THE EFFECT OF INORGANIC NITRATE ON EXERCISE CAPACITY AND METABOLISM IN HEALTH AND DISEASE

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ABSTRACT

Nitric Oxide (NO) is a key signaling molecule involved in various physiological processes, including vasodilation, mitochondrial function, muscle contraction, glucose and calcium homeostasis, and fatigue resistance during exercise. It is now widely recognized that reduced NO bioavailability is associated with the development and presence of cardiovascular and metabolic diseases and is a contributing factor in exercise intolerance. Inorganic dietary nitrate (NO_3^-) has become a broadly used supplement to increase or restore NO bioavailability in health and disease, respectively. The supplementation response during exercise varies between individuals of different health statuses and fitness statuses, and it is also dependent on the dose and supplementation regime. However, the role of sex on the effect of NO_3^- supplementation in exercise outcomes has not been determined. Thus, the focus of the first two manuscripts was to determine the role of sex in the effects of NO_3^- supplementation on different exercise outcomes. **The focus of Manuscript 1 (Chapter 2) was to *explore the role of sex and NO_3^- supplementation on exercise economy and exercise capacity during moderate-intensity exercise and severe-intensity exercise, respectively***. Despite seeing elevations in plasma NO_3^- and NO_2^- concentrations after the ingestion of NO_3^- supplementation in both sexes, only males seem to benefit from supplementation. While females showed no improvement in exercise economy during a submaximal cycle ergometer test, males showed a ~6% reduction in oxygen consumption at the same power output. Males also showed improvements in exercise capacity during a time-to-exhaustion trial within the severe exercise domain. Taken all together, NO_3^- supplementation seems to benefit males but not females during exercise modalities that rely on the cardiovascular system. **The objective of Manuscript 2 (Chapter 3) was to *determine the role of sex in NO_3^- supplementation on skeletal muscle function and fatigue resistance***. During skeletal muscle contractile function, NO_3^- supplementation seems to have differentiated effects in males and females. Males seem to benefit from NO_3^- supplementation during maximal isokinetic knee extension as

the contraction velocity tested increases. Females, on the other hand, showed a detrimental effect on contractile function measured during isokinetic knee extension and when maximal knee extension power (Pmax) and velocity (Vmax) are estimated. Both sexes showed no improvement on a fatigue resistance protocol. Based on our results, using NO_3^- supplementation as an ergogenic in females should be viewed with caution, and warrants further investigation.

Manuscript 3 (Chapter 4) focuses on NO_3^- supplementation as an exercise therapeutic rather than an exercise enhancer in individuals with cardiovascular disease (CVD). Specifically, individuals with peripheral arterial disease (PAD), which is a disease characterized by diminished vascular NO production, has shown to increase exercise capacity and improve symptoms during walking (i.e. longer pain-free walking). However, the underlying mechanisms are not fully understood. **Thus, the objective of Manuscript 3 (Chapter 4) was to *explore the effect of NO_3^- supplementation on skeletal muscle bioenergetics and tissue perfusion of the calf muscles after peak plantar flexion exercise in patients with PAD*.** After following NO_3^- supplementation, patients with PAD showed a significant increase in circulating plasma NO_2^-. This was accompanied by an increase in claudication-free walking but not peak walk time. Advanced MRI techniques allowed us to assess the effect of NO_3^- supplementation on tissue perfusion and metabolism of skeletal muscle during peak plantar flexion exercise. We found that after supplementation, tissue perfusion increases, and oxidative metabolism is improved. All together suggests that NO_3^- supplementation may be acting as a therapeutic in this clinical population, restoring tissue perfusion and metabolism during exercise, as such, improving patients' symptoms during walking.

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APPROVAL OF THE DISSERTATION

This dissertation, “The Effect of Inorganic Nitrate on exercise performance in Health and Disease”, has been approved by the Graduate Faculty of the School of Education and Human Development in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Jason D. Allen, Committee Chair

Arthur L. Weltman, Committee Member

Christopher M. Kramer, Committee Member

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_____Date

DEDICATION

I dedicate this thesis to Pia, Ignacio, Antenor, my Mum, and Dad. Without them, this journey would not have been possible.

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1. INTRODUCTION

Nitric Oxide (NO) is a key signaling molecule involved in various physiological processes, including vasodilation, mitochondrial function, muscle contraction, glucose and calcium homeostasis, and fatigue resistance during exercise (Brown, 2001; Kobzik et al., 1994; Kumar et al., 2022; Percival, 2011; Stamler & Meissner, 2001). It is now widely recognized that reduced NO bioavailability is associated with the development and presence of cardiovascular and metabolic diseases (Förstermann, 2010; Wu & Meininger, 2009) and a contributing factor in exercise intolerance (Lauer et al., 2008). Traditionally, NO is produced by NO synthases (NOS) from converting the amino acid L-Arginine in the presence of oxygen and required co-factors (Moncada & Higgs, 1993). This canonical pathway, however, can be affected in environments with an excess of reactive oxygen and nitrogen species (RONS), inflammation, or reduced availability of oxygen. For example, in cardiovascular and metabolic disease, chronic RONS leads to NOS-uncoupling with decreased eNOS expression (Forstermann & Sessa, 2012), increased superoxide production (Förstermann & Li, 2011), and increased NO consumption by free radicals (Mancardi et al., 2004; Ridnour et al., 2004). Additionally, environments with reduced oxygen supply at the tissue level, like the ones seen during strenuous exercise, also limit the capacity of the enzyme to produce NO. Therefore, interventions that increase NO bioavailability, which do not depend on the enzymatic NOS pathway, hold great scientific promise for improving disease conditions and reducing exercise stress.

1.1 Nitrate-nitrite-NO pathway

In the last two decades, increasing NO bioavailability from exogenous sources has garnered substantial research interest in healthy subjects and those with cardiometabolic disease (Jones et al., 2018; Weitzberg & Lundberg, 2013). A promising approach is via oral inorganic nitrate (NO_3^-) supplementation. Interestingly, NO_3^- can be found in high concentrations in green leafy vegetables such as spinach, celery, and arugula, as well as in

beets (Hord, 2011; Hord et al., 2009). Through this pathway, ingested NO_3^- is absorbed into the circulation in the upper part of the gastrointestinal tract, then sequestered and concentrated in the mouth by salivary glands (Qin et al., 2012). In the oral cavity, commensal bacteria reduce NO_3^- to nitrite (NO_2^-), as a byproduct of ATP production (Qin et al., 2012; Qu et al., 2016). NO_2^- is then swallowed, and although a portion is reduced to NO in the acidic environment of the stomach (Benjamin et al., 1994; Lundberg & Govoni, 2004), the majority is absorbed into circulation to increase the circulating plasma NO_2^- (Piknova et al., 2021; Villar et al., 2021; Webb et al., 2008). In environments of low oxygen availability and low pH (Castello et al., 2006; Millar et al., 1998; Modin et al., 2001), NO_2^- is reduced to NO by agents including deoxy-hemoglobin, deoxy-myoglobin, and molybdenum-containing enzymes (Cosby et al., 2003; Ortiz de Zevallos et al., 2022; Shiva et al., 2007; Zhang et al., 1998). As such, there could be a complementary interaction between the two NO pathways to maintain NO bioavailability. Specifically, the NOS pathway's activity is inhibited in low oxygen conditions, while the NO_3^-- NO_2^--NO pathway is enhanced (Lundberg et al., 2015). This creates the potential for oral NO_3^- consumption to act as a NO backup source when NOSs may be dysfunctional (Forstermann & Sessa, 2012; Kapil et al., 2020). It is essential to highlight that this pathway is highly dependent on the oral microbiome, and not swallowing saliva after supplementation or using strong antibacterial mouthwash abolishes the increase in plasma NO_2^- (Govoni et al., 2008; Woessner et al., 2016).

1.2 Nitrate in exercise economy and exercise tolerance

The first report about the effects of NO_3^- on exercise was by Larsen et al. in 2007 (2007). They gave young, healthy individuals oral sodium nitrate (~ 6 $mmol.day^{-1}$) for three days, which resulted in a ~3-5% reduction in oxygen consumption during submaximal exercise on a cycle ergometer. Subsequently, similar reductions in oxygen consumption (~5%) were shown after ingestion of beetroot juice (BRJ) for three days

which contained ~5.6 NO_3^- mmol.day^{-1} (Bailey et al., 2009). To date, several studies have confirmed these findings during different exercise modalities, such as cycling (Cermak et al., 2012; Flueck et al., 2016; Larsen et al., 2011; Larsen et al., 2010; Porcelli et al., 2016), walking (Kuennen et al., 2015; Lansley et al., 2011), and running (Lansley et al., 2011). However, others report no such benefits in exercise economy after ingesting NO_3^- (Bescós et al., 2011; Muggeridge et al., 2015; Thompson et al., 2014; Wickham et al., 2019).

Two primary cellular targets have been proposed to explain the underlying mechanisms of the NO_3^--NO_2^--NO-mediated reduced oxygen consumption 1) enhanced mitochondrial efficiency (i.e., lower oxygen cost of ATP production) (Larsen et al., 2011; Whitfield et al., 2016), and/or 2) improved muscle contractile efficiency (i.e., lower ATP cost of muscle force production) (Bailey et al., 2019; Bailey et al., 2009; Hernández et al., 2012). Since the Larsen paper, there has been a surge of scientific interest and publications focusing on the physiological and potential ergogenic effects of NO_3^- supplementation (Figure 1 - *Search Term: ((nitrate[Title/Abstract]) AND exercise[Title/Abstract]), followed by visual inspection of studies*). Unfortunately, many of these studies have relatively small sample sizes, differing exercise modalities, subject health and fitness status, and/or sex (Porcelli et al., 2015; Shannon et al., 2022; Woessner et al., 2018), which may often explain equivocal findings regarding submaximal exercise and exercise tolerance/performance.

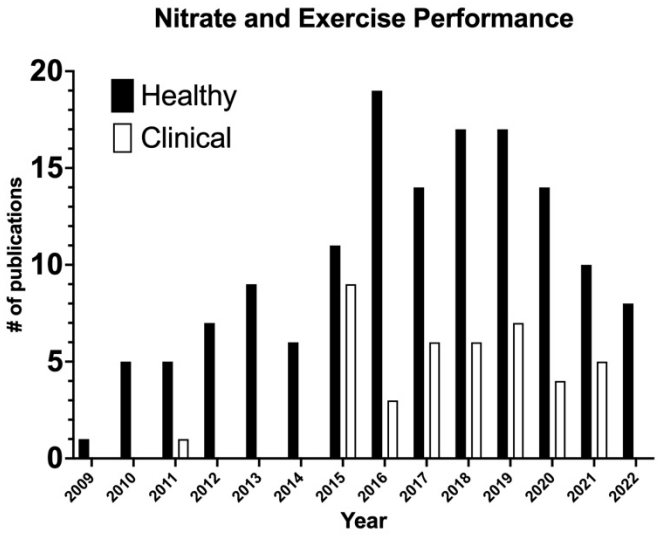

Figure 1. The number of studies assessing the effects of inorganic nitrate on exercise outcomes in health and disease.

Meta-analytic reports generally confirm that exercise capacity measured during a time to exhaustion (TTE) task is enhanced by NO_3^- supplementation (McMahon et al.,

2017; Shannon et al., 2022). Typically, these tests are performed at a work rate above the lactate threshold where no steady state can be reached and is terminated by subjects' volitional exhaustion close to VO_{2peak}. During this higher intensity, relatively short exercise trial, NO_3^- not only improves muscle contractile efficiency, as outlined above, but it also seems to improve tissue perfusion, thus, oxygen delivery, in a fiber type-dependent manner (Ferguson et al., 2013). In support of these ideas, in animal models, dietary NO_3^- supplementation has been shown to increase skeletal muscle blood flow predominantly to type II fibers during exercise, producing increased microvascular and myocyte PO_2 when compared with controls (Ferguson et al., 2013; Ferguson et al., 2015).

1.3 Nitrate in skeletal muscle function and its influence on fatigue resistance.

Within skeletal muscle, NO is mainly produced by neuronal NOS (nNOS) (Percival, 2011), which plays a crucial role in optimizing the muscle's contractile function (Kobzik et al., 1994; Stamler & Meissner, 2001). During muscle contraction, NO initiates posttranslational modifications of the Ryanodine Receptor (RyR), allowing calcium channels to remain open and increasing cytosolic calcium concentration (Kumar et al., 2022). Additionally, increased NO production activates cyclic guanosine monophosphate (cGMP), stimulating myosin light chain phosphorylation and calcium sensitivity, increasing actin-myosin cross-bridge cycling and potentially maximum sarcomere shortening velocity (Maréchal & Gailly, 1999). As such, NO plays a pivotal role in regulating calcium availability and calcium sensitivity within skeletal muscle to maintain optimal contractile function, potentially enhancing twitch force, rate of force development, shortening velocity, and power.

The ergogenic effects of NO_3^- supplementation may be related to NO-mediated improvements in skeletal muscle contractile function (Coggan et al., 2021; Coggan et al., 2018; Coggan et al., 2019; Coggan, Leibowitz, Kadkhodayan, et al., 2015; Coggan, Leibowitz, Spearie, et al., 2015; Coggan & Peterson, 2016, 2018; Gallardo et al., 2020;

Haider & Folland, 2014; Whitfield et al., 2016). Bailey et al. (Bailey et al., 2010), utilizing ^{31}Phosphorus magnetic resonance spectroscopy (^{31}P-MRS), were the first to show that following six days of NO_3^- supplementation in the form of BRJ containing ~5.1 NO_3^- mmol.day^{-1} reduced ATP cost of force production for the same given force output compared to the placebo condition. More recent data show that dietary NO_3^- supplementation can increase maximal speed (i.e., maximal shortening velocity) during an isometric voluntary contraction by 11% and peak power by 6% in young and middle-aged healthy individuals (Coggan, Leibowitz, Kadkhodayan, et al., 2015; Coggan, Leibowitz, Spearie, et al., 2015). Whitfield et al. (2016) showed that in healthy individuals, seven days of dietary NO_3^- (~26mmol NO_3^-.day^{-1}) resulted in increased peak force during induced isometric twitches and accelerated rate of force production. In support of these findings, animal models and ex vivo experiments have shown that 7-day NO_3^- supplementation in mice improves force generated by skeletal muscle accompanied by increased expression of calcium-handling proteins (i.e., calsequestrin-1 and the dihydropyridine receptor) (Hernández et al., 2012). Therefore, the overarching theme of this thesis document is to examine the effects of NO_3^- supplementation on physical function in humans. We explore the effects of supplementation in young healthy individuals with a novel between-sex approach in manuscripts 1 and 2 and then explore the effects in older individuals with PAD in study 3.

1.4 The role of sex in mediating the effects of inorganic nitrate on exercise outcomes

Males and females are physiologically different and can respond differently to various external stressors, including exercise. For example, muscle mass and composition differ between the two sexes. Females have a lower absolute muscle mass and a relatively lower number of type II fibers within the same muscle group than their male counterparts (Callahan et al., 2014; Haizlip et al., 2015). Females also present a reduced capacity to transport and deliver oxygen to working skeletal muscle compared to males (Charkoudian

& Joyner, 2004). This is associated with a smaller heart, thus, a smaller left ventricle (Joyner & Casey, 2015), combined with a ~10-15% lower hemoglobin concentration (Murphy, 2014). Females then show a more considerable arterio-venous oxygen difference during submaximal exercise as a compensatory mechanism (Beltrame et al., 2017). Despite these differences, females still rely on a more oxidative metabolism due to their relatively higher type I fiber number and somewhat higher capillary density (Carter et al., 2001; Høeg et al., 2009; Mauvais-Jarvis, 2015). Moreover, type I fibers have a slower activity of sarcoplasmic reticulum calcium-ATPase producing a slower contraction-relaxation cycle. As such, females cannot produce explosive force to the same extent as their male counterparts; however, this makes females more resistant to muscular fatigue (Bell & Jacobs, 1986; Bemben et al., 1990). A recent study confirmed this by demonstrating that females had a greater time to task failure at the same relative intensity than males (Ansdell et al., 2019).

The underrepresentation of female subjects in dietary nitrate research has been stressed previously (Baranauskas et al., 2022; Senefeld et al., 2020; Wickham & Spriet, 2019). It is not only the physiological differences between sex, which result in different exercise responses, but also young healthy females show higher NO bioavailability. It has been shown that females have higher circulating NO metabolites (i.e., nitrate and nitrite) and a larger nitrate-reducing capacity than males (Kapil et al., 2018). In addition, peak estrogen levels during the late follicular phase of the menstrual cycle are associated with higher serum levels of NO_3^-/ NO_2^- and exhaled NO (Rosselli et al., 1994). These sex differences question the applicability of the current NO_3^- supplementation for females, as the literature is overwhelmingly based on male data (Senefeld et al., 2020). Therefore, studies to investigate the role of sex on NO_3^- supplementation on exercise outcomes are warranted. Our novel approach looking at between-sex differences, is the first study to directly assess the role of sex on NO_3^- supplementation in exercise economy and exercise performance during aerobic exercise (manuscript 1) and muscle contractile function (manuscript 2).

1.5 Inorganic nitrate to increase ambulation in symptomatic Peripheral Arterial Disease

Vascular NO bioavailability is essential for cardiovascular health. A reduction in the ability to produce NO by the vascular endothelium is an early event in the process of atherosclerotic lesion formation and is associated with cardiovascular risk factors and established cardiovascular disease (CVD) (Förstermann, 2010; Wu & Meininger, 2009). Peripheral arterial disease (PAD) is a type of CVD where atherosclerotic lesions develop in the arteries that supply blood to the lower extremities, thus, limiting tissue perfusion (Ouriel, 2001). As such, many patients with PAD suffer from intermittent claudication (IC), defined as pain in one or both legs during walking that is relieved with rest. Among subjects with PAD+IC, 1/3rd have pain during light activity at home, and an additional 1/3rd have pain walking a short distance (one block) (Hiatt, 2001). These patients suffer from a markedly impaired quality of life and a high perception of disability (Olsen et al., 1988). Increased pain-free ambulation is a primary goal of therapy for PAD.

Interestingly, patients with PAD+IC exhibit endothelial dysfunction and reduced endogenous eNOS-derived NO production compared to healthy controls (Polhemus et al., 2015). Treatment options to increase function in PAD are limited, with pharmaceutical approaches mostly ineffective and surgical interventions being invasive, expensive, and unavailable to some patients (Omar et al., 2016; Pabon et al., 2022). In addition, surgical revascularization, although increasing blood flow, does not necessarily translate to improved exercise performance or complete relief of IC symptoms (Regensteiner et al., 1993). As such, oral NO_3^- supplementation may be an attractive approach to increase NO bioavailability in PAD. The efficacy of NO_3^- supplementation as an alternative therapy may be greater than in other clinical populations as PAD+IC present a greater ischemic tissue excursion that favors NO_2^- reduction to NO (Askew et al., 2005; Pipinos et al., 2003). As such, symptomatic PAD (leg ischemia) could be considered an ideal human model to

examine the role of NO_3^- supplementation in the presence of CVD and dysfunctional eNOS.

Many of the underlying mechanisms that may improve exercise performance in patients with PAD+IC following NO_3^- supplementation are the same as for healthy subjects (as outlined above). However, individuals with PAD+IC present several disease-related peripheral maladaptations that lead to pathological tissue dysfunctions, which may benefit from increased NO bioavailability. These include endothelial dysfunction/reduced endogenous NO production (Shantsila et al., 2012; Shechter et al., 2009), capillary density rarefaction (Askew et al., 2005; Chow et al., 2022; Kitzman et al., 2014; Robbins et al., 2011), impaired microcirculation function(Park et al., 2022), skeletal muscle hypoperfusion (Anderson et al., 2009; Groen et al., 2014; Isbell et al., 2007; Sullivan et al., 1990), increased reactive oxygen species (Abdul-Ghani et al., 2009; Hart et al., 2018; Saavedra et al., 2002; Williams et al., 2004) and inflammation (Hart et al., 2018; Kahn et al., 2006), mitochondrial dysfunction (Park et al., 2022; Rosca & Hoppel, 2010), reduced aerobic enzyme activity (Sullivan et al., 1991), and a preferential loss of type I oxidative fibers (Askew et al., 2005; Kitzman et al., 2014). This results in patients with PAD having a more glycolytic phenotype which presents the ideal conditions for NO to have a beneficial effect. As such, in patients with PAD, NO_3^- supplementation may restore reduced levels of bioavailable NO and improve physical performance, fatigue resistance, and recovery. Given the scarcity of other pharmacologic therapeutics for individuals with PAD that delays COT or increases ambulation, novel therapeutics that improve tissue perfusion, mitochondrial respiration, and skeletal muscle strength are of great scientific and clinical interest (Kaso & Annex, 2020).

The first report on the benefits of NO_3^- supplementation in patients with PAD was by Kenjale et al. (Kenjale et al., 2011). They investigated the acute effect of 9 mmol NO_3^- (in the form of BRJ taken 3 hours before testing) on walking exercise capacity in patients with PAD+IC. They showed that claudication onset time increased by 18% (~32 secs) and peak walking time (PWT) by 17% (~65sec) (Kenjale et al., 2011). These improvements were

accompanied by reduced desaturation of oxygenated hemoglobin measured by Near-Infrared spectroscopy (NIRS), suggesting better indices of oxygenation and/or mitochondrial function after NO_3^- supplementation. NIRS has also been used in this population as an indirect measurement of microvascular function, showing significant improvement in the recovery rate of tissue oxygenation index (TOI) (Pekas et al., 2023). Patients showed a much faster recovery rate reaching 100% TOI earlier after consuming dietary NO_3^- (Pekas et al., 2023).

Notwithstanding improvements in non-invasive measures of tissue oxygenation after NO_3^- supplementation, NIRS does not measure tissue perfusion and oxidative metabolism. NIRS measures tissue oxygenation by interacting with non-visible light in the Near-infrared spectra (~650 to 850nm) with hemoglobin and myoglobin, heme-containing molecules, and their oxygenated status (Ferrari et al., 2011; Grassi & Quaresima, 2016). As such, NIRS is limited to a restricted portion of skeletal muscle, and given the heterogeneities of vascular beds and muscle tissue, it is impossible to translate this to the whole muscle group. Therefore, it lacks spatial and temporal resolution. Additionally, muscle composition, fiber orientation within tissue, adipose tissue thickness at the placement location, and skin properties all affect the accuracy of this measurement.

To overcome NIRS limitations for measuring skeletal muscle bioenergetics and/or tissue perfusion, more advanced imaging methodologies have been used in other human studies assessing the efficacy of dietary NO_3^- supplementation. For example, two studies from the same research group have been able to quantify exercise metabolites (i.e., PCr, hydrogen ions, inorganic phosphate) during exercise after NO_3^- supplementation in healthy individuals utilizing a ^{31}P MRS (Bailey et al., 2010; Vanhatalo et al., 2011). They showed that metabolic perturbations during severe exhaustive exercise (which creates hypoxic conditions in the tissue bed analogous to those in patients with symptomatic PAD during mild exercise) are significantly lowered after NO_3^- supplementation (Bailey et al., 2010; Vanhatalo et al., 2011). This was accompanied by increased exercise tolerance and decreased PCr depletion, suggesting improved muscle metabolism/function. Even during

recovery following exhaustive exercise while breathing hypoxic air (~14.5% oxygen), NO_3^- supplementation improved PCr recovery by ~16% compared to the non-supplemented condition in healthy individuals (Vanhatalo et al., 2011). As such, investigating the effects of NO_3^- on the metabolic responses and tissue perfusion of patients' lower limbs during exhaustive exercise with higher spatial and temporal resolution is warranted (Pollak et al., 2012; Sporkin et al., 2022). Therefore, study 3 will expand upon the underlying mechanisms that could explain the improvements in IC and walking capacity after NO_3^- supplementation in the PAD population.

This thesis proposal aims to determine the effect of NO_3^- supplementation on exercise performance in health and disease. The following three manuscripts will investigate the following research questions:

1. **"The effect of inorganic nitrate on exercise economy and endurance performance in young healthy males and females."** This manuscript will explore the role of sex and NO_3^- supplementation on exercise economy and exercise capacity during moderate-intensity exercise and severe-intensity exercise, respectively. All exercise testing will be done on an electronically braked cycle ergometer, and oxygen consumption will be measured.

2. **"Inorganic Nitrate: Sex Differences in Muscle Contractile Function and fatigue resistance."** This manuscript will explore the role of sex in NO_3^- supplementation on skeletal muscle function and fatigue resistance. Skeletal muscle function will be tested during maximal isokinetic knee extension at three angular velocities (i.e., 180, 270, and 360 degrees/second). Muscle fatigue will be tested during repetitive isometric knee extension at 60% maximum voluntary isometric contraction. All testing will be performed on an isokinetic dynamometer.

3. **"The effects of oral inorganic nitrate supplementation on lower limb perfusion and metabolism during exercise in patients with Peripheral Arterial Disease (PAD)."** This manuscript will explore the effect of NO_3^- supplementation on skeletal muscle bioenergetics and tissue perfusion of the calf muscles after peak plantar flexion exercise in patients with PAD. Skeletal muscle bioenergetics will be measured by creatine recovery using Chemical Exchange Saturation Transfer (CEST) images obtained on a 3T MRI scanner. Tissue perfusion will also be measured on the 3T MRI scanner, but images will be obtained by Arterial spin labeling (ASL).

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2. MANUSCRIPT 1: THE EFFECT OF INORGANIC NITRATE ON EXERCISE ECONOMY AND ENDURANCE PERFORMANCE IN YOUNG MALES AND FEMALES.

2.1 Abstract

Inorganic dietary nitrate (NO_3^-) is a widely used supplement purported to provide beneficial effects during submaximal exercise and/or high-intensity, short-duration efforts. Sex may influence the effect of NO_3^- supplementation on exercise outcomes; therefore, the purpose of the present study was to investigate the role of sex on NO_3^- supplementation on bouts of submaximal and maximal exercise.

Methods: In a double-blind, randomized, crossover study, twelve females (23.9±3.6 years) and fourteen males (22.9±3.6 years) completed two 4-minute moderate-intensity exercise bouts followed by a time to exhaustion (TTE) task to failure trial after following 3-days of NO_3^- supplementation (BRJ) or identical NO_3^--depleted placebo (PL). Female subjects were tested during the early follicular phase of the menstrual cycle to control estrogen levels.

Results: After supplementation, both sexes increased plasma NO_3^- and NO_2^- ($p < 0.05$). Females showed a larger increase in plasma NO_2^- than males (F: Δ 264.7 ± 117.2 μM vs M: Δ 161.9 ± 61 μM; $p < 0.05$). During moderate-intensity exercise, BRJ reduced the steady-state VO_2 by ~5% in males ($p < 0.05$) but not in females ($p>0.05$). Similarly, BRJ extended TTE by ~15% in male subjects ($p < 0.05$) but not in females ($p>0.05$).

Conclusion: Dietary NO_3^- supplementation improves exercise economy during moderate-intensity exercise and exercise capacity during severe-intensity TTE in males but not in females. The lack of effect seen in females is despite a larger increase of plasma NO_2^-.

2.2 Introduction

Inorganic nitrate (NO_3^-) supplementation is widely used by recreational and elite athletes as it is purported to provide ergogenic benefits (Burke, 2019; Jones et al., 2018) via reduction to nitric oxide (NO) in low pH and reduced pO_2 tissue environments seen during exercise. Once ingested, NO_3^- is swallowed and absorbed in the small intestine, and while most of it is excreted, about 25% is concentrated in the oral cavity, where it undergoes a two-electron reduction to produce nitrite (NO_2^-), a reaction catalyzed by commensal oral bacteria (Lundberg et al., 2009). NO_2^- is then swallowed, absorbed, and circulates to tissues where it can be reduced to NO by different reducing agents such as deoxygenated hemoglobin, deoxygenated myoglobin, and/or xanthine oxidase (Kapil et al., 2020; Ortiz de Zevallos et al., 2022). The increase in NO bioavailability through NO_3^- supplementation improves exercise economy during submaximal exercise (reduced oxygen consumption for a given workload) (Larsen et al., 2007) and also increases exercise capacity in the severe domain (i.e. above critical power) with most data reported in males (Bailey et al., 2015; Breese et al., 2013; Kelly et al., 2013; Thompson et al., 2014).

Males and females present sex-specific physiologic, morphologic, and anthropometric characteristics which have an impact on exercise endurance and fatigability (Ansdell et al., 2019; Bell & Jacobs, 1986; Bemben et al., 1990; Callahan et al., 2014; Haizlip et al., 2015; Mauvais-Jarvis, 2015). When compared to males, females have reduced oxygen-carrying capacity due to differences in hemoglobin levels, accompanied by lower plasma volume and a smaller left ventricular mass, resulting in a reduced aerobic capacity. Females also present with a lower overall muscle mass and a smaller proportion of type II fibers than their male counterparts (Welle et al., 2008). However, females have a lower PO_2 within skeletal muscle and greater arterial oxygen extraction during exercise (Beltrame et al., 2017). In addition, females demonstrate a ~28-day cycle fluctuation in sex hormones that may impact performance outcomes. Specifically, estrogen increases NO bioavailability by multiple pathways, thus, influencing vascular N-oxides. NO_3^- and NO_2^- have been shown

to be elevated during the late follicular phase of the menstrual cycle (when estradiol is highest) (Ekerhovd et al., 2001; Rosselli et al., 1994). These differences suggest a potential differential effect of NO_3^- supplementation on exercise performance in females compared to males.

The literature, with regards to NO_3^- supplementation and exercise performance, is comprised of studies with small sample sizes consisting of predominantly males (Porcelli et al., 2015; Shannon et al., 2022; Woessner et al., 2018). It is, therefore, unclear if these data are generalizable to females or if there are different responses to supplementation between sexes (Wickham & Spriet, 2019). As such, the purpose of this study was to examine the effect of oral NO_3^- supplementation on exercise economy and exercise capacity in young healthy males and females. The primary hypothesis was that both males and females would exhibit improvements in exercise economy and exercise capacity following dietary NO_3^- supplementation.

2.3 Methods

Experimental Design and Protocol

This study was a randomized, double-blind cross-over design (Figure 1, NCT04588740). Participants reported to the laboratory for initial screening and baseline exercise testing prior to randomization to either inorganic NO_3^- supplementation in the form of beetroot juice (BRJ) or placebo (PL) (See description under *Supplementation procedures*). Female subjects were tested during the early follicular phase (1 – 3 days after menses onset) of the menstrual cycle, as determined by self-report. All study visits were performed by the same researcher, at the same time of day (± 1 hour), and under the same sensory conditions. Females were tested on two consecutive months. Participants were recruited from the University of Virginia and surrounding Charlottesville, Virginia area. All procedures were approved by the Institutional Review Board at the University of Virginia.

Subjects

Subjects were included if they exercised ≤ 3 days/week for less than 30 minutes, were apparently healthy and not aerobically trained (i.e., females ≤ 45 mL/min/kg, males ≤ 50 mL/min/kg), normotensive (<120/80 mmHg), and had no orthopedic limitations to exercise testing. Female subjects must have had regular menstrual periods (minimum of 10-12 menses per year) and were not on any type of oral contraception (n = 10) or were on a monophasic oral contraceptive pill (n = 2) consistently for at least 6 months prior to the study.

Subjects were excluded if they had any known pulmonary, cardiovascular, or metabolic conditions or diseases, any food allergies, if they were taking any medications or supplements that could impact study outcomes in the last 6 months (i.e., pre-workout supplements, L-citrulline, L-arginine, etc.), tobacco use, abnormal blood pressure, currently or recently pregnant or lactating (>1 year), or who contracted Covid-19 during the experimental period.

Screening visit

During the screening visit, anthropometric measurements and resting blood pressure were obtained for all subjects. These measurements were followed by an incremental exercise test on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). The exercise test consisted of a 2-minute warm up at 20 watts followed by 25-watt power output increases every 2 minutes until volitional exhaustion. Subjects were instructed to maintain a pedaling rate of around 80±5 rpm. During all exercise bouts, metabolic measures were obtained via standard open circuit spirometry (Vmax Encore metabolic cart Carefusion), heart rate (HR) was constantly monitored with a HR chest strap linked to a Polar A300 watch, and rating of perceived exertion (RPE) was collected (Borg, 1998). VO_{2peak} was calculated as the highest 30-second average attained before the subject's volitional exhaustion. Gas exchange threshold (GET) was determined by the V-Slope method (Beaver et al., 1986). The power output

associated with 75% of the GET (moderate intensity) and the power output corresponding to 70% of the difference between GET and peak power output (70% Δ - severe intensity) were calculated for subsequent exercise testing.

Supplementation procedures

Subjects were assigned to consume 70mL of beetroot juice (BRJ ~6.5mmol NO_3^-) twice/day (~13mmol total NO_3^-) for ~3 days or an identical NO_3^--depleted placebo (PL). On testing days, subjects were instructed to ingest the last two 70mL shots 2h prior to their laboratory arrival time while remaining otherwise fasted. Both supplements were provided by the same company to guarantee similar taste and appearance (Beet It Pro, James White Drinks, LTD). Female subjects were given additional bottles and were instructed to start consuming the juice the day before the estimated day of menses to consider any changes in the start of the menstrual cycle and guarantee consumption of at least 3 days of supplementation before experimental visits.

Experimental Procedure

During experimental visits, subjects underwent ~10min of supine rest prior to vascular assessments. First, as per manufacturer's instructions, Pulse Wave Analysis (PWA) was performed using a SphygmoCor for which an arm cuff was placed on the subjects' upper arm, aligning the designated markings with the brachial artery. The automated system then measured pulsations recorded at the brachial artery to produce central aortic pressure waveforms. Pulse wave velocity (PWV) was measured via a simultaneous comparison of the carotid pulse (via applanation tonometry) and the femoral pulse via a specialized thigh cuff.

After completion of the vascular tests, subjects completed two bouts of 2-minutes of cycle ergometer exercise at 20watts followed by 4-minutes at 75% GET (moderate-intensity). Each bout was separated by a 5 min passive recovery.

Upon completion of the two moderate-intensity exercise bouts and after an additional 5 minutes of passive recovery, subjects performed an exercise bout at a workload set at 70% Δ GET and VO_{2peak} (severe intensity domain) until exhaustion. They were encouraged to pedal for as long as they could and were blinded to the time expired. When pedaling rate fell by >10rpm despite verbal encouragement by the researchers, the test was terminated, and time to exhaustion (TTE) task was recorded. Data from the two moderate-intensity bouts were averaged to improve signal-to-noise ratio.

Blood sampling – Plasma nitrate and nitrite

Prior to the exercise test, blood was drawn from an antecubital vein into a NO_3^- free syringe (BD Luer-Lok™) (~5mL) for subsequent NO_3^- and NO_2^- analysis. Plasma NO_3^- and NO_2^- were assessed via ozone-based chemiluminescence using a Sievers NOA model 280i (GE Analytical Instruments, Boulder, CO, USA) as previously described (1, 2). Briefly, plasma samples for NO_3^- analysis were deproteinized using cold ethanol precipitation in a 1:3 dilution (plasma:ethanol) followed by a 30-minute incubation before being centrifuged at 14,000g for 10-min. The supernatant was removed for the subsequent NO_3^- analysis in the presence of vanadium chloride in hydrochloric acid at 95°C. The NO_2^- of the undiluted (non-deproteinized) plasma samples was determined by its reduction to NO in the presence of glacial acetic acid and potassium iodide as previously explained (Kenjale et al., 2011).

Statistical analysis

The between-sex demographic data was examined using a two-tailed unpaired Student T-test. A Mixed-model Analysis of variance (ANOVA) with Sidak's adjustments for multiple comparisons was used to determine differences between treatment (PL vs BRJ) and between sex for VO_2, HR, RPE, TTE (sec), and vascular measures. For HR and RPE at several time points within the same TTE test (3-min, 5-min, and at exhaustion) a three-way ANOVA (Supplement*Sex*Timepoint) was used. All statistical analysis was

conducted using GraphPad Prism Version 9.3 (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). GraphPad Prism was also utilized for the creation of all graphs and figures. Biorender (www.biorender.com) was used to create the study design figure. Data are reported as mean ± SD unless otherwise stated, with $p<0.05$ required for statistical significance.

2.4 Results

Participant characteristics

Twelve young healthy female subjects (23.9 $\pm$ 3.6 years) and fourteen young healthy males (22.9 $\pm$ 3.6 years) participated in the study. Although there was no statistical difference in age and/or BMI, as expected, males were taller and heavier and had a higher peak power output and VO_{2peak} during the screening visit (Table 1; $p < 0.01$ for all).

Plasma nitrate and nitrite in males and females

Plasma NO_3^- and NO_2^- data is summarized in Figure 2. During the PL supplementation phase, males and females showed no significant difference in plasma NO_3^- or plasma NO_2^-. After supplementation, both sexes showed a significant increase in plasma NO_3^- and plasma NO_2^- compared to PL ($p \leq 0.001$). Females showed a larger increase in plasma NO_2^- when compared to males (Females: Δ 264.7 ± 117.2 μM vs Males: Δ 161.9 ± 61 μM; $p < 0.05$).

All subjects were given the same absolute inorganic NO_3^- dose (~13mmol ~2 hours prior to testing), which resulted in a significantly lower dose normalized to body weight for males than females for plasma NO_3^- and NO_2^- ($p < 0.001$, figure 2 C and D).

The effect of nitrate supplementation on resting hemodynamics

NO_3^- supplementation did not impact any of the cardiovascular measures depicted in Table 2 (all $p > 0.05$; Table 2). Males had higher pSBP and aSBP than females when compared to the same supplementation condition ($p < 0.05$).

The effect of inorganic nitrate on oxygen consumption during moderate-intensity exercise

Changes between treatments on VO_2 at 20 watts and during moderate-intensity exercise are depicted in Figure 3 and summarized in table 3. At both power outputs, there was a significant interaction effect between sex and supplement ($p \leq 0.01$) for VO_2. VO_2 at 20 watts was significantly reduced after supplementation in males (PL: 932.2 ± 112 vs BRJ 861.9 ± 163.4 $ml.min^{-1}$; $p = 0.02$) but not in females (PL: 723.3 ± 94.7 vs BRJ 756.7 ± 117.6 $ml.min^{-1}$; $p = 0.43$). Similarly, end-exercise VO_2 consumption during 75% GET between treatments was reduced in males (PL: 1420.3 ± 187.3 vs BRJ 1333.5 ± 194.6 $ml.min^{-1}$; $p < 0.05$) but not in females (PL: 1011.2 ± 195.4; BRJ 1017.6 ± 237.6 $ml.min^{-1}$; $p < 0.05$). HR and RPE at end-exercise were not significantly different between PL and BRJ for either sex (data not shown). Submaximal power outputs, as expected, were significantly higher in males than in females (Females: 54.1±16.9 vs Males: 80.9±12.0; $p \leq 0.001$).

The effect of inorganic nitrate on time to exhaustion task in the severe domain

The effect of BRJ on TTE is depicted in Figure 4 and summarized in table 4. BRJ supplementation significantly increased TTE in males (PL: 463.1 ± 184.1; BRJ 531.3 ± 256.2 sec; $p < 0.05$) but not in females (PL: 426.1 ± 142.1s; BRJ 404.1 ± 153.4 sec; $p > 0.05$). HR and RPE at 3-min, 5-min, and at volitional exhaustion were not significantly different between treatments in either sex (data not shown). VO_{2peak} achieved at task failure was not significantly different between treatments (data not shown). Power outputs during the TTE were relative to each participant's peak exercise capacity; thus, on average, men

showed a significantly higher power out for this test (Females: 131.6±35.2; Males: 199.1±26.6; $p \leq 0.001$).

2.5 Discussion

To our knowledge, this is the first study to examine the impact of sex on the effect of oral NO_3^- supplementation on exercise economy and exercise capacity. After controlling for phase of the menstrual cycle, our findings suggest that NO_3^- supplementation, in the form of BRJ, improves submaximal exercise economy as well as TTE exercise capacity in males but not in females. This occurred despite females exhibiting a greater increase in absolute plasma NO_2^- after consuming the same absolute dose of NO_3^-.

Differentiated response to inorganic nitrate supplementation in males and females.

In the present study circulating plasma NO_3^- and NO_2^- during the PL supplementation phase was not different between males and females (Figure 2A and B), which differs from prior reports, in which females show a higher circulating baseline NO_2^-, but not NO_3^-, when compared to males (Kapil et al., 2018). However, previous studies did not control for hormonal fluctuation during the menstrual cycle. Our findings may be related to the fact that females were tested during the early follicular phase, where differences between sexes in circulating levels of estrogen between sex are expected to be minimized. Estrogen levels during the early follicular phase are associated with lower circulating NO_3^- and NO_2^- when compared to the late follicular phase (Rosselli et al., 1994).

After NO_3^- supplementation, absolute plasma NO_3^- increased in both males and females, with absolute plasma NO_2^- 33% higher in females than in males (Figures 2A and 2B). This suggests females might convert NO_3^- to NO_2^- at a greater rate than males, resulting in higher circulating plasma NO_2^-, as previously reported in saliva measures (Kapil et al., 2018). Additionally, lower body mass in females results in the same absolute dose of NO_3^- being 30% greater than in males when normalized to body weight (male: 0.17

mmol/kg BW vs female: 0.21 vs 0.17 mmol/kg BW, respectively). Accordingly, relative (to mass) plasma NO_3^- concentration was higher in females than males (Figures 2C and 2D). While to date, there is insufficient evidence to determine an optimal NO_3^- dosage regimen for females, it appears that females may require lower NO_3^- doses to achieve similar circulating NO_3^- and NO_2^- concentrations than males. Additionally, a higher relative dose may not produce performance benefits during exercise (Gallardo et al., 2020).

Effect of inorganic nitrate on vascular measures in males and females.

The blood pressure lowering effect of NO_3^- supplementation has been reported in clinical populations (Berry et al., 2015; Curtis et al., 2015; Kenjale et al., 2011) as well as in healthy volunteers (Bailey et al., 2017; Kapil et al., 2018; Vanhatalo et al., 2010; Webb et al., 2008; Wylie et al., 2013). Reductions are typically between 4-5 mmHg for systolic blood pressure and between 1-2 mmHg for diastolic blood pressure (Jackson et al., 2018; Siervo et al., 2013) and have been demonstrated following both acute and chronic NO_3^- supplementation (Thompson et al., 2017; Wylie et al., 2016). However, NO_3^- supplementation does not always lower BP (Beijers et al., 2018; Cermak et al., 2012; Gilchrist et al., 2013; Larsen et al., 2010; Shepherd et al., 2015; Wilkerson et al., 2012). The lack of effect in the current study could be related to our sample of young healthy volunteers who present with already low blood pressure. This is in accordance with the principle that the extent to which BP will be lowered by an intervention will be dependent on the basal blood pressure (Law et al., 2003).

Blood pressure in the current study was taken in a supine position compared with seated in most (if not all) other studies. Seated measures may result in higher systolic and diastolic blood pressures (Bartling et al., 2021; Privšek et al., 2018), likely to compensate for the increased effects of gravity (Smith et al., 1970). Perhaps NO_3^- has a greater sympatholytic-like effect when seated.

The effect of NO3- supplementation on oxygen consumption during exercise at moderate intensity in males and females.

In the present study NO_3^- supplementation reduced oxygen cost of submaximal exercise in males (~5%) but not in females (Figures 3A and 3B). This was achieved with no changes in HR or RPE by the end of the submaximal exercise bouts. Our findings in males are similar to previous studies (Larsen et al., 2011; Larsen et al., 2007; Porcelli et al., 2015; Wylie et al., 2013; Wylie et al., 2016), and the lack of effect in females has also been reported (Forbes & Spriet, 2022; Wickham et al., 2019). However, this is the first study designed to directly compare the role of sex in exercise economy following NO_3^- supplementation. Generally, females have a better exercise economy during submaximal exercise when compared to males (Beltrame et al., 2017; Hopker et al., 2010). Non NO-dependent physiological differences that may contribute to these sex difference responses may include differences in contractile function (Wüst et al., 2008), mitochondrial efficiency and function (Miotto et al., 2018; Montero et al., 2018), skeletal muscle antioxidant capacity (Barp et al., 2002; Borrás et al., 2003), and/or capillary density per given muscle area (Høeg et al., 2009; Roepstorff et al., 2006).

Increases in exercise economy following NO_3^- supplementation has been attributed to a reduced ATP cost of cross-bridge formation (Whitfield et al., 2016) and/or improved oxygen cost of ATP resynthesis (Larsen et al., 2011). However, the latter has been recently challenged, suggesting that improvement in excitation-contraction efficiency may be a stronger candidate to explain improvements in exercise economy (Whitfield et al., 2016). Data from animal models and single skeletal muscle fibers show that treatment with NO_3^- / NO_2^- increases intracellular calcium handling and force production (Bailey et al., 2019; Hernández et al., 2012), potentially mediated by the role that NO has in increasing calcium ATPase efficiency (Ishii et al., 1998). As such, the lack of effect of NO_3^- supplementation on submaximal exercise economy in females may be related to the fact that they already show an improved calcium ATPase efficiency (i.e. calcium uptake/ATPase activity) (Harmer et al., 2014) associated with sex-hormones (Fanò et al., 2001). Specifically,

estrogen has a direct impact on calcium handling proteins and supports normal contractile function (Bupha-Intr & Wattanapermpool, 2006).

Another mechanism for improvements in contractile function efficiency following NO_3^- supplementation has been proposed recently by Whitfield et al (Whitfield et al., 2016). Their data suggests that there is an increased production of hydrogen peroxide and reactive oxygen species by mitochondria during submaximal exercise after NO_3^- supplementation. In response to this transient increase in redox molecules, there is a direct impact on force production and mechanical efficiency (Andrade et al., 1998; Andrade et al., 2001). However, the relatively higher antioxidant capacity within skeletal muscle in females could be blunting the transient increase of these important signaling molecules, thus, limiting the associated improvements in contractile function (Barp et al., 2002; Borrás et al., 2003).

Alternatively, intrinsic characteristics of mitochondria as well as better tissue perfusion, may contribute to the lack of effect in females. Females show a higher mitochondrial preference for lipid oxidation (Miotto et al., 2018) and greater capillary density per given muscle area (i.e. better tissue perfusion) (Høeg et al., 2009; Roepstorff et al., 2006). It has been proposed that NO_3^- supplementation shifts skeletal muscle cells to a higher glycolytic activity, therefore, reducing oxygen consumption without improvements in mitochondrial function (Wynne & Affourtit, 2022). Taken together, sex-specific characteristics of skeletal muscle and mitochondria might also explain why males show improvements during submaximal exercise and females do not.

The effect of NO_3^- supplementation on exercise capacity during severe-intensity exercise in males and in females.

A second novel finding of the present study is that NO_3^- supplementation improves exercise capacity in males (~15%) but not in females (Figure 4). Surprisingly, NO_3^- supplementation in females resulted in a non-significant reduction in exercise capacity by ~5%.

Our finding in males is in accordance with most of the literature. In fact, recent meta-analysis suggested that TTE may be the exercise outcome most like to show an increase following NO_3^- supplementation (McMahon et al., 2017; Senefeld et al., 2020). In females, there is a dearth of data to produce clear conclusions (Baranauskas et al., 2022; Senefeld et al., 2020; Wickham & Spriet, 2019). Previously, Wickham et al., reported a lack of response to NO_3^- supplementation on a cycle time trial test (Wickham et al., 2019). The current study is the first study to perform a head-to-head comparison of the effect NO_3^- supplementation between sex.

Our hypothesis that both males and females would increase TTE was based on the fact that severe-intensity exercise elicits a dramatic drop in oxygen tension and pH (Richardson et al., 2006; Richardson et al., 2015; Rossiter et al., 2002) and recruits a higher proportion of type II fibers (MacINTOSH et al., 2000; VØLLESTAD et al., 1984). Thus, it was thought that NO_2^- would be reduced to NO regardless of physiological differences between sexes. This increased NO bioavailability should improve oxygen delivery to exercising muscle, mitochondrial respiration efficiency, and reduce the ATP cost of muscle contraction, ultimately improving exercise capacity (Ferguson et al., 2013; Ferguson et al., 2015; Jones et al., 2018; Lundberg & Weitzberg, 2022).

The physiological explanation for the lack of effect in females is not clear but could be due to a combination of factors. Females have a lower relative proportion of type II fibers as well as a higher capillary density when compared to males (Carter et al., 2001; Roepstorff et al., 2006; Welle et al., 2008), and it has been shown that NO_3^- supplementation is more likely to benefit glycolytic phenotypes (Bailey et al., 2015; Breese et al., 2013) due to their relatively lower oxygen tension during exercise, improved blood delivery in a fiber type-dependent manner (Ferguson et al., 2015) and increased force production .

A potential explanation for the performance decrease may be related to the differences in relative NO_3^- dosage. It has been previously demonstrated that higher doses of inorganic NO_3^- do not necessarily result in higher NO bioavailability or performance (Gallardo et al.,

2020; Wylie et al., 2013). For example, reduced contractile function of skeletal muscle (Gallardo et al., 2020), as well as reductions in peak oxygen consumption (Bescos et al., 2011; Larsen et al., 2010), have been reported previously. Additionally, higher concentrations of circulating NO_3^- and NO_2^- may alter the redox balance of the tissues. Specifically, NO_3^- has been shown to compete with NO_2^- and inhibit XOR's capacity to reduce NO_2^- to NO (Damacena-Angelis et al., 2017; Ortiz de Zevallos et al., 2022; Williams et al., 2023). Additionally, during high-intensity exercise, there may be an increased production of peroxynitrite which could be affecting NO bioavailability within skeletal muscle (Gholami et al., 2019).

Study Limitations

We provided all subjects the same absolute dose, which resulted in a relatively higher NO_3^- dose in females. This dose resulted in ~50% higher circulating NO_3^- and ~80% higher circulating NO_2^- in females when plasma values were normalized to BW. It is possible that the dosing strategy used here may have been too high for females resulting in detrimental rather than beneficial effects during exercise.

The present study used two-step transitions to assess the VO_2 response during moderate-intensity exercise. Tan et al (Tan et al., 2022) have shown that more step transitions may be required to be able to detect the oxygen-lowering effect of NO_3^- supplementation. Finally, the present study only examined supplementation during the early follicular phase of the menstrual cycle. Whether these findings remain consistent during other phases of the menstrual cycle requires additional investigation.

2.6 Conclusion

Data from the present study indicate that males improve exercise economy during submaximal exercise and exercise capacity during a severe-intensity TTE, whereas females

showed no ergogenic benefits and may potentially experience a reduced exercise capacity, although this requires further investigation.

Based on these findings, current suggestions for NO_3^- supplementation, based on studies where samples are exclusively male subjects, are not necessarily applicable to females (Shannon et al., 2022). These data have important practical implications when considering supplementation in females as it seems to affect males and females in different manners, with supplementation in females potentially being detrimental to exercise performance. Future studies should consider different NO_3^- doses (potentially normalized to BW) and study designs, which could help elucidate the underlying mechanisms of the differentiated effect of NO_3^- supplementation in males and females.

2.7 Tables

TABLE 1. Subject Characteristics

	All	**Female**	**Male**	***p*-value**
Number	26	12	14	
Age	23.4 ± 3.5	23.9 ± 3.6	22.9 ± 3.6	0.48
Height (m)	1.68 ± 10.5	1.60 ± 8.1	1.75 ± 5.7	<0.0001
Weight (Kg)	70.3 ± 12.6	61.4 ± 7.4	77.9 ± 11.2	0.0002
BMI	24.7 ± 3.1	24.2 ± 3.5	25.2 ± 2.7	0.41
VO_2 peak (ml/kg/min)	38.7 ± 7.8	34.4 ± 5.9	41.7 ± 7.4	0.006
Peak power output (W)	200.8 ± 55.9	157.1 ± 42.7	238.2 ± 34.7	<0.0001

BMI, body mass index; W, watts; VO_2peak, maximal oxygen uptake. Data presented as mean ± SD. *p*-value = Student T-test male vs female.

TABLE 2. The effect of inorganic nitrate or placebo on cardiovascular measures.

	All Subjects (n = 26)		Female (n = 12)		Male (n = 14)	
	Placebo	**Nitrate**	**Placebo**	**Nitrate**	**Placebo**	**Nitrate**
SBP (mmHg)	114.5 ± 8.7	114.5 ± 7.9	109.5 ± 7.6	108.8 ± 4.1	118.7 ± 7.4*	119.4 ± 7.2*
DBP (mmHg)	66.4 ± 7.5	67 ± 5.9	65.8 ± 8.7	66.1 ± 6.2	67 ± 6.5	67.9 ± 5.8
MAP (mmHg)	79.5 ± 8	79.5 ± 5.7	78.2 ± 9.1	77.5 ± 5.5	80.6 ± 7.2	81.1 ± 5.4
Aortic SBP (mmHg)	99.7 ± 8.1	99.7 ± 6.3	96.2 ± 8.6	95.7 ± 5	102.7 ± 6.4¥	103.1 ± 5.3*
Aortic DBP (mmHg)	67.7 ± 7.6	68.1 ± 5.9	67.7 ± 8.8	66.7 ± 6.4	67.7 ± 6.7	69.4 ± 5.4
PWV (m/s)	4.6 ± 0.9	4.9 ± 0.8	4.4 ± 0.6	4.6 ± 0.7	4.8 ± 1	5 ± 0.9

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, Mean arterial pressure; PWV, Pulse Wave Velocity. Data presented as mean ± SD. ¥ $p < 0.05$ Males vs females - same supplementation condition and, * $p < 0.05$ Males vs females - same supplementation condition.

TABLE 3: The Effects of inorganic nitrate or placebo on oxygen consumption during submaximal.

	All Subjects		Females		Males	
	Placebo	Nitrate	Placebo	Nitrate	Placebo	Nitrate
Warm-Up						
Power output (watts)	20 ± 0		20 ± 0		20 ± 0	
VO_2 ($mL.min^{-1}$)	825.9 ± 156.5	823.2 ± 142.7	714.3 ± 106.8	765.7 ± 102.6	921.6 ± 126.7	872.6 ± 156.8*
Heart Rate (bpm)	100.9 ± 15.6	99.7 ± 13	103.6 ± 15.2	105.3 ± 19.5	96.7 ± 10.5	95.9 ± 9.9
RPE	8 ± 1	8 ± 1	8 ± 1	8 ± 1	8 ± 1	8 ± 2
Moderate-Intensity						
Power output (watts)	68.5 ± 19.7		54.1 ± 16.9		80.9 ± 12.0 ¥	
VO_2 ($mL.min^{-1}$)	1231.5 ± 279.8	1187.7 ± 265.2	1011.2 ± 195.4	1017.6 ± 237.6	1420.3 ± 187.3	1333.5 ± 194.6*
Heart Rate (bpm)	122.9 ± 18.9	121.3 ± 16.8	126.1 ± 20.9	127.2 ± 22.8	119.1 ± 14.6	116.9 ± 11.2
RPE	9.8 ± 1.8	9.5 ± 2	10 ± 1.5	10.2 ± 1.4	9.6 ± 2.1	9 ± 2.2

W, watts; VO_2, oxygen uptake; RPE, Rate of Perceived Exertion. Data presented as mean ± SD. * $p < 0.05$ compared to PL, and ¥ $P < 0.01$ Female vs Males.

TABLE 4: The effects of inorganic nitrate or placebo on exercise capacity during severe intensity exercise.

	All Subjects		Female		Male	
	Placebo	**Nitrate**	**Placebo**	**Nitrate**	**Placebo**	**Nitrate**
Power output (W)	167.9 ± 45.2		131.6 ± 35.2		199.1 ± 26.6 ¥	
Heart Rate						
3 min (bpm)	167 ± 12	169 ± 13	170 ± 13	170 ± 15	165 ± 12	168 ± 11
5 min (bpm)	177 ± 11	179 ± 12	177 ± 12	180 ± 14	177 ± 11	177 ± 11
Peak (bpm)	183± 10	183 ± 10	181 ± 11	182 ± 12	184 ± 9	184 ± 8
RPE						
3 min	17 ± 1	17 ± 2	15 ± 2	15 ± 2	15 ± 2	15 ± 2
5 min	15 ± 2	15 ± 2	17 ± 1	17 ± 2	17 ± 1	17 ± 2
Failure	19 ± 1	19 ± 1	19 ± 1	19 ± 1	19 ± 1	19 ± 1
Exhaustion						
$VO_{2\ Peak}$ ($mL.min^{-1}$)	2641.7 ± 718.1	2625.4 ± 730	2085.5 ± 520.9	2064.1 ± 494.5	3118.5 ± 481.3	3106.5 ± 527.1
TTE (s)	446 ± 166	472.6 ± 224.3	426.1 ± 142.1	404.1 ± 153.4	463.1 ± 184.1	531.3 ± 256.2*

W, watts, VO_2, oxygen uptake. BPM, beats per minute. Data presented as mean ± SD. * $P < 0.05$ compared to PL, and ¥ $P < 0.01$ Female vs Males.

2.8 Figures

FIGURE 1. Study design

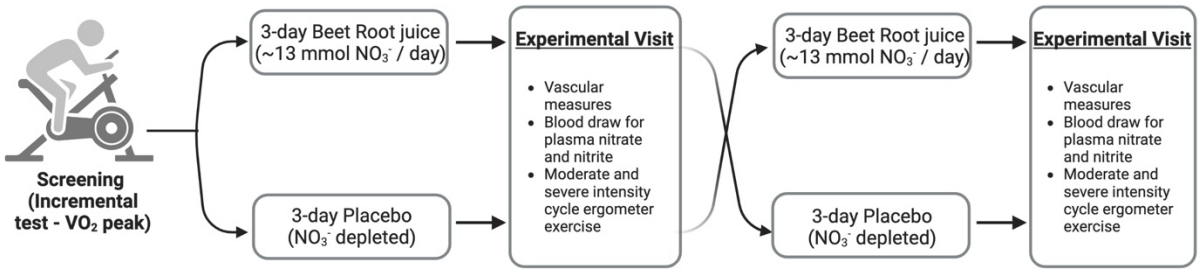


FIGURE 2. ***The effect of inorganic nitrate or placebo on plasma nitrate and nitrite.*** Absolute values for (A) plasma nitrate and (B) plasma nitrite. Plasma concentration relative to body weight (BW) in kg for (C) plasma nitrate and (D) plasma nitrite; Placebo conditions (white bars) and BRJ (grey bars). Values are mean ± SD. ✻ significant difference compared to PL ($p < 0.05$). ✻✻✻ significant difference compared to PL ($p < 0.0001$).

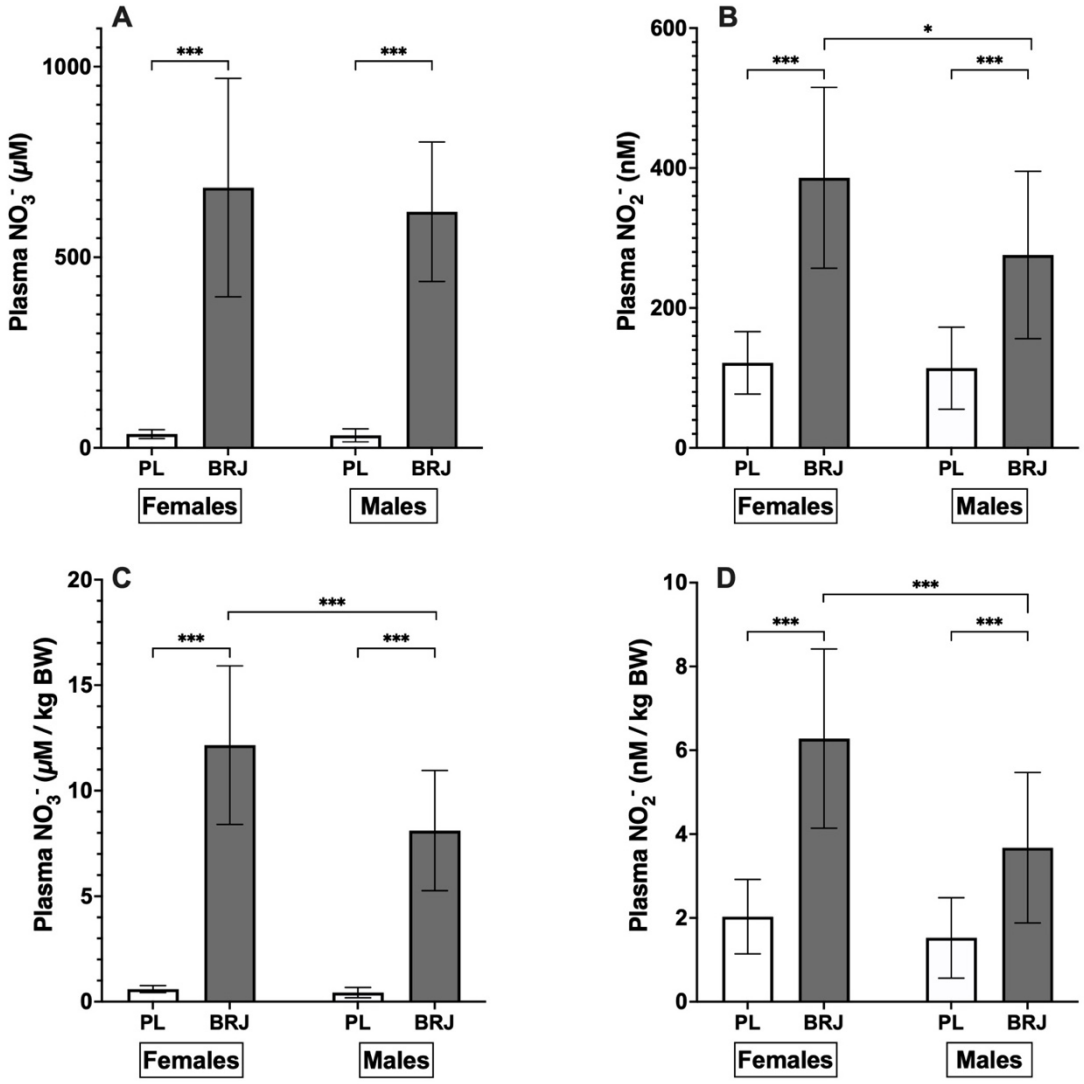


FIGURE 3. The effect of inorganic nitrate on oxygen consumption during moderate intensity exercise. VO_2 consumption at (A) 20W and (B) end-exercise (last 60" of exercise) after beetroot juice (BRJ – closed circles) supplementation relative to placebo (PL – open circles) condition in males and females. Values are mean ± SD. ✻ significant difference compared to PL ($p < 0.05$), ✻✻ significant difference compared to PL ($p < 0.01$).

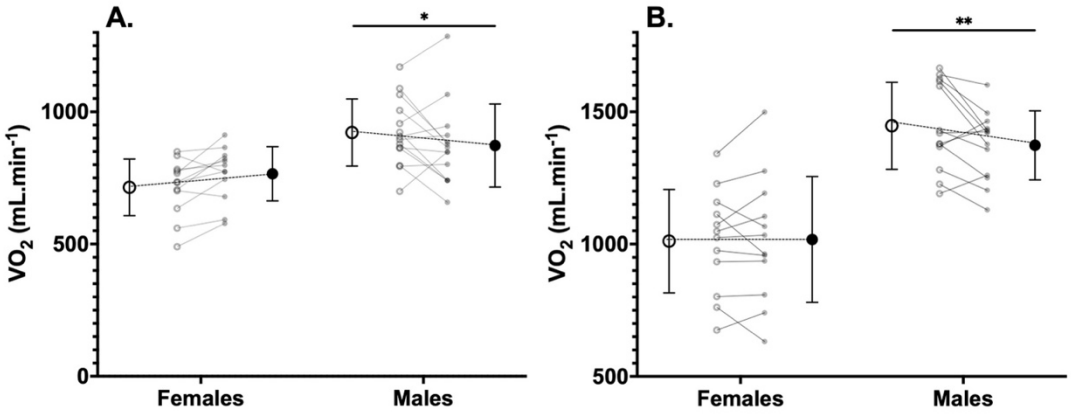
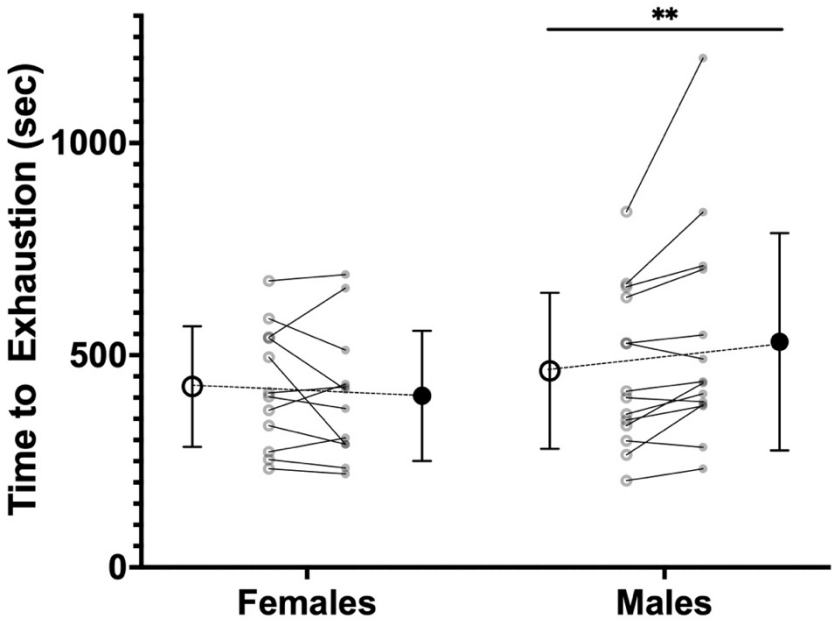


FIGURE 4. ***The effect of inorganic nitrate or placebo on measures during severe-intensity exercise.*** Time-to-exhaustion (seconds) after beetroot juice (BRJ – closed circles) supplementation relative to placebo (PL – open circles) condition in males and females. Values are mean ± SD. ✻✻ significant difference compared to PL ($p < 0.01$).



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3. MANUSCRIPT 2: INORGANIC NITRATE: SEX DIFFERENCES IN MUSCLE CONTRACTILE FUNCTION AND FATIGUE RESISTANCE.

3.1 Abstract

Nitric Oxide (NO) plays a pivotal role in muscle contractile function. Inorganic nitrate (NO_3^-) supplementation has been demonstrated to increase NO bioavailability and potentially act as an ergogenic aid in subjects with NOS dysfunction. Exercise responses to NO_3- in healthy young adults are conflicting, and few studies have examined sex differences.

Purpose: To determine the effects of NO_3^- supplementation on skeletal muscle force development and endurance during knee extension in healthy males and females.

Methods: Seven females (age 23.9±3.6y) and ten males (age 22.9±3.6y) were randomized in a double-blind, placebo-controlled crossover study. Female subjects were tested during the early follicular phase of the menstrual cycle to control estrogen levels. Subjects ingested either 70ml beetroot juice (6.5mmol NO_3-) twice/day (13mmol total) or an identical NO_3^--depleted placebo (PL). On testing days, they ingested (13mmol) 2h prior to arrival. Knee extension contractile function was assessed using a Biodex 4 isokinetic dynamometer at speeds of 180°, 270°, and 360°/sec during 10 consecutive maximal efforts with a 30sec rest between each speed. After 5min rest, a time to fatigue (TTF) protocol consisting of cycles of 3 sec at 60% maximal isometric voluntary contraction and 2-sec rest was performed. An inability to maintain the 60% threshold on 3 occasions despite encouragement was considered TTF.

Results:

Peak power (W/kg) at the two fastest speeds tested (i.e. 270 and 360°/sec) showed a significant effect for sex and for the interaction of sex and treatment, with females showing a reduction with BRJ when compared to PL condition ($p < 0.05$). Estimated maximal knee extension power (Pmax) and maximal knee extension velocity (Vmax) also demonstrated a sex-by-treatment effect ($p < 0.05$). There were no significant differences for sex, treatment or interaction for TTF (females PL; 269.14±161.17 vs BRJ; 277.14±157.50 sec; $p > 0.05$: males PL; 228.20±171.13 vs BRJ;194.13±99.65 sec; $p > 0.05$).

Conclusion: Dietary nitrate supplementation seems to have differentiated effects in males and females. The latter showed reductions in maximal isokinetic knee extension at the highest speed tested.

3.2 Introduction

Dietary inorganic nitrate (NO_3^-) supplementation has been shown to improve nitric oxide (NO) bioavailability in mammals through its absorption and metabolism by the enterosalivary pathway (Weitzberg & Lundberg, 2013). Oral inorganic nitrate is purported increase physical performance and has gained popularity as an ergogenic aid (Burke, 2017, 2019; Jones et al., 2018). However, the effects of NO_3^- supplementation on muscle contractile function remain equivocal, with some studies showing benefits in maximal speed (i.e., maximal shortening velocity) during isometric voluntary contraction and peak power in humans (Coggan, Leibowitz, Kadkhodayan, et al., 2015; Hernández et al., 2012; Kadach et al., 2023; Whitfield et al., 2017), but others finding no improvements (Kokkinoplitis & Chester, 2014; Kramer et al., 2016; Lago-Rodríguez et al., 2020; Wickham et al., 2019). Moreover, the majority of the participants in previous studies are male. To date the effects of NO3- supplementation on skeletal muscle contractile function have not been examined in a sex-dependent manner.

Within skeletal muscle, NO is mainly produced by neuronal NO synthase (nNOS) (Percival, 2011), which plays a crucial role in optimizing the muscle's contractile function (Kobzik et al., 1994; Stamler & Meissner, 2001). During muscle contraction, NO initiates posttranslational modifications of the Ryanodine Receptor (RyR), allowing calcium channels to remain open and increasing cytosolic calcium concentration (Kumar et al., 2022). Additionally, increased NO production activates cyclic guanosine monophosphate (cGMP), stimulating myosin light chain phosphorylation and calcium sensitivity, increasing actin-myosin cross-bridge cycling and potentially maximum sarcomere shortening velocity (Maréchal & Gailly, 1999). As such, increasing NO bioavailability through NO3- supplementation has been considered a simple intervention to enhance muscle contractile function in humans. However, the role of sex on NO_3^- supplementation on "muscle speed and power" is not clear. Most studies have investigated the effects of dietary NO_3^- supplementation on human contractile function with samples that include only

males or low number of females (Coggan, Leibowitz, Kadkhodayan, et al., 2015; Jonvik et al., 2018; Kadach et al., 2023; Whitfield et al., 2017). It is important to note that the physiology of male and female contractile function differs markedly. For example, females have a lower proportion of type II fibers which has direct impact on contractile properties of skeletal muscle (Ivy et al., 1981; Welle et al., 2008). Calcium handling is closely tied to fiber type; as such, type II fibers are associated with greater peak release and re-sequestration of calcium than type I fibers (Baylor & Hollingworth, 2003; Lamboley et al., 2014). This has been related to different levels of calcium handling protein expression (i.e., SERCA and calsequestrin) (Lamboley et al., 2013, 2014). Furthermore, female sex hormones (i.e. estrogen) influence skeletal muscle contractile properties (Haizlip et al., 2015; Sarwar et al., 1996).

Data from human and animal models suggest that dietary NO_3^- interventions seem to favor structural and functional changes preferentially in type II fibers (Bailey et al., 2019; Hernández et al., 2012), resulting in improved performance in situations where a higher proportion of these fibers are recruited (Bailey et al., 2015; Breese et al., 2013; Coggan, Leibowitz, Kadkhodayan, et al., 2015). The above mechanisms, mostly associated with type II fibers, have been suggested to be central to the effects of improved contractile function and delaying the onset of fatigue following NO3- supplementation (Coggan & Peterson, 2018; Hernández et al., 2012). Thus, there may be less potential for this supplement to be ergogenic in contractile function properties and fatigue resistance in females. Therefore, translating results from male subjects to female performance could be misleading as sex-dependent physiological differences could impact the beneficial effect of supplementation. Further research is required to elucidate the relative efficacy of NO_3^- supplementation in enhancing performance in males and females in exercise modalities where force development and power dominate.

In the present investigation we assessed whether sex affects the impact of NO_3^- supplementation on contractile function. We hypothesized that (1) males would improve

contractile function more than females and (2) fatigue resistance would be enhanced to a greater extent in males than in females.

3.3 Methods

Experimental Design and Protocol

This study was a randomized double-blind, placebo-controlled trial (NCT04588740). Participants reported to the laboratory for a screening test before randomization to either ~13mmol NO_3^- in the form of 140ml beetroot juice (BRJ) or identical nitrate-depleted placebo (PLA) (See description under *Supplementation procedures*). Female subjects were tested during the early follicular phase of the menstrual cycle (3 – 5 days since menses onset), as determined by self-report. All study visits were performed by the same researcher, at the same time of day (± 1 hour), and under the same sensory conditions. The washout period between treatments was at least 7 days for both males and females, with females participating in two consecutive months to account for phase of the menstrual cycle. Participants were recruited from the University of Virginia and the surrounding Charlottesville, Virginia, area. The University of Virginia Institutional Review Board approved all procedures and all subjects provided written informed consent.

Subjects

Subjects were included if they exercised ≤ 3 days/week regularly for no more than 30 minutes on average, were apparently healthy, had normal blood pressure (< 120/80 mmHg), and no orthopedic limitations. Only eumenorrheic female subjects (minimum of 10-12 menses per year) not on any contraception (n = 10) other than a monophasic oral contraceptive pill (n = 2 consistently for at least six months before the study) were recruited. Subjects were excluded if they had any known pulmonary, cardiovascular, or metabolic conditions or diseases, any food allergies, if they were taking any medications or supplements that could impact study outcomes in the last six months (i.e., pre-workout supplements, L-citrulline, L-arginine, etc.), tobacco use, abnormal blood pressure, or

currently or recently pregnant or lactating (< one year), or who contracted Covid-19 during the experimental period.

Supplementation procedures

After completing the screening and familiarization visit (see below) subjects were assigned to consume ~6.5mmol NO_3^- in the form of 70ml of beetroot juice shots (BRJ) twice/day (~13mmol total NO_3^-) for ~3 days or an identical NO_3^--depleted placebo (PLA). On testing days, subjects were instructed to ingest the last two 70mL shots 2h prior to their laboratory arrival time. Female subjects were given additional bottles and were asked to start consuming the juice the day before the estimated day of menses to control for changes in the start of the menstrual cycle and guarantee consumption of at least three days of supplementation before experimental visits. The BRJ and PLA beverages were provided by the same company to ensure the same taste and appearance (Beet It Pro, James White Drinks, LTD).

Experimental Procedure

Prior to all experimental visits and supplementation, a full screening and familiarization visit was completed, where subjects performed the experimental procedure to reduce any potential learning effect. All testing was performed on the right leg of subjects using a multimodal dynamometer (Model System 3; Biodex Medical Systems Inc, Shirley, NY) as described previously (Norte et al., 2018). Briefly, subjects sat upright with the right knee at 90° flexion for calibration, and a correction for limb weight was used. The dynamometer was adjusted to place the lever arm's rotation axis alongside the lateral femoral epicondyle. To limit movement, a strap was secured across the hips throughout testing. The protocol included four separate assessments: i) Isokinetic maximal knee extension (Figure 1A), ii) A maximal voluntary isometric contraction sequence performed prior to a fatigue protocol (Pre-MVIC) (Figure 1B-left), iii) a fatigue protocol (Figure 1 b

-middle) and, iv) a repeated MVIC protocol following the fatiguing bouts (Post-MVIC) completion of the fatigue protocol (Figure 1B right).

i) Isokinetic maximal knee extension. Subjects were instructed to "kick out their foot and pull back as hard and fast as possible" for eight- repetitions, each at three different isokinetic velocities (180deg/s, 270deg/s, and 360deg/s), each separated by 30-second rest (Norte et al., 2018). Participants were given real-time visual feedback, and verbal encouragement was given to ensure maximal effort. The highest power generated at each velocity was plotted to obtain a power-velocity relationship which was then fitted to a 2nd-order polynomial as previously described (Coggan, Leibowitz, Kadkhodayan, et al., 2015; Yamauchi et al., 2009). The polynomial function allowed us to estimate subject's maximal knee extensor velocity, Vmax, as well as maximal knee extensor power, Pmax.

ii & iv) Maximal voluntary isometric contraction before (Pre-MVIC) and after (Pre-MVIC) completion of the fatigue protocol. *S*ubjects performed 3 maximal isometric knee extension contractions with a range of motion locked at 90° each separated by a 1-minute rest. For the Pre-MVIC the highest value of the three MVICs was recorded and compared across study visits.

For the Post-MVIC each of the three contractions was recorded and used to calculate recoverability of skeletal muscle power over time when compared to the pre-MVIC value (□□MVIC at 1, 2 and 3 mins).

iii) Fatigue protocol. As shown in Figure 1B, between the pre-MVIC and post-MVIC, subjects performed 3-second duration isometric contractions interspersed with 2-second rest periods between each repetition (i.e., 12 repetitions/minute). The resistance was set to 60% of the screening/familiarization pre-MVIC. Subjects were instructed to perform this protocol until failure, and both visual feedback and verbal encouragement were provided. When subjects could no longer maintain the 60% MVIC as determined by three failed

repetitions (defined as <50% of the assigned workload achieved), the exercise was stopped, and time-to-exhaustion was recorded.

Blood sampling – Plasma nitrate and nitrite

Immediately prior to the exercise test, venous blood was drawn from an antecubital vein into a nitrate-free syringe and separated into five 1ml Eppendorf tubes containing 5uL 1 to 1000 heparin. These tubes were then centrifuged for 3 min at 5000 rpm. The resultant plasma was transferred into separate 1ml Eppendorf tubes and immediately stored at −80°C until analysis. Plasma NO_3^- and NO_2^- were assessed via ozone-based chemiluminescence using a Sievers NOA model 280i (GE Analytical Instruments, Boulder, CO, USA) as previously described per manufacturer's instructions. Briefly, plasma samples for nitrate analysis were deproteinized using cold ethanol precipitation in a 1:3 dilution (plasma: ethanol) followed by a 30-minute incubation before being centrifuged at 14,000g for 10min. The supernatant was removed for the subsequent nitrate analysis in the presence of vanadium chloride in hydrochloric acid at 95°C. The nitrite of the undiluted (non-deproteinized) plasma samples was determined by its reduction to NO in the presence of glacial acetic acid and potassium iodide, as previously described (Kenjale et al., 2011).

Measurements and data analysis procedures

The demographic data were examined using a two-tailed unpaired Student T-test. Mixed-Analysis of variance (ANOVA) with Sidak's adjustments for multiple comparisons was used to determine differences between supplements (PL vs. BRJ) and between sexes for plasma NO_3^- and NO_2^-. Peak power at each velocity, estimated maximal power (Pmax) and maximal knee extensor velocity (Vmax), as well as time-to-fatigue during the fatigue protocol, were also analyzed by Mixed-ANOVA between supplements (PLA vs. BRJ) and between sexes. Specifically, for pre- and post-MVIC (i.e. recoverability after the fatigue protocol at different time points within the same test – Pre, 1min, 2min, and 3min), we used a three-way ANOVA (Supplement*Sex*Timepoint). All statistical analysis was

conducted using GraphPad Prism Version 9.3 (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). GraphPad Prism was also utilized for the creation of all graphs and figures. Data are reported as mean ± SD unless otherwise stated, with $p \leq 0.05$ required for statistical significance.

3.4 Results

Participant Characteristics

Twelve young healthy female subjects (23.9 + 3.6 years) and fourteen young healthy males (22.9 + 3.6 years) participated in the study. There was no statistical difference in age and/or BMI, as expected; males were taller, and heavier and had a higher peak power output as summarized in Table 1.

Plasma nitrate and nitrite

Female and male subjects showed no significant difference in plasma NO_3^- or plasma NO_2^- during the PL visit. After BRJ supplementation, both sexes showed a significant increase in plasma NO_3^- and plasma NO_2^- compared to PL ($p \leq 0.001$, Figure 2). Plasma NO_2- concentration following supplementation was higher for females than males ($p \leq 0.05$).

Measurements of skeletal muscle contractile function

i) Isokinetic maximal knee extension

Males demonstrated a higher peak power than females across all velocities (sex effect, $p < 0.05$), with no effect of treatment (BRJ v PLA) (Figure 3A). Peak power at 270 and 360 deg/sec showed a significant sex-by-treatment interaction ($p \leq 0.05$). This difference is informed by Figure 3B, that shows the change in peak power between PL and BTR (treatment) for each sex. There was a large effect size at 270deg/sec and 360deg/sec (Cohen's D effect size= of 0.92 and 0.80, respectively). Pmax (Figure 3C) and Vmax (Figure 3D) also demonstrated a medium and large effect sizes for the sex-by-treatment effect (ES=0.71 and 0.59, respectively).

ii & iv) Maximal voluntary isometric contraction before (Pre-MVIC) and after (Post-MVIC) completion of the fatigue protocol

Males demonstrated a higher Pre-MVIC (PEAK) than females (sex effect, p=0.01). There were no changes by treatment (p=0.81) or any interaction effects (p=0.49) (Figure 4A).

Absolute Post-MVIC values were greater in males than females at all 3-time points (sex effect p=0.001, data not shown). However, when expressed as a percentage of Pre-MVIC (PEAK), this difference was eliminated, as shown in Figure 5B. Both groups increased Post MVIC as they recovered (time effect, p<.01) at a similar rate (no time*sex effect, p=0.74) Figure 4B.

iii) Fatigue protocol.

Workloads used for the fatigue protocols were based on the pre-supplementation peak MVIC for each cohort and, therefore, absolutely but not relatively higher by design for males (females; 96.1 ± 25.3 Nm and males 124.8 ± 36.9 Nm; $p<0.05$). There was no difference between BRJ and PL for time to fatigue for either (females; $p=0.99$ or males; $p=0.46$), as shown in Figure 5.

3.5 Discussion

To our knowledge this is the first study to investigate whether dietary NO_3^- supplementation has a differentiated effect on skeletal muscle contractile function, specifically in males and females. We found during isokinetic maximal knee extensions at higher velocities (270 and 360 deg/sec) that females appeared to demonstrate reduced power outputs when consuming NO_3^- in the form of BRJ. Furthermore, the medium and large effect sizes for the sex-by-treatment effect for peak power, Pmax, and Vmax support this idea that the response may be reduced in females and requires further investigation. Additionally, we saw no changes in fatigue resistance during an intermittent isometric knee

extension at 60% of MVIC after NO_3^- supplementation in either of the sexes. As a complementary measure, we also assessed the recoverability of contractile function after the fatigue protocol with no significant changes between supplements.

The effect of dietary nitrate on isokinetic maximal knee extension

Our results add to the literature that dietary NO_3^- does not benefit maximal isokinetic strength (Aucouturier et al., 2015; Haider & Folland, 2014; Jonvik et al., 2021). In support of this, a recent meta-analysis showed that contractile function might not be improved after supplementation, regardless of the contractile velocity tested (Lago-Rodríguez et al., 2020). Despite the advances in our understanding of how the effect of NO_3^- supplementation on exercise outcomes might be influenced by fitness level, supplementation regime (i.e., acute vs chronic), and/or dose, sex remains an understudied variable (Shannon et al., 2022; Wickham & Spriet, 2019). In the current study, we expand this by comparing the effect of NO_3^- supplementation in males and females. We saw that peak power for the two fastest angular velocities tested; males showed a 2-3% reduction and females a 3-8% reduction for peak power.

Similarly, estimated values of maximal contraction power (Pmax) and maximal knee extensor velocity (Vmax) showed sex-dependent responses to NO_3^- supplementation. Previous studies reported an improvement in Pmax between 5-6% after NO_3^- supplementation which aligned with our results in males (~5%), (Coggan, Leibowitz, Kadkhodayan, et al., 2015; Gallardo et al., 2020). In contrast, females in our study showed a ~21% reduction which disagrees with any previous reports. Vmax, on the other hand, showed a non-significant 3% improvement for the male subjects and a ~24% reduction for female subjects. Coggan et al (Coggan, Leibowitz, Spearie, et al., 2015; Gallardo et al., 2020) has reported a consistent ~10% increase in Vmax in healthy individuals in a mixed sample where females were also included. Nevertheless, the small sample size in their study made it difficult to determine any sex differences.

The suggestion that NO_3^- might help improve skeletal muscle contractile function is based on the fact that after supplementation, there is an increase in calcium handling proteins in type II fibers but not in type I fibers (Hernández et al., 2012; Ivarsson et al., 2017; Whitfield et al., 2017). This allows an increase in cytosolic calcium concentrations in type II fibers, potentially facilitating the initial force development during muscle contraction of type II fibers (Andrade et al., 1998; Hernández et al., 2012). As such, the lower proportion of type II fibers seen in females would diminish any potential benefits in contractile function after supplementation. Additionally, all subjects were given the same absolute NO_3^- dose; thus, females ingested a higher proportion of dietary NO_3^-. It has been previously reported that higher doses do not necessarily improve performance (Gallardo et al., 2020), and combined with high-intensity exercise, it could result in an increase in peroxynitrite (Gholami et al., 2019). The latter has been shown to markedly reduce maximum force in type I fibers by reducing calcium sensitivity with less effect on type II fibers aligning with our results where females, who typically have a larger proportion of type I fibers(Dutka et al., 2011; Supinski et al., 1999).

The effect of dietary nitrate on fatigue resistance.

Delaying skeletal muscle fatigue is another expected effect of NO_3^- supplementation as it could optimize calcium handling towards the final stages before failure (Andrade et al., 1998; Bailey et al., 2019; Hernández et al., 2012). We found no differences between placebo and NO_3^- supplementation for fatigability during intermittent isometric knee extension exercise (Figure 4). This result is consistent with two studies that report no significant effect of NO_3^- supplementation on fatigability (Coggan et al., 2019; Le Roux-Mallouf et al., 2019) but contrasts another study where NO_3^- supplementation delays muscle fatigue development (Husmann et al., 2019). The latter employed a dynamic exercise of the knee extensor, while we used an isometric knee extension protocol, which could explain the divergent results. Thus, increasing circulating NO_2^- does not necessarily delay the development of fatigue during an isometric knee extension protocol, potentially

due to the involvement of a smaller muscle mass compared to whole-body exercise, where we have reported improvements in cycling performance in male subjects only. It could also be that contractile properties are more responsive to supplementation than measures of fatigue during repeated contractions.

Furthermore, we wanted to assess the recoverability of skeletal muscle after the fatigue protocol, as it has been previously suggested that NO_3^- may accelerate phosphocreatine recovery kinetics following high-intensity knee extensor exercise in hypoxic conditions (Vanhatalo et al., 2011; Vanhatalo et al., 2014). These conditions may enhance the conversion of NO_2^- to NO, thus, improving tissue perfusion and clearing exercise metabolites faster. However, our data show no benefit in the recovery of contractile function at any of the three-time points assessed (Figure 5).

Study limitations

There are a few limitations to the study protocol, including the number of repetitions tested during the isometric knee extension protocol (x10 repetitions per velocity), which could limit our capacity to measure a true maximum effort during this assessment. Also, we only allowed a 30-second rest between each velocity tested; as such, subjects could have experienced some skeletal muscle fatigue by the time they were assessed on the fastest velocity (i.e. 360deg/sec). This could influence males and females differently, given their fiber type composition and fatigability, therefore, decreasing their contractile capacity in this last velocity tested. Nevertheless, the reduction in contractile function in females as velocities were increased could be considered a test of fatigability, where NO_3^- supplementation reduced contractile capacity in females regardless.

3.6 Conclusion

In summary, we investigated the role of sex on the effects of dietary NO_3^- supplementation on maximal isokinetic knee extension contractile function as well as maximal isometric knee extension torque before and after a fatiguing protocol. The effect

of dietary NO_3^- supplementation appears to have differentiated effects in males and females. Until the effects of NO_3^- supplementation are fully elucidated, using BRJ as an ergogenic in females should be viewed with caution.

3.7 Tables

Table 1. Subjects' characteristics

	All	Female	Male	*p*-value
Number	26	12	14	
Age	23.4 ± 3.5	23.9 ± 3.6	22.9 ± 3.6	0.48
Height (m)	1.68 ± 10.5	1.60 ± 8.1	1.75 ± 5.7	**<0.0001**
Weight (Kg)	70.3 ± 12.6	61.4 ± 7.4	77.9 ± 11.2	**0.0002**
BMI	24.7 ± 3.1	24.2 ± 3.5	25.2 ± 2.7	0.41
Pre-supplementation MVIC (Nm)	38.7 ± 7.8	160.2 ± 42.2	208.1 ± 61.5	**0.03**

BMI, body mass index; W, watts; MVIC, maximal voluntary isometric contraction. Data presented as mean ± SD. *p*-value = Student T-test male vs female.

3.8 Figures

FIGURE 1. Experimental protocol schematic. The experimental visit was divided into two major testing blocks; (A) Maximal isokinetic knee extension and (B) pre- and post-MVIC with the fatigue protocol in between.

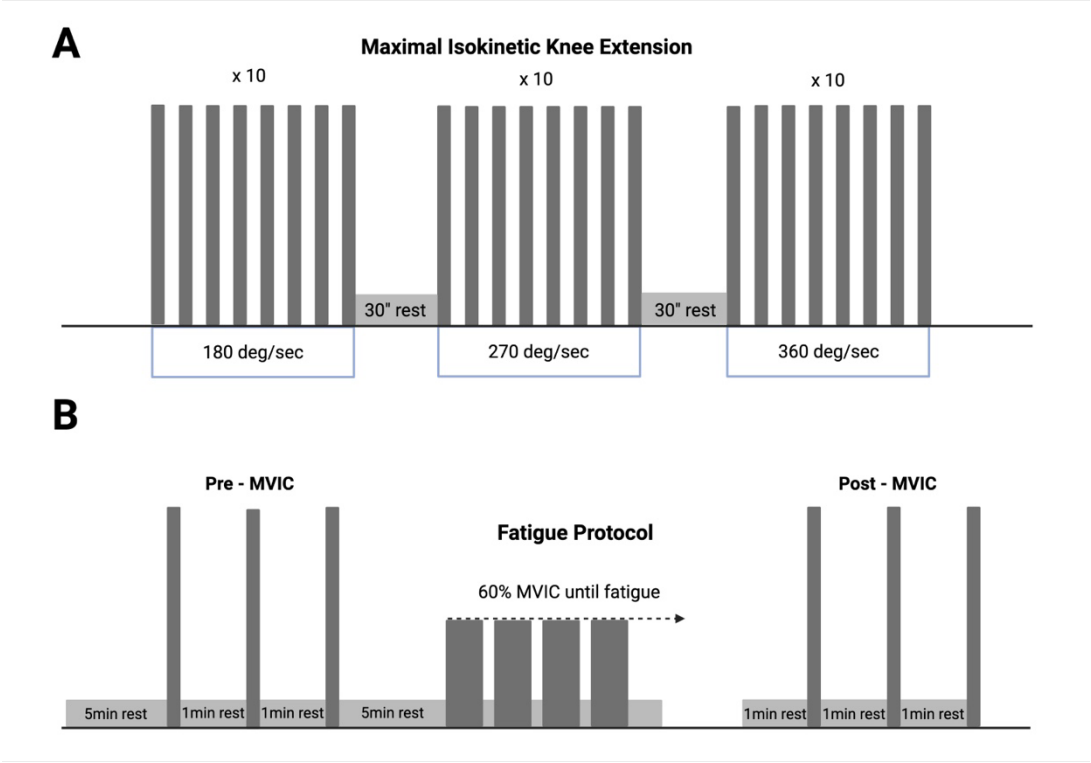


FIGURE 2. Plasma NO_3^- (panel A) and NO_2^- (panel B) concentrations after placebo (PL) supplementation (white bars) and beetroot (BRJ) juice supplementation (grey bars).

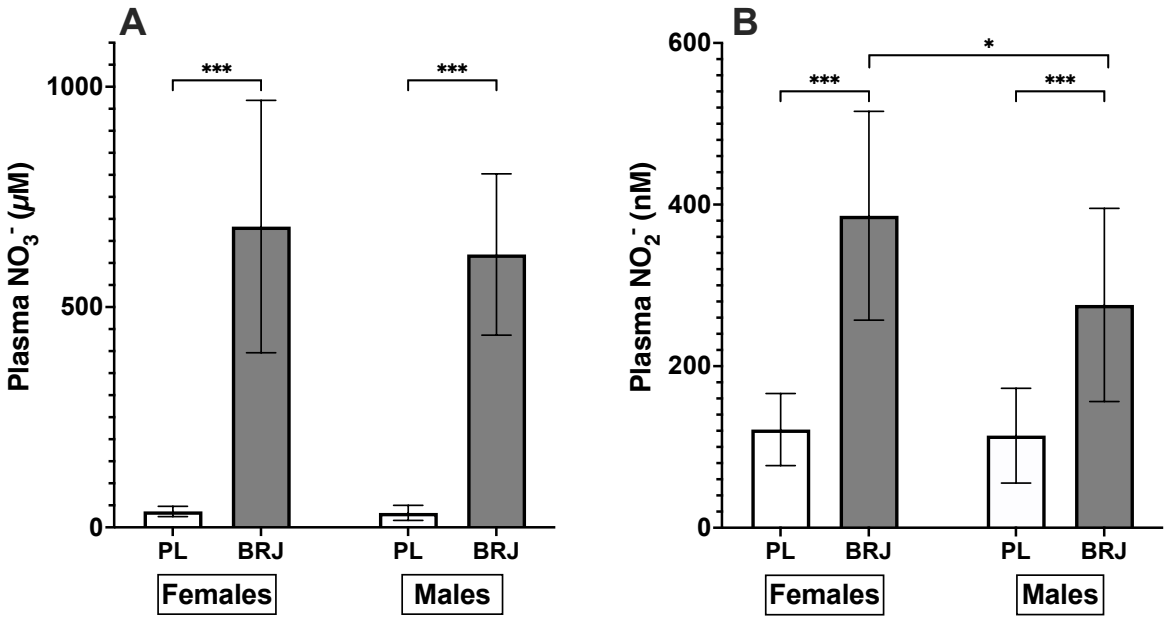


FIGURE 3. The effect of inorganic nitrate on isokinetic peak power and estimated maximal power and velocity. (A) Absolute power values for females (triangles) and males (circles) on PL (white) and BRJ (solid) at the three angular velocities tested. (B) Change in isokinetic peak power between PL and BRJ in females (white bars) and males (grey bars). (C) Estimated maximal knee extensor velocity, Vmax, after PL (white bars) and BRJ (grey bars) in males and females. (D) Estimated maximal knee extensor power, Pmax, after PL (white bars) and BRJ (grey bars) in males and females. Effect sizes (EF) for the sex-by-treatment effect are presented in the graphs. Data presented are mean±SD.

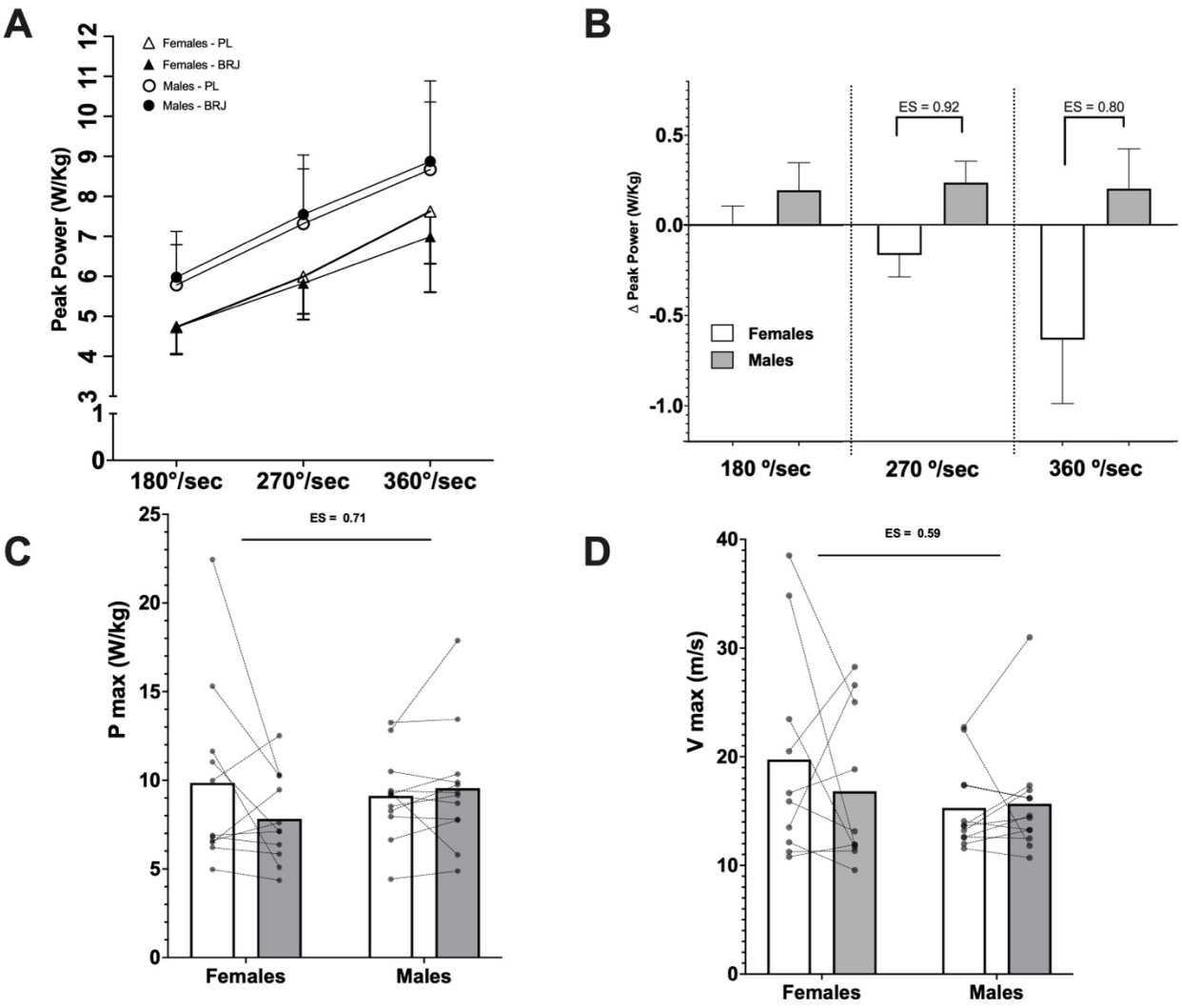


FIGURE 4. Maximal voluntary isometric contraction (A) before the fatigue protocol (Pre-fatigue – PEAK) and (B) after the fatigue protocol expressed as a percentage of Pre-MVIC (1-minute, 2-minute, and 3-minute after fatigue).

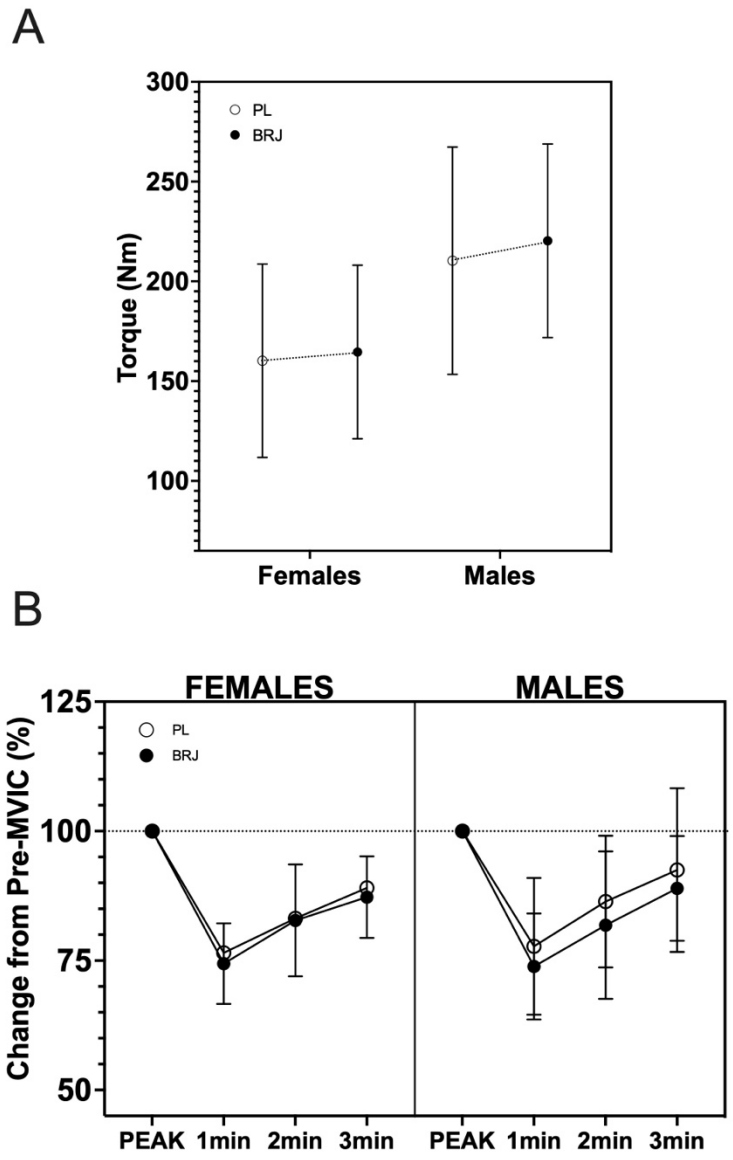
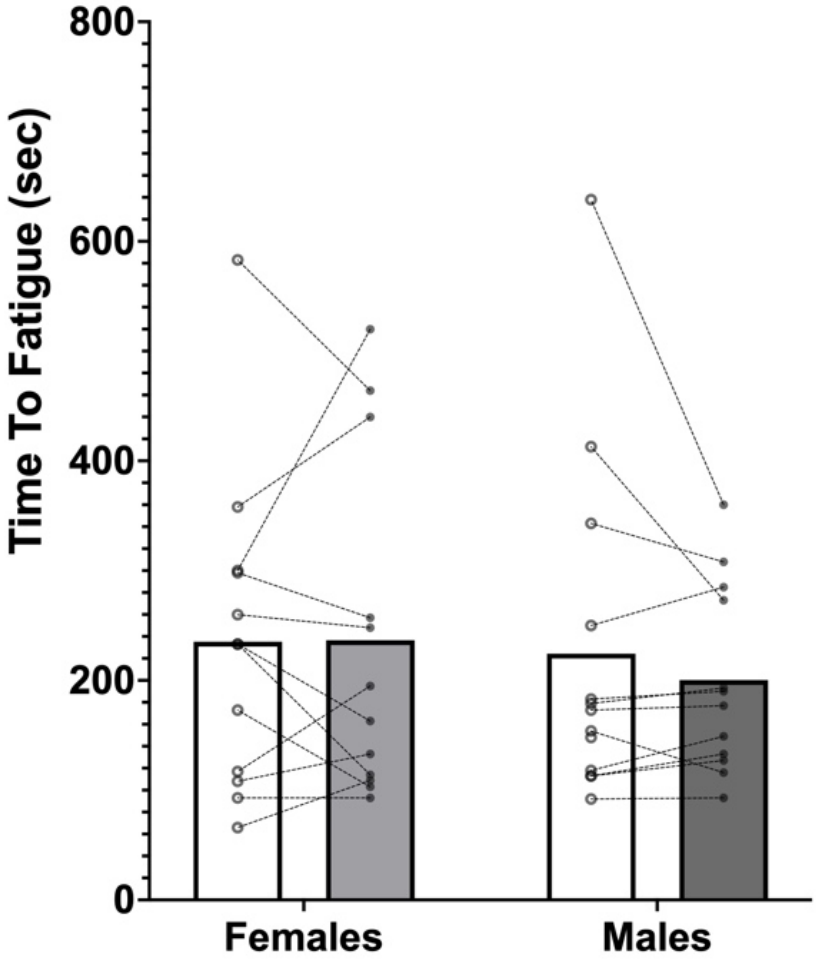


FIGURE 5. Absolute values for time to fatigue for each experimental visit (PL – white bars; BRJ – greys bars).



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4. MANUSCRIPT 3: THE EFFECTS OF ORAL INORGANIC NITRATE SUPPLEMENTATION ON LOWER LIMB PERFUSION AND METABOLISM DURING EXERCISE IN PATIENTS WITH PERIPHERAL ARTERIAL DISEASE (PAD).

4.1 Abstract

Patients with peripheral arterial disease (PAD) fail to adequately supply blood and oxygen (O_2) to working tissues and presents as claudication pain during walking. Oral inorganic nitrate (NO_3^-) supplementation has been shown to restore NO bioavailability in this clinical population resulting in improved claudication symptoms and walking capacity. The underlying mechanisms have not been fully elucidated; therefore, we aimed to determine the effects of NO_3^- supplementation on skeletal muscle perfusion and oxidative capacity measured by advanced magnetic resonance imaging (MRI), Arterial Spin Labelling (ASP) and Chemical Exchange Saturation Transfer (CEST), respectively. In a double-blind, randomized, crossover design, five patients (72.2 ± 5.2 years) with PAD underwent resting blood draws and treadmill exercise testing. On a separate day, plantarflexion exercise until claudication symptoms or exhaustion within the 3T magnet. All subjects consumed ~5 days of NO_3^- supplementation (BRJ) or identical NO_3^--depleted placebo (PL) before the treadmill testing (3 days) and MRI testing (5 days). Following BRJ, plasma NO_2^- concentration increased significantly (410.5 ± 237.5 nM; $p < 0.01$) compared to PL (156.3 ± 45.4 nM). Subjects walked 30% longer before the onset of claudication pain (238.5 ± 197.8 s vs. 312.8 ± 207.3 s; $p = 0.15$, $d = 0.37$) with no changes in peak walking time (589.5 ± 103.6 s vs 585.8 ± 56s; $p = 0.94$) following BR vs PL. Tissue perfusion at peak plantar flexion exercise showed a small increase (17.4 ± 10.5 vs 22.9 ± 17.4 mL/min/100g; $p = 0.30$, $d = 0.40$) and CEST asymmetry decay times were also improved (247.6 ± 145.1 vs 196 ± 99.4; $p = 0.58$, $d = 0.44$). These findings support the hypothesis that NO_2^- -related NO signaling increases peripheral tissue oxygenation in areas of hypoxia and improves symptoms in PAD.

4.2 Introduction

Peripheral arterial disease (PAD) is a form of cardiovascular disease (CVD) caused by atherosclerotic plaques which narrow the conduit arteries of the legs, resulting in reduced blood flow to the lower extremities (Campia et al., 2019). It is estimated that PAD affects over 200 million people worldwide (Fowkes et al., 2017) and 8.5 million people in the USA over the age of 40 (Gerhard-Herman et al., 2017). The most common symptom in patients with PAD is intermittent claudication (IC), which is defined as lower leg pain triggered by walking, and it is only improved with rest (Criqui & Aboyans, 2015; Fowkes et al., 2013). The transient development of IC in response to walking in these patients is due to limited oxygen delivery, the uncoupling of metabolism and perfusion within skeletal muscle, or both (Anderson et al., 2009; Pipinos et al., 2003). Individuals with PAD have severely limited exercise tolerance, which significantly compromises their ability to perform activities of daily living. As such, strategies to improve blood flow and mitochondrial function should have considerable clinical utility in individuals with PAD.

Nitric oxide (NO) is a key signaling molecule that modulates a variety of physiological processes, such as blood flow, mitochondrial respiration, platelet aggregation, and neurotransmission, among others (Lundberg & Weitzberg, 2022). NO is produced by nitric oxide synthases (NOS), which are responsible for the conversion of the amino acid L-arginine to NO in the presence of oxygen and complementary co-factors (Forstermann & Sessa, 2012). Disruption of NOS-derived NO production, specifically vascular NO, has been associated with increased risk factors for the development of CVD (Daiber et al., 2019; Förstermann, 2010; Lundberg et al., 2015). Additionally, plasma nitrite (NO_2^-) concentration, which may act as a marker of NO bioavailability, has been shown to be positively associated with exercise capacity in patients with PAD (Allen et al., 2010). As such, interventions that improve NO bioavailability may be of particular importance in this clinical population where endogenous NO production is impaired.

Interestingly, in the last few decades, consumption of inorganic nitrate (NO_3^-), abundant in green leafy vegetables and beets, has been a focus for potential NO production when endothelial (e)NOS-derived NO is impaired (Lundberg et al., 2011; Woessner et al., 2018). Upon oral supplementation, NO_3^- is rapidly absorbed in the upper gastrointestinal tract into the circulation, which is then concentrated in the oral cavity, where commensal oral bacteria are responsible for reducing it to the more reactive NO_2^- anion (Bryan et al., 2022). NO_2^- is subsequently swallowed, and while a portion is reduced to NO by the high acidity of the stomach, the remaining is absorbed into the circulation and can be further reduced to NO in blood and other tissue compartments by enzymatic and non-enzymatic pathways (Gladwin & Kim-Shapiro, 2008; Kelley et al., 2006; Shiva et al., 2007). The last reduction step (i.e., NO_2^- to NO) is enhanced in conditions of a low pH (Modin et al., 2001) and low oxygen availability (Castello et al., 2006) found within peripheral tissue of patients with PAD. Dietary NO could potentiate an alternative and complementary source of NO to enhance vascular eNOS function in these patients.

To date, NO_3^- supplementation in patients with PAD has shown promising results. Acute NO_3^- supplementation (2-3h before testing) improves exercise capacity and delays claudication onset time (COT) by ~17-20% and 18%, respectively (Kenjale et al., 2011; Pekas et al., 2021). These functional improvements have been accompanied by improved indirect indices of gross tissue perfusion measured by near-infrared spectroscopy (NIRS) (Kenjale et al., 2011; Pekas et al., 2021). To date, direct measurements of the response of micro- and macrovascular function in to NO_3^- supplementation during exercise in a temporal and spatial manner have not been reported.

In addition to abnormal tissue delivery of blood flow in patients with PAD, oxygen utilization may be limited by reduced mitochondrial function (Park et al., 2022; Pipinos et al., 2006; Pipinos et al., 2003). Mitochondrial function is associated with poor peak walking time (Anderson et al., 2009; Gonzalez et al., 2016), and mitochondrial respiration has been shown to increase following dietary NO_3^- supplementation in some (Larsen et al.,

2011) but not all studies (Whitfield et al., 2016). Currently, the effects of inorganic nitrate supplementation on active skeletal muscle metabolism during exercise are unclear.

In the present study, we examined the effects of inorganic nitrate supplementation on 1) exercise treadmill walking performance, along with calf tissue perfusion and muscle oxidative metabolic energetics; and 2) after peak calf plantarflexion exercise in patients with symptomatic PAD. It was hypothesized that NO_3^- supplementation, in comparison to placebo, would 1) delay the onset of claudication pain while treadmill walking, 2) improve calf muscle perfusion and 3) accelerate skeletal muscle creatine recovery kinetics after calf plantarflexion exercise.

4.3 Methods

Experimental Design and Protocol

This study was a randomized, double-blind cross-over design (Figure 1, NCT05036213). Participants reported to the laboratory for initial screening and baseline exercise testing prior to randomization to either NO_3^- supplementation (~13mmol) in the form of 140ml beetroot juice (BRJ) or nitrate-depleted placebo (PLA) (See description under *Supplementation procedures*). All subjects were recruited from vascular clinics at the University of Virginia Medical center (Charlottesville, VA). The University of Virginia Institutional Review Board approved all procedures, and all subjects provided written informed consent.

Subjects

Five subjects completed this study (Table 1). All subjects had a history of stable intermittent claudication for three or more months. All subjects were receiving antiplatelet and lipid-lowering therapy unless medically contraindicated by their physician. There was no difference in subject medication regimens between visits. Exclusions were based on past medical history of gangrene, impending limb loss, or osteomyelitis; lower extremity

vascular surgery, angioplasty, lumbar sympathectomy within three months of enrollment; severe peripheral neuropathy; any condition other than PAD that limits walking, unstable angina, history of significant left-main or three-vessel coronary artery disease (>70% stenosis, unprotected by grafts), or recent myocardial infarction (6 weeks); or chest pain during treadmill exercise, which appears before the onset of claudication, or >3 mm ST depression during exercise. Subjects with a history of renal insufficiency (GFR < 30) were excluded to avoid any potential issues with elevated potassium and phosphorus clearance from the beetroot beverage. All subjects were instructed to avoid antibacterial mouthwash products as they may interfere with the metabolism of NO_3^- to NO_2^- by oral bacteria (Govoni et al., 2008; Woessner et al., 2016).

Screening visit

All subjects completed a physical examination and had their medical history reviewed by a cardiologist. Once it was determined that subjects were eligible for the study, they underwent maximal cardiopulmonary treadmill exercise testing (CPX) with 12-lead ECG monitoring. We utilized the Gardner graded treadmill protocol as it is specifically designed for a claudication-limited population (Gardner et al., 1991). Expired gases were analyzed continuously using open circuit spirometry (Vyntus CPX metabolic cart Vyaire, Yorba Linda, CA). The subject indicated the point at which they initially felt claudication pain (COT), and the point at which they could not continue was recorded as peak walking time (PWT).

Supplementation procedures

After completion of the screening visit, subjects were randomized to consume 70mL of beetroot juice (BRJ ~6.5mmol NO_3^-) twice/day (~13mmol total NO_3^-) for ~5 days or an identical NO_3^--depleted placebo (PL). On testing days, subjects were instructed to ingest the last two 70mL shots two 2h prior to their laboratory arrival time while remaining otherwise fasted and withholding medications that could influence BP and endothelial

function testing (including angiotensin-converting enzyme inhibitors, beta, alpha, and Ca^{2+} channel blockers, etc., unless contraindicated). Each treatment was tested ~7 days apart, to allow a sufficient washout period of NO_3^- from the patients' system. The same company provided both BRJ and PL supplements to ensure similar taste and appearance (Beet It Pro, James White Drinks, LTD).

Vascular and Exercise testing

On day ~3 of supplementation, subjects were scheduled for vascular and CPX testing. Upon arrival at the Clinical Research Unit (CRU), subjects underwent ~10min of supine rest before vascular assessments. Pulse Wave Analysis (PWA) was assessed using a SphygmoCor (Version 8.0; AtCor Medical, Sydney, NSW, Australia) per the manufacturer's instructions. An arm cuff was placed on the subject's upper arm, aligning the designated markings with the brachial artery. The automated system then recorded pulsations at the brachial artery to produce central aortic pressure waveforms. This was followed by Pulse wave velocity (PWV), which was measured by simultaneously comparing the carotid pulse (via applanation tonometry) and the femoral pulse via a specialized thigh cuff. The subjects completed a maximal graded treadmill exercise test, as described previously (see *2.3 Screening visit* for details).

Magnetic Resonance Imaging testing

On day ~5 of supplementation, subjects reported to the 3T MRI scanner (Prisma, Siemens Medical Solutions, Erlangen, Germany), where they completed two different imaging protocols after plantarflexion peak exercise. The procedure consisted of the patient laying supine feet first inside the MRI scanner. The leg of interest (lowest ABI) was placed into an MR-compatible flexion ergometer (Trispect, Ergospect GmbH, Innsbruck, Austria). Patients were asked to begin plantar flexion exercise in time with a metronome set at 60 beats per minute until the onset of claudication symptoms or calf exhaustion. First, CEST imaging was taken pre-exercise and immediately at the cessation

of exercise (Sporkin et al., 2022). Chemical Exchange Saturation Transfer (CEST), is a novel MRI technique allowing the acquisition of images of skeletal muscle bioenergetics with high spatial resolution. This recently developed alternative imaging technique (Haris et al., 2014) has been validated to detect muscle energetics in patients with PAD sensitively (Sporkin et al., 2022) and has a 3-fold higher signal-to-noise ratio than other MR spectrometry methods (Kogan, Haris, Debrosse, et al., 2014; Kogan, Haris, Singh, et al., 2014).

Following a 15min rest period, the exercise protocol was repeated with ASL perfusion imaging performed immediately at exercise cessation (Lopez et al., 2015). Arterial Spin Labeling (ASL) is a novel MRI technique in which protons in arterial blood are imparted with a magnetic tag allowing for more localized calf muscle blood flow changes following oral inorganic nitrate supplementation.

Blood sampling – Plasma nitrate and nitrite

Prior to the treadmill exercise test, blood was drawn from an antecubital vein into a NO_3^- free syringe (BD Luer-Lok^{TM}) (~5mL) for subsequent NO_3^- and NO_2^- analysis. Plasma NO_3^- and NO_2^- were assessed via ozone-based chemiluminescence using a Sievers NOA model 280i (GE Analytical Instruments, Boulder, CO, USA) as previously described. Briefly, plasma samples for NO_3^- analysis were deproteinized using cold ethanol precipitation in a 1:3 dilution (plasma: ethanol) followed by a 30-minute incubation before being centrifuged at 14,000g for 10-min. The supernatant was removed for the subsequent NO_3^- analysis in the presence of vanadium chloride in hydrochloric acid at 95°C. The NO_2^- of the undiluted (non-deproteinized) plasma samples was determined by its reduction to NO in the presence of glacial acetic acid and potassium iodide, as previously described (Kenjale et al., 2011).

Statistical analysis

Differences in plasma NO_3^- and NO_2^-, COT, PWT, VO_{2peak}, and hemodynamic and vascular-derived measures were analyzed with a two-tailed, paired samples t-test. Estimated tissue perfusion from ASL images and CEST asymmetry decay were also analyzed with a two-tailed, paired samples t-test. Significant effects were further explored using simple contrasts, with the level adjusted via a Bonferroni correction. As this is an underpowered pilot study, the analyses also included effect sizes (Cohen's d). Data are presented as means±SD unless otherwise stated. Statistical significance was accepted when $p<0.05$.

4.4 Results

Subject Characteristics.

Five male patients with PAD diagnosed with an ABI lower than <0.9, participated in the study. Demographics, smoking status, and medications are summarized in Table 1.

Plasma Nitrite concentrations

The BRJ supplementation was well-tolerated by all subjects. Venous plasma NO_3^- and NO_2^- concentrations were significantly higher following BRJ supplementation when compared to the PL condition ($p<0.04$, Figure 2).

Exercise Tolerance

The mean COT and PWT response following PL and BRJ are shown in Figure 3, A and B, respectively. Ingestion of BRJ resulted in a ~74sec (~30%) longer mean exercise time before the subjects reported the onset of claudication pain (Fig 3A, p=0.146, Cohen's d = 0.37). PWT was not changed after BRJ supplementation compared to the PL condition (Fig 2B, p=0.94). Heart rate (HR) (PL: 119.3 ± 4.2 vs. BRJ 121.8 ± 14.8 bpm, $p>0.05$) and VO_2peak (PL: 15.1 ± 1.6 vs. BRJ 16.2 ± 1.1 $mL.kg^{-1}.min^{-1}$, $p>0.05$) did not change significantly between treatments.

Quantitative calf muscle perfusion measured by Arterial Spin Labeling

Tissue perfusion at peak plantar flexion exercise is depicted in Figure 4. Following BRJ supplementation, there was a small increase in tissue perfusion when compared with the PL condition (Δ5.50 mL/min/100g, Cohen's $d = 0.4$).

Skeletal muscle bioenergetics measured by Chemical Exchange Saturation Transfer (CEST)

CEST asymmetry ($CEST_{asym}$) decay times at peak plantar flexion exercise for the overall calf and the individual gastrocnemius are shown in Table 2 and Figure 5, respectively. During the BRJ condition, $CEST_{asym}$ decay (creatine recovery kinetics) for the gastrocnemius suggested a small beneficial effect (Δ-63.0±231.46 sec) on mitochondrial oxidative metabolism (Cohen's d =0.44).

Vascular function and hemodynamic measures

Measures of brachial blood pressures or aortic blood pressures are shown in Figure 6, BRJ supplementation resulted in a reduction in brachial (Δ-10.5mmHg, p=0.23, Cohen's $d = 0.67$) (Figure 6A) and aortic systolic blood pressure (Δ-10.25mmHg, p=0.17, Cohen's $d = 0.72$) with no changes in diastolic pressure or MAP. Measures of arterial stiffness also showed a reduction in Augmentation index (Aix, Δ-2.5%, $p = 0.13$, Cohen's $d = 1.25$), and the Augmentation index adjusted to 75 beats/min (Aix@75bpm, Δ-3.5%, $p = 0.45$, $d = 0.41$) (Figure 6C). PWV showed small changes following BRJ supplementation (Δ-0.87 ± 1.7 m/s, $p > 0.05$, Cohen's $d = 0.81$) (Figure 6D).

4.5 Discussion

Our pilot data suggest that increasing circulating plasma NO_2^- concentration (~3-fold, Figure 2) through NO_3^- supplementation (~13mmol) in the form of BRJ, delays

claudication onset time in patients with PAD during a CPX test. To better understand the mechanisms underlying this effect, we examined the role of localized calf muscle perfusion and bioenergetics using advanced Magnetic Resonance Imaging (MRI), after peak plantar flexion exercise (Bock et al., 2018; Kenjale et al., 2011; Pekas et al., 2021).

Our preliminary data suggest that after NO_3^- supplementation, there is an improvement in tissue perfusion (Figure 4), alongside an increase in skeletal muscle mitochondrial oxidative efficiency and/or a greater capillary to myocyte oxygen-diffusive capacity as suggested by a decreased $CEST_{asym}$ decay recovery time. Additionally, we noted a decrease in peripheral and central blood pressure measures, suggesting a potential beneficial hemodynamic effect of NO_3^- supplementation in patients with PAD.

Claudication onset time and peak walking capacity

Acute ingestion of BRJ, without exercise training, increased COT by ~30% (Δ+74sec, Cohen's d = 0.4); this result is in accordance with our previous report, where acute BRJ supplementation (~13 NO_3^- mmol) delayed COT in patients with PAD by ~18% (Kenjale et al., 2011). This is a clinically significant improvement as three months of supervised exercise training, a class 1A recommendation for symptomatic PAD, improved COT by 66% (Δ+138sec) in our hands (Allen et al., 2010). Despite the improvements in COT, there were no changes in peak walking time, contrasting our previous findings (Kenjale et al., 2011) and those reported by others (Bock et al., 2018; Pekas et al., 2021), but is in agreement with van der Avoort (van der Avoort et al., 2021).

Pain-free walking is a primary aim for PAD treatment and there are limited therapeutic options beyond surgery and exercise training. Currently, only two medications, cilostazol and pentoxifylline, are FDA-approved to treat PAD-associated walking impairment. However, benefits from cilostazol are modest and approximately 30% of patients discontinue cilostazol due to adverse effects (Dawson et al., 1998; Girolami et al., 1999; Money et al., 1998; Stevens et al., 2012). Recent evidence also suggests a lack of efficacy for pentoxifylline (Gerhard-Herman et al., 2017). Our data suggest NO_3^- supplementation

is an easy-to-administer intervention to increase vascular NO bioavailability and improve pain-free walking in patients with symptomatic PAD.

Skeletal muscle tissue perfusion measured by MRI – ASL

The present study found an increase in skeletal muscle tissue perfusion by ~32% (Δ5.50 mL/min/100g, Cohen's d = 0.4, Figure 4). This adds to the current literature that suggests that NO_3^- supplementation improves tissue perfusion in patients with PAD (Kenjale et al., 2011; Pekas et al., 2021). Previous work has utilized NIRS to measure indices of tissue oxygenation; however, this technique does not account for blood flow rate. As such, the current study provides a superior measure of bulk blood flow and perfusion of exercising skeletal muscle with a high spatial and temporal resolution.

An important difference between our current data and previous studies is that we employed a supine plantar-flexion exercise model for blood flow and metabolism assessment, whereas, previous studies have utilized treadmill walking. Treadmill walking recruits a larger mass of skeletal muscle tissue when compared to plantarflexion exercise. Additionally, the upright position during treadmill walking (versus supine position within the MRI) has greater hydrostatic (gravitational) pressures in the lower limbs. Therefore, small improvements in tissue perfusion in our study may potentially present a larger increase in other compartments within the calf muscles during walking.

Skeletal muscle tissue bioenergetics measured by MRI –CEST

Improvements in COT and exercise capacity in patients with PAD are not solely explained by improvements in bulk blood flow delivery (Anderson et al., 2009). Oxygen delivery needs to be coupled with the rate of oxidative metabolism within active tissue to see functional improvements (Anderson et al., 2009). As such, a third important finding of this study was the speeding of the $CEST_{asym}$ decay by ~24% (Δ-63sec, Cohen's d = 0.4, Figure 5). This is consistent with previous studies where dietary NO_3^- supplementation improved PCr recovery kinetics by ~16% in healthy individuals when exercised in hypoxia

(i.e., ~14.5% fractional oxygen) (Vanhatalo et al., 2011). The recovery kinetics of PCr has been closely associated with the maximal rate of oxidative ATP reconstitution (Arnold et al., 1984; Kemp et al., 1993). Improved maximal oxidative mitochondrial capacity could be associated with increased mitochondrial density, oxidative enzyme capacity, and oxygen delivery at the myocyte (Dudley et al., 1987; Holloszy, 1967; Holloszy & Coyle, 1984). Interestingly, improvements in mitochondrial efficiency (i.e., P/O) in healthy humans have been shown after 3 days of NO_3^- supplementation (Larsen et al., 2011), so this is a potential mechanistic explanation for the changes observed in the present study. The effect of a potential change in mitochondrial oxidative capacity on myocyte O_2 uptake is represented by the schematic in Figure 7B.

An alternative explanation for improving the $CEST_{asym}$ decay is better matching of specific capillary perfusion to the myocyte's local metabolic rate irrespective of changes in bulk flow. This would increase the PO_2 gradient between specific capillaries and myocytes and increase O_2 diffusion. It has been shown that NO_3^- supplementation improves microcirculatory function in PAD (Pekas et al., 2023), which is likely the key site for hypoxia and reduction of NO_2^- to NO (Maher et al., 2008). In support of this idea, previous data following 12 weeks of exercise training in patients with PAD suggests that VO_{2peak} improvements only happen after improvements in capillary density (Duscha et al., 2011).

In addition to great diffusion between the capillary and adjacent myocytes NO bioavailability may also help in a more efficient distribution of O_2 across myocytes. This occurs due to a reversible inhibition of NO from NO_2^- reduction at peak exercise could also produce a reversible inhibition of cytochrome c oxidase (Brown, 2001; Brown & Cooper, 1994; Gladwin & Kim-Shapiro, 2008; Shiva, 2013). As a result, the distribution of intracellular oxygen is more efficiently modulated, allowing deeper areas of muscle tissue to become better oxygenated, driven by a larger oxygen pressure difference (Hagen et al., 2003; Thomas et al., 2001; Victor et al., 2009). We have also previously shown in rodent models that circulating nitrite increased blood flow specifically to capillaries in fast twitch muscles which had lower myocyte PO_2 levels (Ferguson et al., 2015). Patients with PAD

show a preferential rarefaction of slow-twitch type 1 fibers to exhibit a more glycolytic phenotype (Askew et al., 2005).

Interestingly, NO_3^- supplementation does not affect PCr recovery in healthy individuals during small muscle mass exercise in normoxia where oxygen delivery is not a limitation (Lansley et al., 2011; Vanhatalo et al., 2014). This reinforces the fact that patients with PAD present a potentially ideal tissue environment during ambulation to enhance the efficacy of NO_3^- supplementation. This simple therapeutic to restore NO, results in improved symptoms and walking capacity.

Hemodynamic and vascular measures

Dietary NO_3^- supplementation reduced brachial SBP (Δ-10.5mmHg, Cohen's d = 0.67) and aortic SBP (Δ-10.25mmHg, Cohen's d = 0.72) with no changes in brachial DBP, brachial MAP or aortic DBP (Figure 6, A and B). These results add to our previous study in patients with PAD where NO_3^- supplementation significantly reduced brachial DBP but not in SBP at rest (Kenjale et al., 2011). Reductions in SBP and DBP do not always happen together after NO_3^- supplementation, although it is now widely accepted that NO_3^- supplementation can reduce blood pressures in healthy (Vanhatalo et al., 2010; Webb et al., 2008), older healthy individuals (Rammos et al., 2014) and hypertensive patients (Ghosh et al., 2013). SBP reduction after NO_3^- supplementation has been suggested to be between 2-5mmHg (Kapil et al., 2010). Our data show a ~10 mmHg, which agrees with data from healthy individuals (Webb et al., 2008) and hypertensive patients after taking BRJ (Ghosh et al., 2013). This is of clinical relevance as it has been shown that reductions of 2-5mmHg in blood pressure might reduce the incidence of stroke by 22% and coronary artery disease by 16% (Pescatello et al., 2004; Sanders, 2007).

Furthermore, we also found that central arterial stiffness markers accompanied these reductions in brachial and aortic blood pressures. Specifically, we found a reduction in the Augmentation index (Aix, Δ-2.5%, Cohen's d = 1.25), and the Augmentation index

adjusted to 75 beats/min (Aix@75bpm, Δ-3.5%, Cohen's $d = 0.41$). One previous study in patients with PAD reported no significant improvements in Aix@75bpm; however, dietary NO_3^- supplementation has been shown to reduce markers of arterial stiffness in other clinical populations (Pekas et al., 2021). PWV also showed a small improvement following NO_3^- supplementation, which contrasts with previous data PAD patients (Bahra et al., 2012; Pekas et al., 2021). Improvements in measures of arterial stiffness are of clinical significance in this population as it has been shown that arterial stiffness and the formation of atherosclerotic lesions are associated with and affect individuals with PAD (Popele et al., 2001; Zahner et al., 2017).

Limitations

The current study presents a few different limitations. The number of subjects recruited in this trial is low; as such, it is likely underpowered to identify differences between treatments. Another limitation would be the lower intra-class correlation (ICC) of ASL after exercise (ICC = 0.87) compared to cuff occlusion hyperemia (0.98) being the latter more reproducible (Lopez et al., 2015). Additionally, we have also speculated that one of the underlying mechanisms for the improvements in $CEST_{asym}$ decay could be due to improved O_2 diffusion within skeletal muscle tissue, which with the current techniques, we were not able to measure.

4.6 Conclusions

In conclusion, dietary NO_3^- supplementation via BRJ increased plasma NO_2^- concentration and claudication pain -free walking during a graded maximal exercise treadmill test. Advanced MRI techniques suggest these changes appear to be mediated by improvements in bulk blood flow to the lower limbs as well as an increase in maximal mitochondrial oxidative capacity. Given the lack of current effective pharmacological approaches to increase ambulation in patients with symptomatic PAD, these are clinically

meaningful benefits. In addition, we observed reductions in central and peripheral systolic blood pressures as well as improvements in measures of arterial stiffness. Our findings suggest dietary NO_3^- supplementation is an easy-to-administer and may present a viable clinical option to increase ambulation in patients with symptomatic PAD.

4.7 Tables

TABLE 1. Subject characteristics

Variable	All Subject
n (males)	5 (5)
Age	72.2 ± 5.2
Weight	91 ± 18
BMI	29.1 ± 4.1
Height	176.4 ± 8.7
ABI	0.67 ± 0.08
Smoker	
Current	4 (80%)
Medications	
ASA/Plavix (blood thinner)	4 (80%)
Alpha-blocker	1 (20%)
Ca^{2+} channel blockers	1 (20%)
Ace-inhibitors	3 (60%)
Proton pump inhibitor	1 (20%)
Statin	5 (100%)
Phosphodiestarase-3 inhibitor	1 (20%)

BMI, Body Mass Index, ABI, Ankle brachial index. Data presented as mean ± SD. Medications are presented as number of subjects and percentage (%).

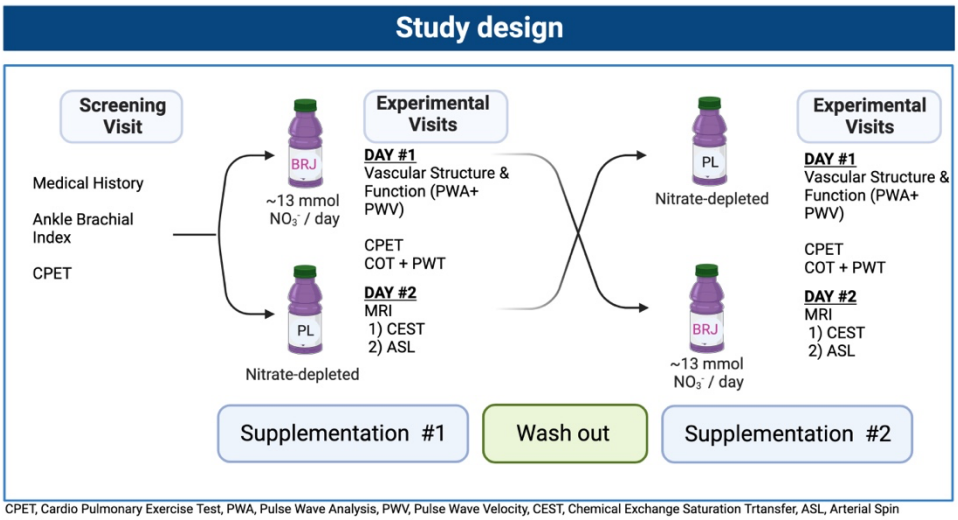
TABLE 2. $CEST_{asym}$ Results

	Placebo (n = 5)	BRJ (n = 5)	Difference	*P*-value
Calf $CEST_{asym}$ decay constant (s), mean±SD	247.6 ± 145.1	196 ± 99.4	-51.5 ± 195.4	0.587
Tibialis anterior $CEST_{asym}$ decay constant (s), mean±SD	181.5 ± 77.9	160.4 ± 102.1	-21.2 ± 143.4	0.758
Tibialis posterior $CEST_{asym}$ decay constant (s), mean±SD	214.9 ± 116.2	210 ± 211.4	-5 ± 249.7	0.967
Gastrocnemius $CEST_{asym}$ decay constant (s), mean±SD	263.5 ± 169.5	200.6 ± 113	-63 ± 231.5	0.576

$CEST_{asym}$ indicates chemical exchanges saturation transfer asymmetry; and BRJ, beetroot juice.

4.8 Figures

FIGURE 1. Study Design



CPET, Cardio Pulmonary Exercise Test, PWA, Pulse Wave Analysis, PWV, Pulse Wave Velocity, CEST, Chemical Exchange Saturation Trtansfer, ASL, Arterial Spin Labelling, NO_3^-, inorganic nitrate.

FIGURE 2. Plasma nitrite concentrations. * Significantly different from Placebo. Data presented Mean±SD.

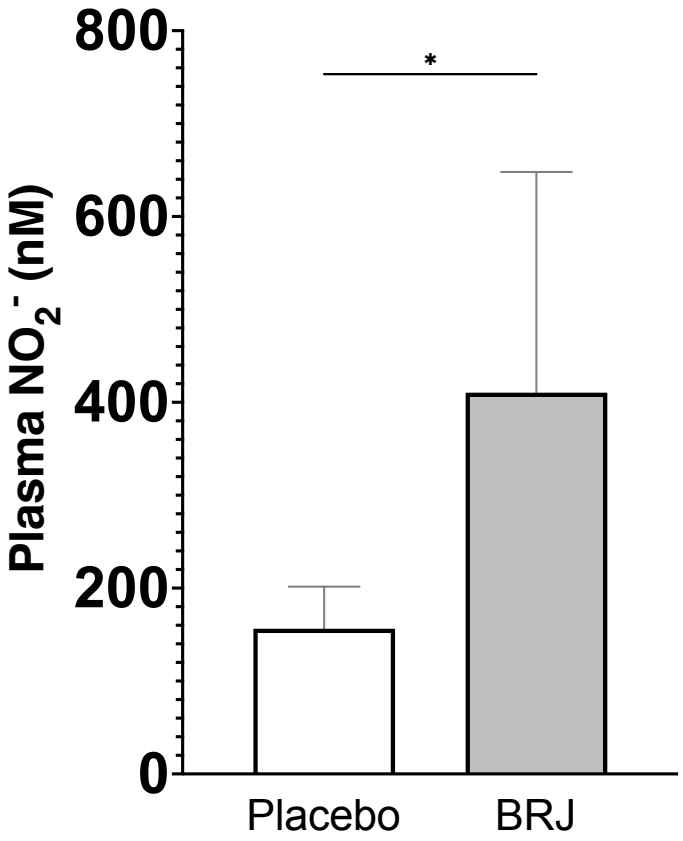


FIGURE 3. Absolute change in (A) claudication onset time (COT) and (B) peak walk time during a maximal CPX following placebo (PLA) and beetroot juice (BRJ) supplementation. Data are presented as mean±SD.

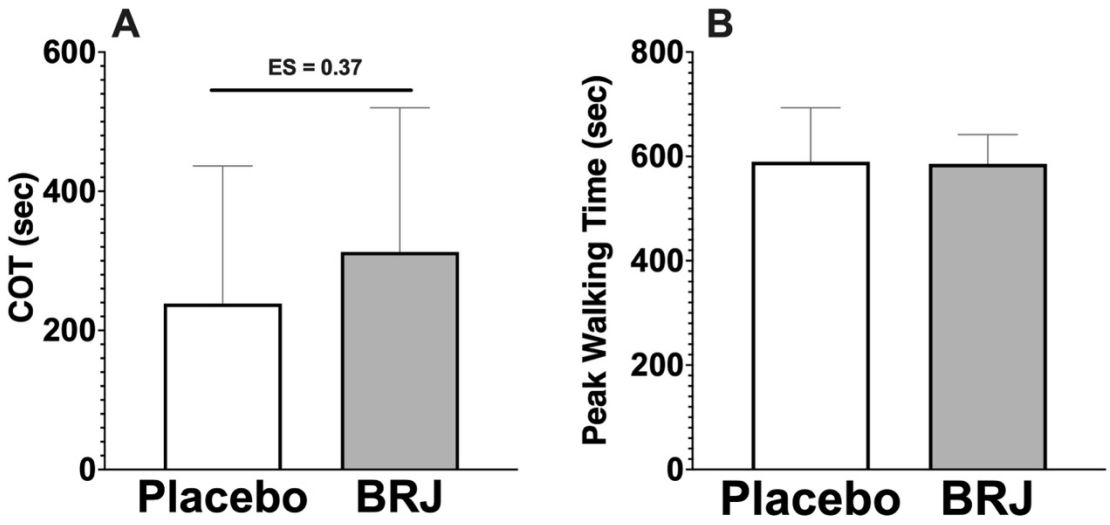


FIGURE 4. Change in skeletal muscle perfusion in response to exercise hyperemia after placebo (PLA) and beetroot juice (BRJ) supplementation. The change shown in the graph is the effect of BRJ compared to the PLA experimental visit. Data are presented as mean±SD.

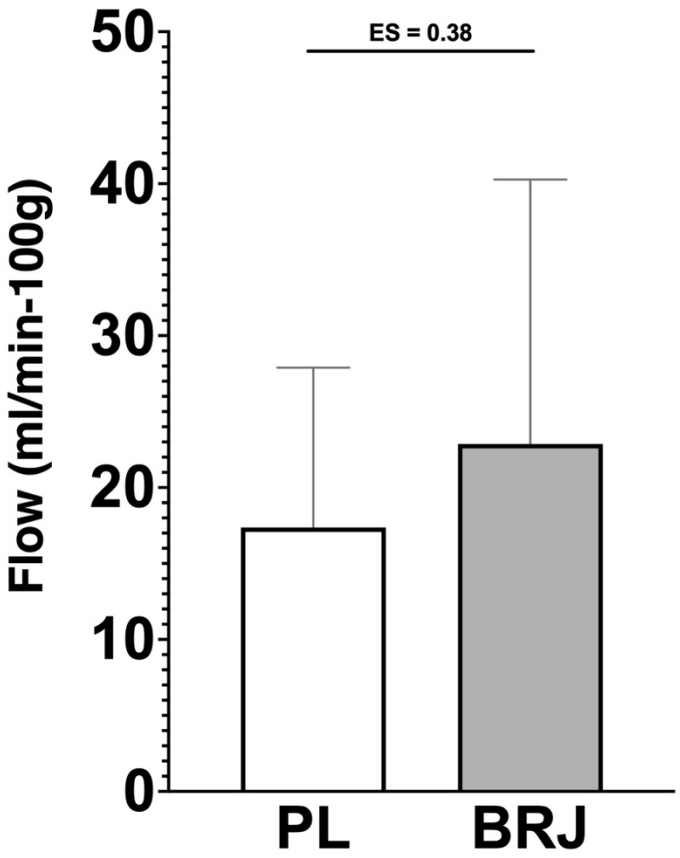


FIGURE 5. Change in CEST asymmetry ($CEST_{asym}$) decay constants after BRJ compared to PL supplementation in the gastrocnemius muscle.

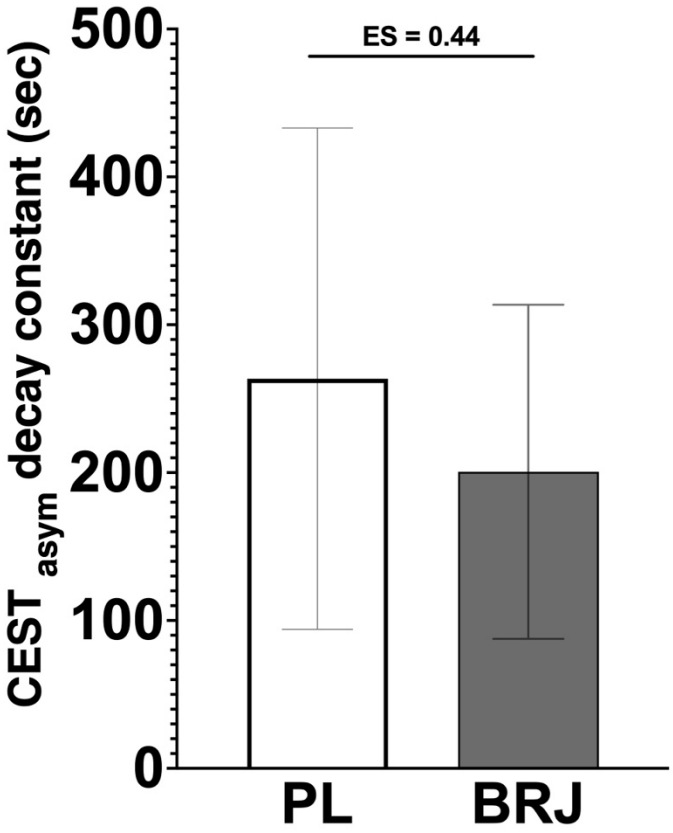
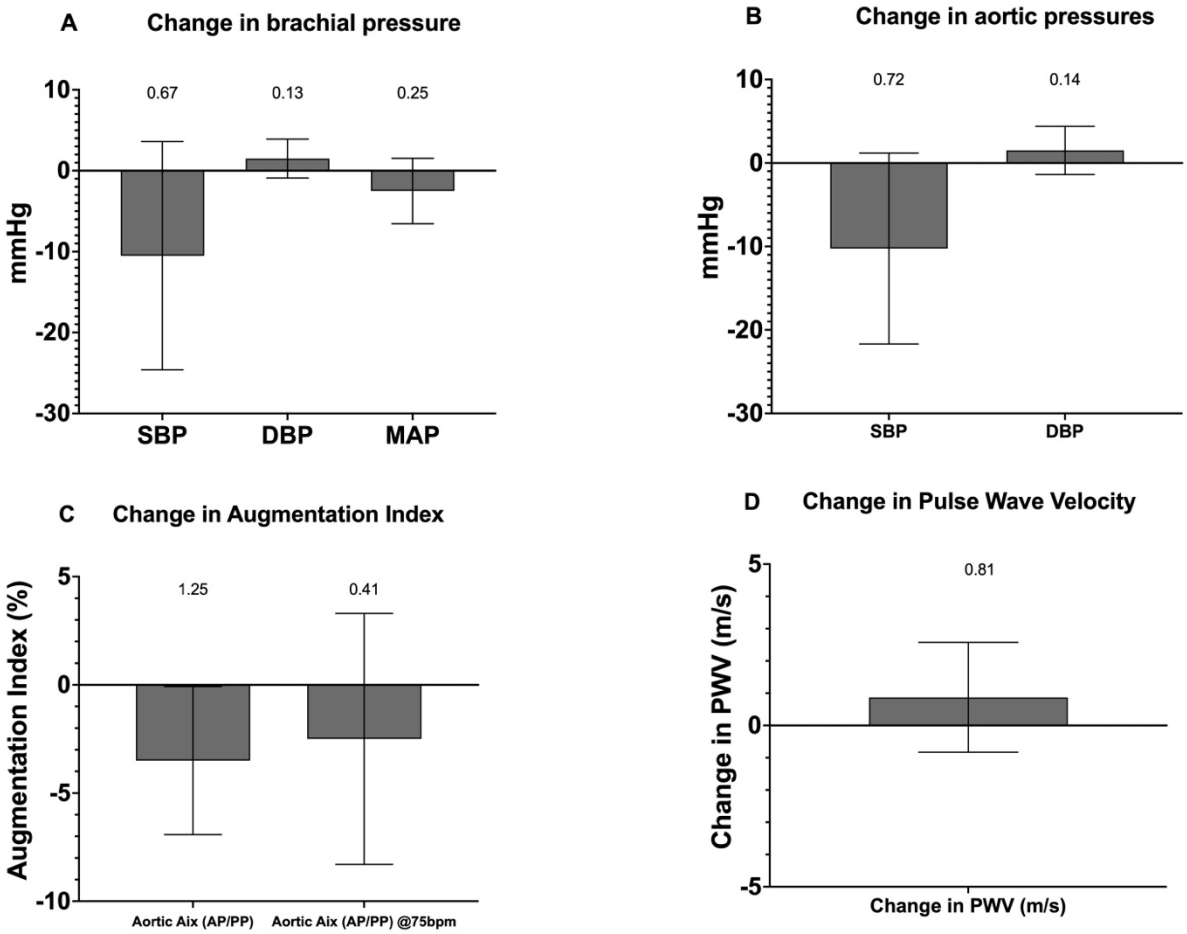


FIGURE 6. Change in (A) brachial pressures, (B) changes in aortic pressures and (C) augmentation index, and (D) changes in pulse wave velocity (PWV) after BRJ compared to PL supplementation. Data are presented as mean±SD. Effect sizes are expressed on top of each measurement.



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5. SUMMARY AND FINAL COMMENTS

Oral inorganic nitrate supplementation (NO_3^-) is widely recognized to increase nitric oxide (NO) bioavailability in humans, which in the last few decades has attracted a vast amount of research interest due to its cardiovascular health benefits as well as its ergogenic effect during exercise in health and disease. The current thesis contributes to the body of literature by examining the role of sex in the effects of dietary NO_3^-- supplementation on exercise outcomes and the underlying mechanisms for exercise improvements in clinical populations (i.e. patients with peripheral arterial disease - PAD) following NO_3^- supplementation.

Herein we present data that shows a potential differentiated effect of dietary NO_3^- supplementation on exercise outcomes in males and females. First, we showed that males, but not females, reduced oxygen consumption by ~6% during submaximal exercise (i.e. below the gas exchange threshold). We also showed that males improved exercise capacity by ~15% during a time-to-exhaustion trial within the severe intensity domain, while females showed a ~5% non-significant reduction. Furthermore, skeletal muscle contractile function follows a similar pattern: males showed an improvement in isokinetic knee extension peak power, and females showed decreased peak power during the same experimental test. These findings show sex differences in response to NO_3^- supplementation on exercise outcomes despite both sexes significantly increasing plasma nitrate and nitrite concentration after supplementation. The underlying explanations for these differences need to be clarified. Still, we could speculate that sex-related differences in skeletal muscle fiber composition, cardiovascular system, and circulating sex hormones may partly explain the different responses to supplementation between males and females.

Dietary NO_3^- supplementation is also considered an exercise therapeutic intended to restore NO deficiencies in clinical patients. The present thesis presents pilot data investigating the mechanisms underlying the beneficial effects of NO_3^- supplementation on exercise capacity and intermittent claudication in patients with PAD. After peak plantar

flexion exercise, we examined the role of localized calf muscle perfusion and bioenergetics using advanced Magnetic Resonance Imaging (MRI). Specifically, we provide evidence that NO_3^- supplementation delays claudication onset time and is accompanied by improved tissue perfusion, increased skeletal muscle mitochondrial oxidative efficiency, and/or greater capillary to myocyte oxygen-diffusive capacity. Given the lack of current effective pharmacological approaches to increase ambulation in patients with symptomatic PAD, these findings are of great importance.