of some other studies (Bescós et al. 2011, Breese et al. 2013).

The lack of ergogenic effects of beetroot juice supplementation in the moderate intensity domain does not limit the relevance of the results of this study. The effects of beetroot juice supplementation during high-intensity exercise is indeed noteworthy, considering that the latter is considered a very effective intervention for weight reduction and improved health status in obesity (Wewege et al. 2017).

The question could be raised if the NO_3^- dose utilized in the present study was enough to induce significant changes in NO_2^- and NO bioavailability. Whereas NO is too labile to be determined (James et al. 2015), in the $NO_3^- \rightarrow NO_2^- \rightarrow$ NO pathway, NO_2^- would obviously be "closer" to the physiological relevant molecule (NO). Plasma [NO_2^-] could not be determined in the present study. Ample previous experimental evidence, however, demonstrates that following dietary NO_3^- supplementation the increases in plasma [NO_2^-] and [NO_3^-] are closely correlated (James et al. 2015). In the present study, the daily NO_3^- dose (5 mmol) was towards the low end of the efficacy spectrum (Wylie et al. 2013, Wylie et al. 2016), particularly considering that we were treating obese patients with an elevated body mass. The treatment, however, was continued for 6 days, i.e. for a period substantially longer than that adopted in previous "acute" supplementation studies (Muggeridge et al. 2013, Kenjale et al. 2011). The end result was that in all patients of the present study plasma [NO_3^-] substantially increased after the supplementation. On average the plasma [NO_3^-] increase was about 7-fold, reaching values quite similar to or even higher than those obtained in previous studies in which dietary NO_3^- supplementation proved to be effective (Kapil et al. 2010, James et al. 2015). Moreover, although in the present study plasma [NO_3^-] was inversely correlated with body mass, no correlation was observed between plasma [NO_3^-] and the main outcome measure, the time to exhaustion during severe-intensity exercise.

Another issue we should take into consideration relates to the recruitment pattern of muscle fibers. It is known that additional fibers, mainly type 2 fibers, are recruited with time during heavy- and severe-intensity exercise in temporal association with the slow component of the

$\dot{V}O_2$ kinetics (Jones et al. 2011). Although it has been previously demonstrated that additional recruitment of fibers is not necessary for the development of the slow component (Zoladz et al. 2008), in several experimental conditions the slow component may be linked to a shift in fiber-type recruitment (15). Beetroot juice supplementation seems to be more effective in type 2 compared to type 1 fibers (Jone et al. 2016). Indeed, the reduction of NO_2^- to NO, mainly responsible of the ergogenic effects of NO_3^- supplementation, seems to be augmented in conditions of low pH and PO_2, typical conditions associated with heavy- and severe-intensity exercise, in which type 2 fibers are predominantly activated. In rats running at 70% of $\dot{V}O_2peak$ blood flow was predominantly elevated in fast twitch muscle after beetroot juice ingestion (Ferguson et al. 2013). Thus, it is plausible that in presence of lower concentrations of NO, heavy and severe-intensity domains exercise could exacerbate tissue hypoxia within type 2 muscle fibers, resulting in a lower "metabolic stability" and fatigue development (Grassi et al. 2011). Good metabolic stability during exercise is associated with smaller perturbations of the levels of metabolites related to muscle inefficiency and fatigue (see Grassi et al. 2011), such as decreases (compared to rest) in [PCr], and increases in [Pi], [ADPfree], [AMPfree], and [IMPfree] etc. (Grassi et al. 2011). Thus, good metabolic stability is directly associated with a greater exercise tolerance. On the other hand, when intramuscular NO bioavailability is increased (as after NO_3^- supplementation) the improved matching between O_2 delivery and O_2 uptake could improve "metabolic stability", reduce metabolic inefficiency and fatigue, and improve enhance exercise tolerance (Grassi et al. 2011).

Physical training of obese patients usually involves activities on cycle-ergometer due to the reduced load on hip, ankle and knee joints compared to walking or running. However, cycling requires a lower metabolic demand compared to the other exercises, and this may limit the weight-loss process. Thus, future interventional studies to be carried out in obese patients should evaluate the ergogenic aid of dietary NO_3^- supplementation during walking and running. The effects of dietary NO_3^- supplementation on voluntary physical activities would also be an interesting topic of investigation. In this study, we did not evaluate the effects of NO_3^- supplementation on blood pressure. Inorganic NO_3^- and beetroot juice consumption are associated with a significant reduction in systolic and diastolic blood pressure (see e.g. Bailey et al. 2009, Kapil et al 2010); this effect would be particularly useful in subjects at high risk for cardiovascular diseases, such as obese and overweight patients. Finally, in this study we increased NO bioavailability by beetroot juice supplementation providing an amount of 100 kcal per day (proteins 4 g, carbohydrates 21 g, fats 0.1 g, fibers 0.6 g, sodium 0.3 g). Although relatively small, this amount could become a problem during long-term supplementation in the

obese population. Future studies should therefore consider other sources of NO_3^- for chronic supplementations, such as nitrate-rich vegetables, as previously utilized in healthy subjects (Porcelli et al. 2016).

Perspectives and significance

The results of the present study suggest that short-term (6 days) dietary NO_3^- supplementation in obese adolescents is safe and effective in improving exercise tolerance during severe-intensity exercises. The effect may be related to improved efficiency of oxidative metabolism (i.e. reduction of slow component), and it needs to be confirmed by longer-term studies. By contrasting the vicious circle of early fatigue → reduced exercise tolerance → reduced physical activity → obesity → early fatigue, the intervention could represent a useful adjunct (similar to respiratory muscle endurance training, see our recent work) (Salvadego et al. 2017) in the control of obesity in adolescents and possibly also in adult obese patients.

Acknowledgements

We thank all the participants for their contributions to this research project. We acknowledge Mrs A. Seddone (head nurse) and the nursing staff at the Division of Auxology for their professional collaboration in blood sampling and monitoring during the execution of the tests. We also thank Dr. Teresa Lorenzon for her valuable help.

Grants

The project was partially supported by Progetti di Ricerca Corrente, Istituto Auxologico Italiano, IRCCS, Milan and Piancavallo (project's reference code: 01C407-2014; acronym: SUPNITESEROB) and by Progetto di interesse Invecchiamento CNR WP3.7

Disclosure

No conflicts of interest, financial or otherwise, are declared by the authors.

5. FINAL CONSIDERATIONS

In this thesis, I presented results of the studies I have conducted during my PhD aimed to broaden the knowledge about the relationship between increased NO bioavailability and physiological responses to exercise, ranging from mechanistic insights in animal model to new clinical applications and effects on altitude performance.

The study carried out on in vivo canine gastrocnemius muscle model add insights on the complex mechanisms involved in the ergogenic benefits of nitrate supplementation. The results suggest no direct effects of increased NO bioavailability on muscle mitochondrial efficiency if type 1 muscle fiber are predominately recruited. Further researches are needed to better clarify the mechanistic basis of NO effects on muscle contraction and more in general on exercise tolerance, in order to pave the way to new potential application of NO_3^- supplementation.

In the second work, I investigated the effects of dietary NO_3^- supplementation on exercise performance at altitude. After beetroot juice supplementation O_2 consumption decreased and time to exhaustion increased in both cycling and arm cranking exercise, indicating dietary nitrate supplementation as a valid aid to counteract exercise intolerance occurring at altitude. Interestingly, in the same study we demonstrated that hypobaric hypoxia affects plasma [NO_3^-] and [NO_2^-] per se, by suggesting that changes in NO bioavailability are part of the physiological adaptation implemented by the body to counteract the decrease in PO_2.

Finally, in the third study, we evaluated the potential clinical applications of beetroot juice in a population of patients characterized by exercise intolerance. Beetroot juice supplementation was able to improve, at least in severe intensity domain, exercise performance in obese adolescents.

In conclusion, dietary NO_3^- supplementation has positive effects on exercise performance at least altitude environment and in obese subjects. Even if the mechanistic bases of NO related decrement in O_2 cost of exercise need to be elucidated and seems not to be related to effects on mitochondrial efficiency when aerobic metabolism is the main contributor to force generation, enhanced NO bioavailability should be considered as a valid ergogenic aid. In my future I hope to have the opportunity to conduct some more experiments on this topic in order to better identify the mechanistic bases of the ergogenic benefits of dietary NO_3^- supplementation and evaluate potential beneficial effects on exercise intolerance of different populations.

6. REFERENCES

Allen JD, Giordano T, Kevil CG. Nitrite and nitric oxide metabolism in peripheral artery disease. Nitric Oxide 26:217–222, 2012.

Allen JD, Miller EM, Schwark E, Robbins JL, Duscha BD, Annex BH. Plasma nitrite response and arterial reactivity differentiate vascular health and performance. Nitric Oxide 20:231–237, 2009.

Ameredes BT, Brechue WF, Stainsby WN. Mechanical and metabolic determination of $\dot{V}O_2$ and fatigue during repetitive isometric contractions in situ. J Appl Physiol 84:1909–1916, 1998.

Andrade FHMR, Reid MB, Westerblad H. Contractile response of skeletal muscle to low peroxide concentrations: myofibrillar calcium sensitivity as a likely target for redoxmodulation. FASEB J 15:309–311, 2001.

Arne M, Janson C, Janson S, Boman G, Lindqvist U, Berne C, Emtner M. Physical activity and quality of life in subjects with chronic disease: chronic obstructive pulmonary disease compared with rheumatoid arthritis and diabetes mellitus. Scand J Prim Health Care 27(3):141-7, 2009.

Bahadoran Z, Ghasemi A, Mirmiran P, Azizi F, Hadaegh F. Beneficial effects of inorganic nitrate/nitrite in type 2 diabetes and its complications. Nutr Metab 16:12-16, 2015.

Bailey SJ, Fulford J, Vanhatalo A, Winyard PG, Blackwell JR, DiMenna FJ, Wilkerson DP, Benjamin N, Jones AM. Dietary nitrate supplementation enhances muscle contractile efficiency during kneeextensor exercise in humans. J Appl Physiol 109:135–48, 2010.

Bailey SJ, Winyard P, Vanhatalo A, Blackwell JR, Dimenna FJ, Wilkerson DP, Tarr J, Benjamin N, Jones AM. Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. J Appl Physiol 107:1144–55, 2009.

Bakker E, Engan H, Patrician A, Schagatay E, Karlsen T, Wisløff U, Gaustad SE. Acute dietary nitrate supplementation improves arterial endothelial function at high altitude: A double-blinded randomized controlled cross over study. Nitric Oxide 50:58-64, 2015.

Beijers RJHCG, Huysmans SMD, van de Bool C, Kingma BRM, Verdijk LB, van Loon LJC, Meex SJR, Gosker HR, Schols AMWJ. The effect of acute and 7-days dietary nitrate on mechanical efficiency, exercise performance and cardiac biomarkers in patients with chronic obstructive pulmonary disease. Clin Nutr 2017.

Benjamin N, O'Driscoll F, Dougall H, Duncan C, Smith L, Golden M, McKenzie H. Stomach NO synthesis. Nature 368(6471):502 1994.

Berry MJ, Justus NW, Hauser JI, Case AH, Helms CC, Basu S, Rogers Z, Lewis MT, Miller GD. Dietary nitrate supplementation improves exercise performance and decreases blood pressure in COPD patients. Nitric Oxide 48:22–30, 2015.

Bescós R, Ferrer-Roca V, Galilea PA, Roig A, Drobnic F, Sureda A, Martorell M, Cordova A, Tur JA, Pons A. Sodium nitrate supplementation does not enhance performance of endurance athletes. Med Sci Sports Exerc 44:2400–9, 2012.

Bescós R, Rodriguez FA, Iglesias X, Ferrer MD, Iborra E, Pons A. Acute administration of inorganic nitrate reduces V̇O2peak in endurance athletes. Medicine and Med Sci Sports Exerc 43(10): 1979–1986, 2011.

Bock JM, Treichler DP, Norton SL, Ueda K, Hughes WE, Casey DP. Inorganic nitrate supplementation enhances functional capacity and lower-limb microvascular reactivity in patients with peripheral artery disease. Nitric Oxide 2018.

Bryas NS, Loscalzo J. (Editors) Nitrate and Nitrite in Human Health and Disease, Humana Press 2011

Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes 54(6):1615–25, 2005.

BjornbergJ, Albert U, Mellander S. Resistance responses in proximal arterial vessels, arterioles and veins during reactive hyperaemia in skeletal muscle and their underlying regulatory mechanisms. Acta Physiol Scand 139: 535–550, 1990.

Borg GA. Perceived exertion: a note on history and methods. Med Sci Sports Exerc 5: 90–93, 1973.

Boushel R, Fuentes T, Hellsten Y, Saltin B. Opposing effects of nitric oxide and prostaglandin inhibition on muscle mitochondrial VO2 during exercise. Am J Physiol Regul Integr Comp Physiol 303(1):R94-100, 2012.

Boveris A, Costa LE, Poderoso JJ, Carreras MC, Cadenas E., Regulation of mitochondrial respiration by oxygen and nitric oxide. Ann N Y Acad Sci 899:121–135, 2000.

Bradbury KE, Appleby PN, Key TJ. Fruit, vegetable, and fiber intake in relation to cancer risk: Findings from the European Prospective Investigation into Cancer and Nutrition (EPIC). Am J Clin Nutr 100:394S–398S, 2014.

Breese BC, McNarry MA, Marwood S, Blackwell JR, Bailey SJ, Jones AM. Beetroot juice supplementation speeds O_2 uptake kinetics and improves exercise tolerance during severe-intensity exercise initiated from an elevated metabolic rate. Am J Physiol Regul Integr Comp Physiol 305: 1441-50, 2013.

Breese BC Poole DC, Okushima D, Bailey SJ, Jones AM, Kondo N, Amano T, Koga S.. The effect of dietary nitrate supplementation on the spatial heterogeneity of quadriceps deoxygenation during heavy-intensity cycling. Physiol Rep 5(14), 2017.

Brotto MAP, Nosek TM. Hydrogen peroxide disrupts calcium release from the sarcoplasmic reticulum of rat skeletal muscle fibres. J Appl Physiol 81:731–737, 1996.

Cacciari E, Milani S, Balsamo A, Spada E, Bona G, Cavallo L, Cerutti F, Gargantini L, Greggio N, Tonini G, Cicognani A. Italian cross sectional growth charts for height, weight and BMI (2 to 20 yr). J Endocrinol Invest 29: 581–593, 2006.

Calbet JA, Holmberg HC, Rosdahl H, van Hall G, Jensen-Urstad M, Saltin B. Why do arms extract less oxygen than legs during exercise? Am J Physiol Regul Integr Comp Physiol 289(5):R1448-58, 2005.

Campos HO, Drummond LR, Rodrigues QT, Machado FSM, Pires W, Wanner SP, Coimbra CC. Nitrate supplementation improves physical performance specifically in non-athletes during

prolonged open-ended tests: a systematic review and meta-analysis. Br J Nutr 119(6):636-657, 2018.

Carlström M, Larsen FJ, Nyström T, Hezel M, Borniquel S, Weitzberg E, Lundberg JO. Dietary inorganic nitrate reverses features of metabolic syndrome in endothelial nitric oxide synthase-deficient mice. Proc Natl Acad Sci USA 107:17716–20, 2010.

Carriker CR, Mermier CM, McLain TA, Mermier CM, Van Dusseldorp TA, Johnson KE, Beltz NM, Vaughan RA, McCormick JJ, Cole NH, Witt CC, Gibson AL. Effect of Acute Dietary Nitrate Consumption on Oxygen Consumption During Submaximal Exercise in Hypobaric Hypoxia. Int J Sport Nutr Exerc Metab 26(4):315-22, 2016.

Casey DP, Treichler DP, Ganger CT IV, Schneider AC, Ueda K. Acute dietary nitrate supplementation enhances compensatory vasodilation during hypoxic exercise in older adults. J Appl Physiol 118:178–186, 2015.

Cerretelli P, Marzorati M, Marconi C. Muscle bioenergetics and metabolic control at altitude. High Alt Med Biol 10(2):165-74, 2009.

Clerc P, Rigoulet M, Leverve X, Fontaine E. Nitric oxide increases oxidative phosphorylation efficiency. J Bioenerg Biomembr 39(2):158-66, 2007.

Craig JC, Broxterman RM, Smith JR, Allen JD, Barstow TJ. Effect of dietary nitrate supplementation on conduit artery blood flow, muscle oxygenation, and metabolic rate during handgrip exercise. J Appl Physiol 125(2):254-262, 2018.

Cermak NM, Gibala MJ, van Loon LJ. Nitrate supplementation's improvement of 10-km timetrial performance in trained cyclists. Int J Sport Nutr Exerc Metab 22:64–71, 2012.

Cermak NM, Hansen D, Kouw IW, van Dijk JW, Blackwell JR, Jones AM, Gibala MJ, van Loon LJ. A single dose of sodium nitrate does not improve oral glucose tolerance in patients with type 2 diabetes mellitus. Nutr Res 35: 674–680, 2015.

Coggan AR, Peterson LR, Dietary Nitrate Enhances the Contractile Properties of Human Skeletal Muscle. Exerc Sport Sci Rev 46(4):254-261, 2018.

Culotta E, Koshland DE. No news is good news. Science 258(5090):1862–1865, 1992

Cumpstey AF, Hennis PJ, Gilbert-Kawai ET, Fernandez BO, Poudevigne M, Cobb A, Meale P, Mitchell K, Moyses H, Pöhnl H, Mythen MG, Grocott MPW, Feelisch M, Martin DS; Xtreme Alps research group. Effects of dietary nitrate on respiratory physiology at high altitude - Results from the Xtreme Alps study. Nitric Oxide 71:57-68, 2017.

Curtis KJ, O'Brien KA, Tanner RJ, Polkey JI, Minnion M, Feelisch M, Polkey MI, Edwards LM, Hopkinson NS. Acute dietary nitrate supplementation and exercise performance in COPD: a double-blind, placebo-controlled, randomised controlled pilot study. PLoS One 10(12): e0144504, 2015.

Dua AK, Dua N, Murrant CL. Skeletal muscle contraction-induced vasodilator complement production is dependent on stimulus and contraction frequency. Am J Physiol Heart Circ Physiol 297:H433-442, 2009.

Eggebeen J, Kim-Shapiro DB, Haykowsky M, Morgan TM, Basu S, Brubaker P, Rejeski J, Kitzman DW. One week of daily dosing with beetroot juice improves submaximal endurance

and blood pressure in older patients with heart failure and preserved ejection fraction. JACC Heart Fail 4: 428–437, 2016.

Erzurum SC, Ghosh S, Janocha AJ, Xu W, Bauer S, Bryan NS, Tejero J, Hemann C, Hille R, Stuehr DJ, Feelisch M, Beall CM. Higher blood flow and circulating NO products offset high-altitude hypoxia among Tibetans. Proc Natl Acad Sci 104:17593–8, 2007.

Faiss R, Pialoux V, Sartori C, Faes C, Dériaz O, Millet GP. Ventilation, oxidative stress, and nitric oxide in hypobaric versus normobaric hypoxia. Med Sci Sports Exerc 45:253–60, 2013.

Feelisch M, Fernandez BO, Bryan NS, Garcia-Saura MF, Bauer S, Whitlock DR, Ford PC, Janero DR, Rodriguez J, Ashrafian H. Tissue processing of nitrite in hypoxia: an intricate interplay of nitric oxide-generating and -scavenging systems. J Biol Chem 283(49):33927-34, 2008.

Ferguson SK, Hirai DM, Copp SW, Holdsworth CT, Allen JD, Jones AM, Musch TI, Poole DC. Impact of dietary nitrate supplementation via beetroot juice on exercising muscle vascular control in rats. J Physiol 591: 547–57, 2013.

Ferguson SK, Holdsworth CT, Wright JL, Fees AJ, Allen JD, Jones AM, Musch TI, Poole DC. Microvascular oxygen pressures in muscles comprised of different fiber types: Impact of dietary nitrate supplementation. Nitric Oxide 48, 38–43, 2015.

Friis AL, Steenholt CB, Løkke A, Hansen M. Dietary beetroot juice - effects on physical performance in COPD patients: a randomized controlled crossover trial. Int J Chron Obstruct Pulmon Dis 12:1765-1773, 2017.

Folland JP, Maas H, Jones DA. The influence of nitric oxide on in vivo human skeletal muscle properties. Acta Physiol Scand 169(2):141-8, 2000.

Fulford J, Winyard PG, Vanhatalo A, Bailey SJ, Blackwell JR, Jones AM. Influence of dietary nitrate supplementation on skeletal muscle metabolism and force production during maximal voluntary contractions. Pflüger Arch 465:517-28, 2013.

Gilchrist M, Winyard PG, Aizawa K, Anning C, Shore A, Benjamin N. Effect of dietary nitrate on blood pressure, endothelial function, and insulin sensitivity in type 2 diabetes. Free Radic Biol Med 60: 89–97, 2013.

Gilchrist M, Winyard PG, Benjamin N. Dietary nitrate-good or bad? Nitric Oxide 22:104–109, 2010.

Gould N, Doulias PT, Tenopoulou M, Raju K, Ischiropoulos H. Regulation of protein function and signaling by reversible cysteine S-nitrosylation. J Biol Chem 288:26473-9, 2013.

Govoni M, Jansson EA, Weitzberg E, Lundberg JO. The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. Nitric Oxide 19:333–337, 2008.

Grassi B, Hogan MC, Kelley KM, Howlett RA, Gladden LB.. Effects of NOS inhibition by l-NAME on oxygen uptake kinetics in isolated canine muscle in situ. J Physiol 568:1021–1033, 2005.

Grassi B, Porcelli S, Salvadego D, Zoladz JA. Slow VO_2 kinetics during moderate-intensity exercise as markers of lower metabolic stability and lower exercise tolerance. Eur J Appl Physiol 111(3):345-55, 2011.

Grassi B, Rossiter HB, Hogan MC, Howlett RA, Harris JE, Goodwin ML, Dobson JL, Gladden LB. Faster O_2 uptake kinetics in canine skeletal muscle in situ after acute creatine kinase inhibition. J Physiol 589(Pt 1):221-33, 2011.

Grassi B, Rossiter HB, Zoladz JA. Skeletal muscle fatigue and decreased efficiency: two sides of the same coin? Exerc Sport Sci Rev 43(2): 75-83, 2015.

Gray DS, Bray GA, Gemayel N, Kaplan K. Effect of obesity on bioelectrical impedance. Am J Clin Nutr 50(2): 255-260, 1989.

Green DJ, Maiorana A, O'Driscoll G, Taylor R. Effect of exercise training on endothelium-derived nitric oxide function in humans. J Physiol 561:1–25, 2004.

Haider G, Folland JP. Nitrate supplementation enhances the contractile properties of human skeletal muscle. Med Sci Sports Exerc 46:2234-43, 2014.

Hennis PJ, Mitchell K, Gilbert-Kawai E, Bountziouka V, Wade A, Feelisch M, Grocott MP, Martin DS. Effects of dietary nitrate supplementation on symptoms of acute mountain sickness and basic physiological responses in a group of male adolescents during ascent to Mount Everest Base Camp. Nitric Oxide 60:24-31, 2016.

Hernàndez A, Schiffer TA, Ivarsson N, Cheng AJ, Bruton JD, Lundberg JO, Weitzberg E, Westerblad H. Dietary nitrate increases tetanic $[Ca^{2+}]_i$ and contractile force in mouse fast-twitch muscle, J Physiol 59:3575–3583, 2012.

Hickner RC, Fisher JS, Ehsani AA, Kohrt WM. Role of nitric oxide in skeletal muscle blood flow at rest and during dynamic exercise in humans. Am J Physiol Heart Circ Physiol 273:H405–H410, 1997.

Hirai DM, Zelt JT, Jones JH, Castanhas LG, Bentley RF, Earle W, Staples P, Tschakovsky ME, McCans J, O'Donnell DE, Neder JA. Dietary nitrate supplementation and exercise tolerance in patients with heart failure with reduced ejection fraction. Am J Physiol Regul Integr Comp Physiol 312: R13–R22, 2017.

Hoon MW, Fornusek C, Chapman PG, Johnson NA. The effect of nitrate supplementation on muscle contraction in healthy adults. Eur J Sport Sci 8:712-9, 2015.

Hoon MW, Jones AM, Johnson NA, Blackwell JR, Broad EM, Lundy B, Rice AJ, Burke LM. The effect of variable doses of inorganic nitrate-rich beetroot juice on simulated 2,000-m rowing performance in trained athletes. Int J Sports Physiol Perform 9:615–20, 2014.

James PE, Willis GR, Allen JD, Winyard PG, Jones AM. Nitrate pharmacokinetics: Taking note of the difference. Nitric Oxide 48: 44-50, 2015.

Janocha AJ, Koch CD, Tiso M, Ponchia A, Doctor A, Gibbons L, Gaston B, Beall CM, Erzurum SC. Nitric oxide during altitude acclimatization. N Engl J Med 365:1942–1944, 2011.

Jensen L, Bangsbo J, Hellsten Y. Effect of high intensity training on capillarization and presence of angiogenic factors in human skeletal muscle. J Physiol 557:571–82, 2004.

Jones A. Dietary nitrate supplementation and exercise performance, Sports Med 44: S35–S45, 2014.

Jones AM. Influence of dietary nitrate on the physiological determinants of exercise performance: a critical review. Appl Physiol Nutr Metab 39:1019-1028, 2014.

Jones AM, Ferguson SK, Bailey SJ, Vanhatalo A, Poole DC. Fiber-type specific effects of dietary nitrate. Exerc Sport Sci Rev 44: 53-60, 2016.

Jones AM, Grassi B, Christensen PM, Krustrup P, Bangsbo J, Poole DC. Slow component of V̇O2kinetics: mechanistic bases and practical applications Med Sci Sports Exerc 43(11): 2046-62, 2011.

Jones AM, Thompson C, Wylie LJ, Vanhatalo A. Dietary Nitrate and Physical Performance. Annu Rev Nutr 38:303-328, 2018.

Kapil V, Milsom AB, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, Arghandawi S, Pearl V, Benjamin N, Loukogeorgakis S, Macallister R, Hobbs AJ, Webb AJ, Ahluwalia A. Inorganic nitrate supplementation lowers blood pressure in humans: Role for nitrite-derived NO. Hypertension 56: 274-281, 2010.

Kelly J, Vanhatalo A, Bayley SJ, Wylie LJ, Tucker C, List S, Winyard PG, Jones AM. Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O_2 uptake dynamics during exercise in hypoxia and normoxia. Am J Physiol Regul Integr Comp Physiol 307:R920–R930, 2014.

Kenjale AA, Ham KL, Stabler T, Robbins JL, Johnson JL, Vanbruggen M, Privette G, Yim E, Kraus WE, Allen JD. Dietary nitrate supplementation enhances exercise performance in peripheral arterial disease. J Appl Physiol 110: 1582–1591, 2011

Kerley CP, Cahill K, Bolger K, McGowan A, Burke C, Faul J, Cormican L Dietary nitrate supplementation in COPD: An acute, double-blind, randomized, placebo-controlled, crossover trial. Nitric Oxide 44:105–111, 2015

Khalifi S, Rahimipour A, Jeddi S, Ghanbari M, Kazerouni F, Ghasemi A. Dietary nitrate improves glucose tolerance and lipid profile in an animal model of hyperglycemia. Nitric oxide 44:24–30, 2015.

Khoo NK, Mo L, Zharikov S, Kamga-Pride C, Quesnelle K, Golin-Bisello F, Li L, Wang Y, Shiva S. Nitrite augments glucose uptake in adipocytes through the protein kinase A-dependent stimulation of mitochondrial fusion. Free Radic Biol Med 70: 45–53, 2014.

Kim JK, Moore DJ, Maurer DG, Kim-Shapiro DB, Basu S, Flanagan MP, Skulas-Ray AC, Kris-Etherton P, Proctor DN. Acute dietary nitrate supplementation does not augment submaximal forearm exercise hyperemia in healthy young men. Appl Physiol Nutr Metab 40:122–128, 2015.

Kinnunen S, Manttari S. Specific effects of endurance and sprint training on protein expression of calsequestrin and SERCA in mouse skeletal muscle. J Muscle Res Cell Motil 33:123–30, 2012.

Kobzik L, Reid MB, Bredt DS, Stamler JS. Nitric oxide in skeletal muscle. Nature 372:546–548, 1994.

Kobayashi J, Othake K, Uchida H. NO-rich diet for lifestyle-related diseases, Nutrients 7: 4911-4937, 2015.

Kraus RM, Houmard JA, Kraus WE, Tanner CJ, Pierce JR, Choi, MD, Hickner RC. Obesity, insulin resistance, and skeletal muscle nitric oxide synthase, J Appl Physiol 113(5): 758-65, 2012.

Kruse NT, Ueda K, Hughes WE, Casey DP. Eight weeks of nitrate supplementation improves blood flow and reduces the exaggerated pressor response during forearm exercise in peripheral artery disease. Am J Physiol Heart Circ Physiol 2018.

Lamarra N, Whipp BJ, Ward SA, Wasserman K. Effect of interbreath fluctuations on characterizing exercise gas exchange kinetics. J Appl Physiol 62: 2003–2012, 1987.

Lamb IR, Novielli NM, Murrant CL. Capillary response to skeletal muscle contraction: evidence that redundancy between vasodilators is physiologically relevant during active hyperaemia. J Physiol 596(8):1357-1372, 2018.

Lansley KE, Winyard PG, Bailey SJ, Vanhatalo A, Wilkerson DP, Blackwell JR, Gilchrist M, Benjamin N, Jones AM. Acute dietary nitrate supplementation improves cycling time trial performance. Med Sci Sports Exerc 43(6):1125–31, 2011.

Lanzarone E, Gelmini F, Fumero A, Carini M, Costantino ML, Fumero R, Alfieri O. Preservation of endothelium nitric oxide release during beating heart surgery with respect to continuous flow cardiopulmonary bypass. Perfusion 25(2):57-64, 2010.

Larsen F J, Weitzberg E, Lundberg JO, Ekblom B. Effects of dietary nitrate on oxygen cost during exercise. Acta Physiol 191:59–66, 2007.

Larsen FJ, Schiffer TA, Borniquel S, Sahlin K, Ekblom B, Lundberg JO, Weitzberg E. Dietary inorganic nitrate improves mitochondrial efficiency in humans. Cell Metab 13(2): 149–59, 2011.

Lazzer S, Salvadego D, Porcelli S, Rejc E, Agosti F, Sartorio A, Grassi B. Skeletal muscle oxygen uptake in obese patients: functional evaluation by knee-extension exercise. Eur J Appl Physiol 113(8): 2125-32, 2013.

Leong P, Basham JE, Yong T, Chazan A, Finlay P, Barnes S, Bardin PG, Campbell D. A double blind randomized placebo control crossover trial on the effect of dietary nitrate supplementation on exercise tolerance in stable moderate chronic obstructive pulmonary disease. BMC Pulmonary Medicine 15:52, 2015.

Levett DZ, Fernandez BO, Riley HL, Martin DS, Mitchell K, Leckstrom CA, Ince C, Whipp BJ, Mythen MG, Montgomery HE, Grocott MP, Feelisch M; Caudwell Extreme Everest Research Group. The role of nitrogen oxides in human adaptation to hypoxia. Sci Rep 1:109, 2011.

Lundberg JO, Gladwin MT, Ahluwalia A, Benjamin N, Bryan NS, Butler A, Cabrales P, Fago A, Feelisch M, Ford PC, et al. Nitrate and nitrite in biology, nutrition and therapeutics. Nat Chem Biol 5:865–869, 2009.

Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov 7:156–67, 2008.

Lundberg JO, Weitzberg E, Lundberg JM, Alving K. Intragastric nitric oxide production in humans: measurements in expelled air. Gut 35:1543–1546, 1994.

Marconi C, Marzorati M, Sciuto D, Ferri A, Cerretelli P. Economy of locomotion in high-altitude Tibetan migrants exposed to normoxia. J Physiol 569:667–75, 2005.

Maréchal G, Beckers-Bleukx G. Effect of nitric oxide on the maximal shortening velocity of a mouse skeletal muscle. Pflügers Arch 436:906-13, 1998.

Martin DS, Gilbert-Kawai ET, Meale PM, Fernandez BO, Cobb A, Khosravi M, Mitchell K, Grocott MP, Levett DZ, Mythen MG, Feelisch M; Xtreme Alps Research Group. Design and conduct of ''Xtreme Alps'': a double-blind, randomised controlled study of the effects of dietary nitrate supplementation on acclimatisation to high altitude. Contemp Clin Trials 36:450–9, 2013.

Masschelein E, Van Thienen R, Wang X, et al. Dietary nitrate improves muscle but not cerebral oxygenation status during exercise in hypoxia. J Appl Physiol 113:736–45, 2012.

McConell GK, Bradley SJ, Stephens TJ, Canny BJ, Kingwell BA, Lee-Young RS. Skeletal muscle nNOS protein content is increased by exercise training in humans. Am J Physiol Regul Integr Comp Physiol 293:821–28, 2007.

McDonough P, Behnke BJ, Padilla DJ, Musch TI, Poole DC. Control of microvascular oxygen pressures in rat muscles comprised of different fibre types. J Physiol 563:903-13, 2005.

McMahon NF, Leveritt MD, Pavey TG. The effect of dietary nitrate supplementation on endurance exercise performance in healthy adults: a systematic review and meta-analysis. Sports Med 47(4):735–56, 2017.

McNarry MA, Lambrick D, Westrupp N, Faulkner J. The influence of a six-week, high-intensity games intervention on the pulmonary oxygen uptake kinetics in prepubertal obese and normal-weight children. Appl Physiol Nutr Metab 40(10): 1012-8, 2015.

Milkowski, A, Garg, HK, Coughlin, JR, et al. Nutritional epidemiology in the context of nitric oxide biology: A risk-benefit evaluation for dietary nitrite and nitrate. Nitric Oxide 15:110–119, 2010.

Modin A, Björne H, Herulf M, Alving K, Weitzberg E, Lundberg JO. Nitrite-derived nitric oxide: a possible mediator of 'acidic-metabolic' vasodilation. Acta Physiol Scand 171(1):9-16, 2001.

Moncada S, Higgs A.The L-arginine-nitric oxide pathway. N Engl J Med 329:2002–12, 1993.

Mowry AV, Donoviel ZS, Kavazis AN, Hood WR. Mitochondrial function and bioenergetic trade-offs during lactation in the house mouse (Mus musculus). Ecol Evol 7(9):2994-3005, 2017.

Muggeridge DJ, Howe CC, Spendiff O, Pedlar C, James PE, Easton C. A single dose of beetroot juice enhances cycling performance in simulated altitude. Med Sci Sports Exerc 46(1):143–50, 2014.

Muggeridge DJ, Howe CC, Spendiff O, Pedlar C, James PE, Easton C. The effects of a single dose of concentrated beetroot juice on performance in trained flatwater kayakers, Int J Sport Nutr Exerc Metab 3: 498–506, 2013.

National Center for Health Statistics. Health, United States, 2015: With Special Feature on Racial and Ethnic Health Disparities. Hyattsville, MD. 2016.

Nisoli E, Carruba MO, Nitric oxide and mitochondrial biogenesis. J Cell Sci 119:2855–2862, 2006.

Nisoli E, Falcone S, Tonello C, Cozzi V, Palomba L, Fiorani M, Pisconti A, Brunelli S, Cardile A, Francolini M, Cantoni O, Carruba MO, Moncada S, Clementi E. Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals. Proc Natl Acad Sci USA 101:16507–16512, 2004.

Omar SA, Artime E, Webb AJ. A comparison of organic and inorganic nitrates/nitrites. Nitric Oxide 26:229–40, 2012.

Pawlak-Chaouch M, Boissiere J, Gamelin FX, Cuvelier G, Berthoin S, Aucouturier J. Effect of dietary nitrate supplementation on metabolic rate during rest and exercise in human: a systematic review and a meta-analysis. Nitric Oxide 29:53:65–76, 2016.

Peeling P, Cox GR, Bullock N, Burke LM. Beetroot juice improves on-water 500 M time-trial performance, and laboratory-based paddling economy in national and international-level kayak athletes. Int J Sport Nutr Exerc Metab 25:278–84, 2015.

Piepoli MF, Coats AJ. The "skeletal muscle hypothesis in heart failure" revised. Eur Heart J 34: 486–488, 2013.

Polgar J, Johnson MA, Weightman D, Appleton D. Data on fibre size in thirty-six humanmuscles. An autopsy study. J Neurol Sci 19:307–18, 1973.

Poole DC, Hirai DM, Copp SW, Musch TI. Muscle oxygen transport and utilization in heart failure: implications for exercise (in)tolerance. Am J Physiol Heart Circ Physiol 302(5):H1050-63, 2012.

Poole DC, Jones AM. Measurement of the maximum oxygen uptake $\dot{V}O_2$max: $\dot{V}O_2$peak is no longer acceptable. J Appl Physiol. 22(4): 997-1002, 2017.

Porcelli S, Pugliese L, Rejch E, Pavei G, Bonato M, Montorsi M, La Torre A, Rasica L, Marzorati M. Effects of short-term high-nitrate diet on exercise performance. Nutrients 8(9): E534, 2016.

Porcelli S, Ramaglia M, Bellistri G, Pavei G, Pugliese L, Montorsi M, Rasica L, Marzorati M. Aerobic fitness affects the exercise performance responses to nitrate supplementation. Med Sci Sports Exerc 47(8): 1643–1651, 2015.

Radegran G, Saltin B. Nitric oxide in the regulation of vasomotor tone in human skeletalmuscle. Am J Physiol 276:H1951-1960, 1999.

Reid MB. Role of nitric oxide in skeletal muscle: synthesis, distribution and functional importance. Acta Physiol Scand 162:401–409, 1998.

Sacks FM, Svetkey LP, Vollmer WM, Appel LJ, Bray GA, Harsha D, Obarzanek E, Conlin PR, Miller ER 3rd, Simons-Morton DG, Karanja N, Lin PH, Aickin M, Most-Windhauser MM, Moore TJ, Proschan MA, Cutler JA; DASH-Sodium Collaborative Research Group. Effects on blood pressure of reduced dietary sodium and the Dietary Approaches to Stop Hypertension (DASH) diet. N Engl J Med 344: 3–10, 2001.

Salvadego D, Lazzer S, Busti C, Galli R, Agosti F, Lafortuna C, Sartorio A, Grassi B. Gas exchange kinetics in obese adolescents. Inferences on exercise tolerance and prescription, Am J Physiol Regul Integr Comp Physiol 299: R1298–R1305, 2010.

Salvadego D, Sartorio A, Agosti F, Tringali G, Patrizi A, Isola M, LoMauro A, Aliverti A, Grassi B. Respiratory muscle endurance training reduces the O2 cost of cycling and perceived exertion in obese adolescents. Am J Physiol Regul Integr Comp Physiol. 313(4): R487-R495, 2017.

Salvadego D, Sartorio A, Agosti F, Tringali G, Patrizi A, Mauro AL, Aliverti A, Grassi B. Acute respiratory muscle unloading by normoxic helium-O2 breathing reduces the O2 cost of cycling and perceived exertion in obese adolescents. Eur J Appl Physiol 115: 99-109, 2015.

Salvadori A, Fanari P, Fontana M, Buontempi L, Saezza A, Baudo S, Miserocchi G, Longhini E. Oxygen uptake and cardiac performance in obese and normal subjects during exercise. Respiration 66: 25–33, 1999.

Scherrer U, Vollenweider L, Delabays A, Savcic M, Eichenberger U, Kleger GR, Fikrle A, Ballmer PE, Nicod P, Bärtsch P. Inhaled nitric oxide for high-altitude pulmonary edema. N Engl J Med 334(10):624-9, 1996.

Shannon OM, McGawley K, Nybäk L, Duckworth L, Barlow MJ, Woods D, Siervo M, O'Hara JP. ''Beet-ing'' the Mountain: A Review of the Physiological and Performance Effects of Dietary Nitrate Supplementation at Simulated and Terrestrial Altitude. Sports Med 47(11):2155-2169, 2017.

Shepherd AI, Gilchrist M, Winyard PG, Jones AM, Hallmann E, Kazimierczak R, Rembialkowska E, Benjamin N, Shore AC, Wilkerson DP. Effects of dietary nitrate supplementation on the oxygen cost of exercise and walking performance in individuals with type 2 diabetes: a randomized, double-blind, placebo-controlled crossover trial. Free Radic Biol Med 86: 200–208, 2015.

Shepherd AI, Wilkerson DP, Dobson L, Kelly J, Winyard PG, Jones AM, Benjamin N, Shore AC, Gilchrist M. The effect of dietary nitrate supplementation on the oxygen cost of cycling, walking performance and resting blood pressure in individuals with chronic obstructive pulmonary disease: a double blind placebo controlled, randomised control trial. Nitric Oxide 48:31–37, 2015.

Siervo M, Jackson SJ, Bluck LJC. In-vivo nitric oxide synthesis is reduced in obese patients with metabolic syndrome: application of a novel stable isotopic method. J Hypertension 29: 1515–1527, 2011.

Sperandio PA, Oliveira MF, Rodrigues MK, Berton DC, Treptow E, Nery LE, Almeida DR, Neder JA. Sildenafil improves microvascular O2 delivery-to-utilization matching and accelerates exercise O2 uptake kinetics in chronic heart failure. Am J Physiol Heart Circ Physiol 303(12): H1474-80, 2012.

Stamler JS, Meissner G. Physiology of nitric oxide in skeletal muscle. Physiol Rev 81:209-237, 2001.

Tengan CH, Rodrigues GS, Godinho RO. Nitric oxide in skeletal muscle: role on mitochondrial biogenesis and function. Int J Mol Sci 13(12): 17160-84, 2012.

Tesch PA, Karlsson J. Muscle fiber types and size in trained and untrained muscles of elite athletes. J Appl Physiol 59:1716–20, 1985.

Thomas GD, Hansen J, Victor RG. Inhibition of alfa2-adrenergic vasoconstriction during contraction of glycolytic, not oxidative, rat hindlimb muscle. Am J Physiol Heart Circ Physiol 266: H920–H929, 1994.

Thomas GD, Victor RG. Nitric oxide mediates contractioninduced attenuation of sympathetic vasoconstriction in rat skeletal muscle. J Physiol 506: 817–826, 1998.

Toledo JC, Augusto O. Connecting the chemical and biological properties of nitric oxide. Chem Res Toxicol 25, 975–989, 2012.

Tschakovsky ME, Joyner MJ. Nitric oxide and muscle blood flow in exercise. Appl Physiol Nutr Metab 33:151-161, 2008.

Umbrello M, Dyson A, Feelisch M, Singer M. The key role of nitric oxide in hypoxia: hypoxic vasodilation and energy supply-demand matching. Antioxid Redox Signal 19:1690-710, 2013.

Van Hall G, Jensen-Urstad M, Rosdahl H, Holmberg HC, Saltin B, Calbet JA. Leg and arm lactate and substrate kinetics during exercise. Am J Physiol Endocrinol Metab 284(1):E193-205, 2003.

Vanhatalo A, Fulford J, Bailey SJ, et al. Dietary nitrate reduces muscle metabolic perturbation and improves exercise tolerance in hypoxia. J Physiol 589:5517–5528, 2011.

Vaughan RA, Gannon NP, Carriker CR. Nitrate-containing beetroot enhances myocyte metabolism and mitochondrial content. J Tradit Complement Med 6(1):17-22, 2015.

Ward ME. Effect of inhibition of nitric oxide synthesis on the diaphragmatic microvascular response to hypoxia. J Appl Physiol 81:1633–1641, 1996.

Wasserman K, Whipp BJ. Exercise physiology in health and disease. Am Rev Respir Dis 112: 219-49, 1975.

Wewege M, van den Berg R, Ward RE, Keech A. The effects of high-intensity interval training vs. moderate-intensity continuous training on body composition in overweight and obese adults: a systematic review and meta-analysis. Obes Rev 18(6): 635 - 646, 2017.

Whitfield J, Gamu D, Heigenhauser GJF, van Loon LJC, Spriet LL, Tupling AR, Holloway GP. Beetroot juice increases human muscle force without changing Ca^{2+}-handling proteins. Med Sci Sport Ex 49(10):2016-2024, 2017.

Whitfield J, Ludzki A, Heigenhauser GJ, Senden JM, Verdijk LB, van Loon LJ, Spriet LL, Holloway GP. Beetroot juice supplementation reduces whole body oxygen consumption but does not improve indices of mitochondrial efficiency in human skeletal muscle. J Physiol 594(2):421–435, 2016.

Woessner MN, McIlvenna LC, Ortiz de Zevallos J, Neil CJ, Allen JD. Dietary nitrate supplementation in cardiovascular health: an ergogenic aid or exercise therapeutic? Am J Physiol Heart Circ Physiol 314(2):H195-H212, 2018.

Woessner M, Smoliga JM, Tarzia B, Stabler T, Van Bruggen M, Allen JD. A stepwise reduction in plasma and salivary nitrite with increasing strengths of mouthwash following a dietary nitrate load. Nitric Oxide 54: 1-7, 2016.

Woessner MN, VanBruggen MD, Pieper CF, Sloane R, Kraus WE, Gow AJ, Allen JD. Beet the Best? Dietary Inorganic Nitrate to Augment Exercise Training in Lower Extremity Peripheral Artery Disease with Intermittent Claudication. Circ Res 2018.

Wolzt M, MacAllister RJ, Davis D, Feelisch M, Moncada S, Vallance P, Hobbs AJ. Biochemical characterization of S-nitrosohemoglobin. Mechanisms underlying synthesis, no release, and biological activity. J Biol Chem 274:28983–28990, 1999.

Wylie LJ, de Zevallos OJ, Isidore T, Nyman L, Vanhatalo A, Bailey SJ, Jones AM. Dose-dependent effects of dietary nitrate on the oxygen cost of moderate-intensity exercise: Acute vs. chronic supplementation. Nitric Oxide 57: 30-39, 2016.

Wylie LJ, Kelly J, Bailey SJ, Blackwell JR, Skiba PF, Winyard PG, Jeukendrup AE, Vanhatalo A, Jones AM. Beetroot juice and exercise: pharmacodynamic and dose–response relationships. J Appl Physiol 115(3): 325–336, 2013.

Zamani P, Rawat D, Shiva-Kuma P, Geraci S, Bhuva R, Konda P, Doulias PT, Ischiropoulos H, Townsend RR, Margulies KB, Cappola TP, Poole DC, Chirinos JA. Effect of inorganic nitrate on exercise capacity in heart failure with preserved ejection fraction. Circulation 131:371-80, 2015.

Zamani P, Tan V, Soto-Calderon H, Beraun M, Brandimarto JA, Trieu L, Varakantam S, Doulias PT, Townsend RR, Chittams J, Margulies KB, Cappola TP, Poole DC, Ischiropoulos H, Chirinos JA. Pharmacokinetics and pharmacodynamics of inorganic nitrate in heart failure with preserved ejection fraction. Circ Res 120(7): 1151-1161, 2017.

Zoladz JA, Gladden LB, Hogan MC, Nieckarz Z, Grassi B. Progressive recruitment of muscle fibers is not necessary for the slow component of V̇O2kinetics. J Appl Physiol 105: 575-80, 2008.