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No Longer Beeting Around the Bush: A Review of Potential Sex Differences with Dietary Nitrate Supplementation

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Running Head: Potential Sex Differences Dietary Nitrate Supplementation

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Invited review for the Applied Physiology, Nutrition, and Metabolism Award for Nutrition Translation – Sex-Specific Responses to Nutrition

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Abstract

Over the last decade there has been substantial interest in the health and athletic performance benefits associated with acute and chronic dietary nitrate (NO_3^-) supplementation. Dietary NO_3^-, commonly found in leafy green and root vegetables, undergoes sequential reduction to nitrite and nitric oxide (NO) via the enterosalivary circulation. Importantly, NO has been shown to elicit a number of biological effects ranging from blood pressure reduction to improved exercise economy and athletic performance. However, a common absence within biological research is the lack of female participants, which is often attributed to the added complexity of hormonal fluctuations throughout the menstrual cycle. Despite mounting evidence supporting significant anthropometric, metabolic, and physiological differences between the sexes, this problem extends to the field of dietary NO_3^- supplementation where women are underrepresented as research participants. This review examines the existing dietary NO_3^- supplementation research with regards to dietary NO_3^- pharmacokinetics, resting blood pressure, exercise economy and performance, and mechanisms of action. It also provides evidence and rationale for potential sex differences in response to dietary NO_3^- supplementation and future directions for this field of research.

- Dietary NO_3^- supplementation has been shown to have positive impacts on health and athletic performance in generally male populations. However, women are underrepresented in dietary NO_3^- supplementation research.
- The present evidence suggests that sex differences exist in response to dietary NO_3^- supplementation and this review highlights avenues for future research.

Keywords: dietary nitrate, sex differences, beetroot juice, nitric oxide, blood pressure, exercise economy, performance, excitation-contraction coupling

Introduction

Dietary nitrate (NO_3^-) is a bioactive compound that has been received both negatively and positively in the media and scientific literature for several decades. In the 1970s dietary NO_3^- (found in vegetables and drinking water) and dietary nitrite (NO_2^-) (found in processed meats) received substantial backlash for their potential to be metabolized to toxic and carcinogenic N-nitroso compounds (Gilchrist et al. 2010). However, over the last decade, dietary NO_3^- and its metabolites have been at the forefront of nutritional research for their beneficial effects on human health and athletic performance (Bailey et al. 2012; Jones et al. 2018). The evidence supporting adverse effects of dietary NO_3^- is largely unfounded and has sparked debate within the scientific community as to whether it is harmful or beneficial to human health (Katan 2009; McNally et al. 2016).

Most of the dietary NO_3^- we consume (~80%) is found in leafy green (spinach, arugula, kale) and root (beetroot, yams, carrots) vegetables (Hord et al. 2009). Once ingested, dietary NO_3^- enters the enterosalivary circulation, which ultimately results in the sequential reduction of NO_3^- to NO_2^- and further to nitric oxide (NO) (Webb et al. 2008). This oxygen (O_2)-independent process is known as the NO_3^--NO_2^--NO pathway and is facilitated by anaerobic bacteria that reside on the dorsal surface of the tongue (Webb et al. 2008).

Importantly, NO is a potent signalling molecule that elicits biological effects on numerous tissues and is involved in an array of physiological processes including, but not limited to vasodilation, calcium-handling, mitochondrial efficiency, and neurotransmission (Bailey et al. 2012). These effects have significant implications for health and exercise performance.

NO can also be produced endogenously through the oxidation of L-arginine to form L-citrulline and NO (Bailey et al. 2012), catalyzed by nitric oxide synthase (NOS). In this review,

we focus on the provision of exogenous dietary NO_3^- as a nutritional intervention. This pathway allows for the formation of NO in scenarios of reduced O_2 availability or impairment of the endogenous NOS enzyme (Bailey et al. 2012). Concentrated beetroot juice (BRJ), sodium nitrate ($NaNO_3$), and potassium nitrate (KNO_3) are potent sources of exogenous NO_3^- that facilitate the production of NO through the O_2-independent pathway and have been shown to elevate plasma [NO_3^-] and [NO_2^-] (Kapil et al. 2010; Larsen et al. 2010; Wylie et al. 2013).

Addressing the Sex Gap

It wasn't until the 1980s and 1990s that leading health organizations recognized the major sex disparity in the evidence backing medicinal and therapeutic health strategies. Furthermore, only in 1993 did the National Institute of Health, "the world's largest single funder of biomedical research", mandate the inclusion of both men and women in clinical research under the Revitalization Act (Mazure and Jones 2015). Notably, it took until 2000 for medical and scientific communities to acknowledge that "women are not just small men" and therefore sex differences may exist in health-related research (Regitz-Zagrosek 2012). Despite this realization, the added complexities of hormonal fluctuations throughout the menstrual cycle often deters researchers from including women as participants in scientific investigations (Costello et al. 2014). This may explain why 26 years have passed since the Revitalization Act, and yet sex differences in human physiology, metabolism, and medicine remain largely unstudied, and calls to include women in research are continually put forward to the scientific community (Bruinvels et al. 2017).

However, it is important to recognize that since the Revitalization Act, studies have identified sex differences with respect to anthropometric, physiologic and metabolic outcomes. A review by Brown (2008) highlighted the role of the sex hormones estrogen and testosterone in

determining these differences, where they act on an array of tissues and organs to affect their structure as well as physiological and metabolic function (Table 1).

Table 1 about here.

Unfortunately, despite recognition of these differences, it is difficult to accurately match male and female subjects to assess sex differences. Many studies simply match subjects on the basis of descriptive population characteristics with little regard for relative fitness status or lean body mass (bm). Often the best approach for exercise and supplementation research is to match subjects for fitness status relative to lean bm and deliver a dose relative to total bm. These considerations should be evaluated prior to facilitating any experimental design regarding sex differences.

Bridging the Gap

Despite evidence supporting biological sex differences, the gap in the literature extends to dietary NO_3^- supplementation research. In stark contrast to over 100 studies in strictly male populations, there are only 7 studies with exclusively female participants that have investigated the effects of dietary NO_3^- supplementation on blood pressure, exercise economy and performance. The aims of this review are to: 1) examine selected areas of dietary NO_3^- supplementation research that have been performed using males and females, 2) identify existing sex differences, and 3) highlight future research questions and directions for this field.

Supplemental Nitrate Dose-Response/Pharmacokinetics

Pharmacokinetic profiles and dose-response curves are essential to understand if a supplement reaches the blood in significant quantities to affect the target tissue(s). To date, only two studies have characterized the dose-response and pharmacokinetic profiles for NO_3^- supplementation. Wylie et al. (2013) reported a dose-dependent relationship with regards to

plasma [NO_3^-] and [NO_2^-] following administration of 4.2, 8.4, and 16.8 mmoles (mmol) dietary NO_3^- administered as BRJ in healthy males. They found peak plasma [NO_3^-] occurred 1 h post-ingestion of BRJ and peak plasma [NO_2^-] occurred ~2-2.5 h post-ingestion. Kapil et al. (2010) also reported that plasma [NO_3^-] and [NO_2^-] increased in a dose-dependent manner following the administration of 4, 12, and 24 mmol KNO_3. Plasma [NO_3^-] rose 30 min post-ingestion, peaked at 3 h, and remained elevated for 24 h. Plasma [NO_2^-] rose significantly 1.5 h post-ingestion, peaked at 2.5 h, and remained elevated above baseline at 24 h. Interestingly, females had significantly greater baseline plasma [NO_2^-] compared to men, despite having similar baseline plasma [NO_3^-]. Absolute plasma [NO_3^-] and [NO_2^-] rose significantly higher in females compared to males, which may be partly explained by a larger dose of KNO_3/kg bm. Although statistical differences were lost, these trends remained when the results were normalized to bm. Recent work by Kapil et al. (2018) suggests that, compared to males, females have an enhanced ability to reduce NO_3^- to NO_2^- at baseline and following NO_3^- supplementation due to increased oral bacterial activity. This research suggests there may be sex differences in NO_3^- metabolism, specifically in the enterosalivary circulation. However, it is important to consider the possibility that non-sex-specific differences may be driving these responses. For example, Jonvik et al. (2016) found that highly trained females had greater dietary NO_3^- intake compared to males. Furthermore, little is known regarding differences in other dietary and lifestyle factors that may influence plasma NO_3^- and NO_2^- levels. Future work should consider sex differences in mouthwash utilization and other oral hygiene habits, as these factors may significantly impact the oral microbiome that is essential for the conversion of dietary NO_3^- to NO_2^- and further to NO.

Importantly, future work should make direct comparisons between the pharmacokinetic responses of males and females to BRJ supplementation and use varying doses of dietary NO_3^- normalized to kg bm. This would determine whether sex-specific responses are due to a larger dose/kg bm and/or intrinsic differences in NO_3^- metabolism.

Key Points

- Plasma [NO_3^-] and [NO_2^-] increase in a dose-dependent manner following dietary NO_3^- supplementation in men and women.
- Following an acute dose of dietary NO_3^-, plasma [NO_3^-] and [NO_2^-] remained significantly elevated above baseline for up to 24 h.
- Women appear to have elevated baseline plasma [NO_2^-] and experience greater relative increases in plasma [NO_3^-] and [NO_2^-] following dietary NO_3^- supplementation than men. However, this may reflect larger doses of dietary NO_3^-/kg bm.

Blood Pressure

High blood pressure (BP) is a leading health issue in North America, and drug and dietary therapies have been investigated to combat this health epidemic (Lawes et al. 2008). Importantly, dietary NO_3^- supplementation is considered a powerful nutritional tool in targeting this health problem. For the purpose of this review, we have highlighted the BP lowering effects of dietary NO_3^- supplementation in healthy and trained populations. In the literature, dietary NO_3^- supplementation has demonstrated BP lowering effects in healthy populations and these effects have been established for systolic (SBP) and diastolic blood pressure (DBP). However, the effects of dietary NO_3^- supplementation on resting BP in trained athletic populations largely demonstrates no response to supplementation. Furthermore, it is crucial to highlight the major

disparity in the number of healthy as well as trained males and females recruited in these studies, and to address the potential sex differences.

Healthy Adults

Several reviews have reported reductions of ~6 mmHg in SBP and ~3 mmHg in DBP following dietary NO_3^- supplementation in healthy individuals (Siervo et al. 2013; Ashor et al. 2017). A total of 21 studies have measured BP in healthy individuals, which included 343 male and only 73 female subjects. Although 43% of these studies included men and women, these numbers clearly highlight the sex-based gap that exists in the dietary NO_3^- BP research.

Two studies made direct comparisons between men and women. Kapil et al. (2010) found that females had significantly lower baseline SBP and DBP. This may suggest that women have enhanced endogenous production of NO_3^- or a greater capacity to reduce NO_3^- to NO_2^-. Furthermore, despite a larger dose of KNO_3/kg bm and a greater subsequent rise in absolute and relative [NO_3^-] and [NO_2^-] in females, males had more profound reductions in SBP and DBP. This may be attributed to women having lower baseline BP values or that women have less robust responses to dietary NO_3^- supplementation. Irrespective of sex, the beneficial effects of KNO_3 on BP responded in a dose-dependent manner following the administration of 4, 12 and 24 mmol KNO_3. Coles and Clifton (2012) also reported a greater reduction in SBP and DBP in men compared to women following 15 mmol NO_3^- delivered as BRJ, despite no sex differences in baseline SBP and DBP. The lack of baseline differences between the sexes for blood pressure may be explained by the fact that the women were slightly older than the males, and BP is known to increase with age (Pinto 2007). These authors did not measure plasma [NO_3^-] or [NO_2^-]. Lastly, two studies measured the BP response to dietary NO_3^- in exclusively female participants. Collofello et al. (2014) demonstrated a ~5 mmHg reduction in SBP and non-

significant reductions in DBP and mean arterial pressure (MAP) in young, healthy females following a single 5 mmol dose of NO_3^- as BRJ. However, Pospieszna et al. (2016) found no effect of a single dose of BRJ or carrot juice, both high in dietary NO_3^-, on SBP or DBP. These results may be explained by the training status of the female collegiate swimmers in this study. Taken together, it appears that both healthy males and females demonstrate significant reductions in SBP and possibly DBP, but these effects are more robust in males compared to females. It is unclear if this is due to differences in baseline plasma [NO_3^-] and [NO_2^-], baseline SBP and DBP, NO_3^- metabolism, or other factors. The field of dietary NO_3^- research would benefit from a comprehensive study investigating plasma [NO_3^-], [NO_2^-], and BP responses of men and women over a 24 h period following both an absolute as well as a relative dose of dietary NO_3^- normalized to kg bm. This data would help identify potential sex differences in the SBP and DBP responses to NO_3^- ingestion.

Trained Athletic Populations

Several studies have investigated the effects of dietary NO_3^- supplementation on the resting BP response of trained athletes with the majority demonstrating no beneficial effects on resting SBP, DBP or MAP regardless of dose or duration (Bond et al. 2012; Cermak et al. 2012a; Wilkerson et al. 2012; Nyakayiru et al. 2017a). However, others have shown significant reductions in SBP (~5 mmHg) following dietary NO_3^- supplementation (Larsen et al. 2007; Lansley et al. 2011b), with Larsen et al. (2007) also reporting a significant reduction in DBP (~ 4 mmHg). It is possible that dietary NO_3^- supplementation is less efficacious in trained populations due to lower resting BP levels, which may coincide with enhanced endothelial NOS expression and activity as well as elevated endogenous NO_3^- production (Cornelissen and Smart 2013; Boorsma et al. 2014). The positive effects of dietary NO_3^- supplementation on resting BP may be

explained by lower aerobic fitness levels (~55 mL O_2/kg/min) compared to the studies where no effects were seen (~62 mL O_2/kg/min). Currently, the effects of dietary NO_3^- supplementation on BP in trained female populations has not been thoroughly investigated and future work is needed.

Key Points

- Healthy, young adults demonstrate BP reductions of ~6 mmHg in SBP and ~3 mmHg in DBP following acute and chronic dietary NO_3^- supplementation.
- Women appear to have lower resting SBP and DBP compared to men and demonstrate less pronounced reductions in SBP and DBP following dietary NO_3^- supplementation.
- Dietary NO_3^- supplementation does not appear to reduce resting BP in trained populations, but females are severely underrepresented in this research.

Exercise Economy and Performance

For decades it was believed that the O_2 cost of submaximal exercise at a given absolute power output is fixed, regardless of age, health and fitness status and is unaltered by physical, nutritional or pharmacological interventions (Poole and Richardson 1997). However, work in the past 10-12 years with dietary NO_3^- supplementation has challenged this basic tenet by demonstrating an ~3-5% reduction in the O_2 cost of exercise at a given power output (exercise economy) during moderate intensity submaximal exercise in physically active males (Affourtit et al. 2015; Jones et al. 2018).

Early Work

The pioneering work by Larsen et al. (2007) in the laboratory of Dr. Bjorn Ekblom reported that supplementing 9 well-trained male cyclists/triathletes with 0.1 mmol $NaNO_3$/d for 3

d decreased O_2 uptake by 3-5% during submaximal cycling exercise between 45-80% VO_{2peak}. However, there was no improvement in the ability to cycle to exhaustion at VO_{2peak}.

Subsequent work from the laboratory of Dr. Andrew Jones demonstrated that BRJ ingestion containing 5.5 mmol NO_3^-/d for 6 d in 8 recreationally active males decreased end-exercise submaximal VO_2 compared to placebo (PL) (1.45 vs. 1.52 L/min) by ~5% but not during severe exercise (BRJ - 3.82 vs. PL – 3.87 L/min) (Bailey et al. 2009). Time to exhaustion (TTE) during intense exercise was improved in the BRJ (675 s) vs. the PL (585 s) trials. Additional work from the same laboratory extended the exercise economy and performance findings to demonstrate that the acute BRJ effects were still present after 5 and 15 d of supplementation (5.2 mmol NO_3^-/d) in 5 recreationally active males and 3 females (Vanhatalo et al. 2010). Lansley et al. (2011a) also showed that O_2 uptake was reduced in 9 recreationally active males during walking, and moderate and severe running compared to a PL after 6 d of BRJ supplementation. Importantly, this study used BRJ that had the NO_3^- removed as a PL, demonstrating that NO_3^- was indeed the active ingredient in BRJ. The same group also demonstrated that the acute ingestion of 6.2 mmol NO_3^- in BRJ improved cycling time trial (TT) performance in 9 male cyclists over 4 (2.8%) and 16.1 km (2.7%) vs. a PL (Lansley et al. 2011b). Other research groups were able to corroborate the exercise economy/performance findings in trained and recreationally active male subjects (Bond et al. 2012; Cermak et al. 2012a; Porcelli et al. 2015; Whitfield et al. 2016; DeCastro et al. 2019).

There is a major sex gap in the BRJ exercise research with few studies that included females in the subject pool or examined exclusively female populations. The first study suggesting that women may respond positively to acute and chronic dietary NO_3^- supplementation demonstrated a ~5% reduction in the O_2 cost of moderate intensity exercise in 5

male and 3 female recreationally active subjects following 2.5 h, 5 d and 15 d of supplementation (Vanhatalo et al. 2010). Although individual data was not provided and there was no discussion of potential sex differences regarding the responses to BRJ supplementation, it is likely that the female subjects responded positively to this intervention. A second study with a large group of recreationally active subjects (19 men, 15 women) demonstrated that the ingestion of 6 mmol NO_3^- decreased O_2 cost during submaximal exercise by 3%, 2 h post-ingestion and following 7 and ~28 d of supplementation (Wylie et al. 2016), suggesting that BRJ was effective in the women.

Table 2 about here.

To date, only 3 studies have investigated the effects of BRJ supplementation on exercise economy and performance in an exclusively recreationally active or sedentary female population (Table 2). Bond Jr. et al. (2014) explored the effects of acute BRJ supplementation on submaximal VO_2 in 12 sedentary women (VO_{2peak}, 26.1 $\pm$ 3.3 mL/kg/min) during the luteal phase of the menstrual cycle. The women were provided with either ~12 mmol dietary NO_3^- or orange juice (negligible NO_3^- content) 2 h prior to exercise testing for 5 min at each of 40, 60 and 80% VO_{2peak}. BRJ had no effect on VO_2 at rest, but reduced VO_2 by ~15% at 40, 60, and 80% VO_{2peak}. Rienks et al. (2015) examined the effects of acute BRJ supplementation on work performed during a rating of perceived exertion (RPE) clamp protocol in 10 recreationally active females (VO_{2peak}, 36.1 + 4.7 mL/kg/min, no control for menstrual cycle). The subjects ingested 12.9 mmol dietary NO_3^- 2.5 h prior to cycling and VO_2 was measured during a 20 min cycling protocol where subjects exercised at a self-selected intensity (RPE = 13, somewhat hard). There was no effect of BRJ on the work completed or total VO_2. However, VO_2 was reduced by 4% during a 5 min cooldown where the subjects cycled for 5 min at 75 W. A recent study by

Wickham et al. (2019) examined the effects of acute and chronic BRJ supplementation on submaximal cycling VO_2 and TT performance in 12 recreationally active females using hormonal contraceptives. The subjects supplemented acutely (2.5 h prior) and chronically (8 d) with 280 mL BRJ/d (~26 mmol NO_3^-) or a NO_3^- free PL. On days 1 and 8, participants cycled for 10 min at 50 and 70% VO_{2peak} and completed a 4 kJ/kg bm TT. Plasma [NO_3^-] and [NO_2^-] increased significantly following acute and chronic BRJ but VO_2 at 50 or 70% VO_{2peak} and TT performance were unaffected. These results were surprising given the large doses of NO_3^- used in this study.

The sparse literature that exists examining the effects of BRJ on exercise economy and performance with untrained/recreationally trained female subjects is equivocal. More research is needed with groups of recreationally active females in varying menstrual states, with varied NO_3^- doses and durations, as well as different exercise modalities and durations. It is not clear why BRJ is not as effective in females compared to recreationally active males and the potential sex differences are discussed in a later section.

Trained Subjects

The initial work by Larsen et al. (2007) and later work by Lansley et al. (2011a) demonstrated improved exercise economy and performance in groups of well-trained male athletes. Interestingly, a large number of more recent studies using a variety of well-trained male athletes have not reported improvements in exercise economy and performance in cycling (Cermak et al. 2012b; Wilkerson et al. 2012; Christensen et al. 2013; Glaister et al. 2015; McQuillan et al. 2016; Nyakayiru et al. 2017a) cross-country skiing (Peacock et al. 2012) and running (Boorsma et al. 2014; Vasconcellos et al. 2017).

An interesting study by Porcelli et al. (2015) investigated the effects of 6 d of $NaNO_3$ (5.5 mmol/d) supplementation on plasma [NO_3^-] and [NO_2^-], the O_2 cost of exercise and TT performance in 21 males with either low, moderate, or high aerobic fitness. Subjects with high aerobic fitness had higher baseline plasma [NO_3^-] compared to individuals with low or moderate aerobic fitness, but the less fit subjects demonstrated the greatest increases in both relative and absolute plasma [NO_3^-] and [NO_2^-]. The individuals with low and moderate fitness also experienced 10 and 7% reductions in the O_2 cost of submaximal exercise and a significant improvement in 3 km running TT performance. However, there was no difference in the O_2 cost of exercise or 3 km running TT performance for individuals with high aerobic fitness. It is possible that the individuals with low and moderate aerobic fitness experienced O_2 cost and performance benefits with dietary NO_3^- due to the dramatic elevation in plasma [NO_2^-] following supplementation compared to individuals with high aerobic fitness. Similar results were obtained by Carriker et al. (2016) who found a reduced O_2 cost of exercise at 45% and 60% VO_{2max} in 5 males with low aerobic fitness but no effect in 6 males with high aerobic fitness. However, the extent of improvement in exercise economy in untrained compared to trained individuals is unclear. Future work should determine whether untrained individuals become as efficient or more efficient than trained individuals following dietary NO_3^- supplementation.

Logan-Sprenger and Logan (2016) reported that 8 elite well-trained triathletes (4 men, 4 women) did not improve their 30 km TT cycling performance following an acute dose of BRJ (19.4 mmol NO_3^-) compared to a PL trial. Lane et al. (2014) reported that an acute dose of 8.4 mmol NO_3^- in BRJ delivered both 8-12 h before as well as 130 min prior had no effect on ~30 km TT performance in 12 well-trained female cyclists. Another study gave 13 female team-sport athletes 6 mmol dietary NO_3^-, 3 h prior to exercise and reported no effect of BRJ

supplementation on repeated sprint performance in the follicular phase of the menstrual cycle (Buck et al. 2015). Similarly, Glaister et al. (2015) gave 14 well-trained female cyclists ~7.3 mmol dietary NO_3^- as BRJ, ~2.5 h prior to exercise and again reported no effect on 20 km cycling TT performance.

Pospieszna et al. (2016) explored the effects of chronic BRJ supplementation on 6 sets of 50 m sprints and 800 m swim time in 11 female collegiate swimmers. The subjects participated in two, 8 d supplementation periods where they received 0.5 L of BRJ and chokeberry juice or 0.5 L of carrot juice with added KNO_3. Each supplement delivered 10.5 mmol of dietary NO_3^-/d. The dietary NO_3^- groups improved repeated sprint performance by 3.1% and 2.1% (sprints 4-6), and time to complete the 800 m swim. It should be noted that this study did not have a PL condition. Conversely, Lowings et al. (2017) found no beneficial effect of acute BRJ supplementation (~12.5 mmol NO_3^-) on repeated sprint swimming performance in ten trained swimmers (5 men, 5 women).

The evidence in well-trained women suggests that BRJ supplementation is not effective for improving athletic performance (similar to well-trained males) (Table 2). None of the studies with well-trained females measured exercise O_2 uptake to determine if exercise economy was improved with BRJ and most did not control for menstrual status. Future research in trained females needs to measure VO_2 during submaximal exercise and control for menstrual status to determine the effects of dietary NO_3^- supplementation on exercise economy in this population. Furthermore, Pospieszna et al. (2016) was the only group to investigate chronic BRJ supplementation, and no study to date has combined acute and chronic supplementation with well-trained female participants. Studies are needed to determine whether the effects of dietary

NO_3^- supplementation on submaximal exercise economy and athletic performance differ acutely and chronically in trained female populations.

Key Points

- BRJ supplementation (>5 mmol NO_3^-) decreased the O_2 cost of submaximal exercise (~3-5%) and often improved exercise performance, both acutely and chronically in recreationally trained males.
- The effect of BRJ supplementation on O_2 economy and performance has been studied much less in females and the existing results are equivocal.
- BRJ supplementation in well-trained males and females does not appear to improve O_2 economy during submaximal exercise, and TTE and TT performance. More work with trained female athletes is needed.

Mechanisms of Action

Dietary NO_3^- supplementation has been shown to elicit a number of biological effects due to its sequential reduction to NO_2^- and NO (Bailey et al. 2012). Due to the widespread effects of NO, several mechanisms of action have been proposed, and it is likely that they may work concomitantly to produce the robust biological effects associated with dietary NO_3^- supplementation. However, the mechanistic studies to date include cell culture work as well as animal and human work involving almost exclusively male subjects and it is clear that our knowledge of the mechanisms behind dietary NO_3^- supplementation in females is severely lacking.

Blood Pressure Regulation

BP is regulated through many complex pathways and signaling cascades. It is imperative to understand the relationship between NO and BP regulation, specifically how NO interacts

with these pathways and signaling cascades to influence the BP response. NO is capable of binding to guanylyl cyclase, which converts guanosine triphosphate to cyclic guanosine monophosphate (cGMP) and ultimately causes vasodilation through a number of pathways (Maréchal and Gailly 1999). cGMP is capable of inhibiting calcium (Ca^{2+}) entry into the cell, ultimately decreasing intracellular Ca^{2+} concentrations, and promoting relaxation (Blatter and Wier 1994). Alternatively, cGMP can activate potassium (K^{+}) channels leading to hyperpolarization and relaxation (Archer et al. 1994) and activate cGMP-dependent kinase which activates myosin light chain kinase, ultimately dephosphorylating myosin light chains resulting in smooth muscle relaxation (Etter et al. 2001). Human studies demonstrated increased cGMP levels following dietary NO_3^- supplementation (Bode-Böger et al. 1999, Larsen et al. 2010; Kapil et al. 2010) and two studies also reporting significantly reduced BP (Bode-Böger et al. 1999; Kapil et al. 2010). However, no studies have compared cGMP levels following dietary NO_3^- supplementation in men and women. This information would highlight potential sex differences that may exist in dietary NO_3^- conversion through the NO_3^--NO_2^--NO pathway, and the potential for BP reduction.

Reduction in the Oxygen Cost of Exercise

Following acute and chronic NO_3^- supplementation, there is a consistent 3–5% reduction in the O_2 cost of steady-state submaximal exercise in recreationally active males (Bailey et al. 2012). Originally, it was proposed that there may be three potential explanations for the reduced O_2 cost of submaximal exercise following dietary NO_3^- supplementation: 1) inhibition of mitochondrial ATP production requiring increased anaerobic energy production from the glycolytic and phosphocreatine pathways, 2) improved mitochondrial phosphate/oxygen (P/O) ratio suggesting a decrease in the O_2 required to produce the same amount of ATP, and 3)

improved excitation-contraction coupling suggesting an increase in force production per ATP consumed (Bailey et al. 2010).

Over the last decade, many studies have been performed to identify the leading mechanism of action explaining the ergogenic effects of dietary NO_3^- supplementation. It is possible that dietary NO_3^- supplementation elicits its beneficial effects in humans through an improvement in excitation-contraction coupling (Haider and Folland 2014; Hoon et al. 2015; Coggan et al. 2015; Whitfield et al. 2017; Coggan and Peterson, 2018), as there is equivocal evidence to support the effects of dietary NO_3^- supplementation on mitochondrial efficiency and function (Larsen et al. 2011; Whitfield et al. 2016).

i) Mitochondrial Efficiency

The electron transport chain (ETC) is comprised of a series of complexes that utilize electrons from NADH and $FADH_2$ to facilitate the movement of protons (hydrogen (H^+) ions) from the mitochondrial matrix to the intermembrane space (Holloway 2017). This creates an electrochemical gradient that ultimately drives the synthesis of ATP from ADP and inorganic phosphate via ATP synthase. Importantly, this pathway is dependent on the final electron acceptor, O_2. The efficiency of ATP synthesis is partially dependent on proton leak and electron slippage (Holloway 2017). Proton leak occurs when H^+ ions are distributed via alternative pathways that do not contribute to ATP synthesis, such as uncoupling proteins. Electron slippage occurs when electrons are moved from the mitochondrial matrix to the intermembrane space without the pumping of H^+ ions, resulting in a decreased membrane potential needed to drive ATP synthesis (Holloway 2017). The efficiency of the ETC is expressed as the phosphate/O_2 (P/O) ratio, or the rate of ATP synthesis over the rate of O_2 consumed (Larsen et al. 2011).

It is possible that dietary NO_3^- supplementation reduces the O_2 cost of submaximal exercise by directly affecting mitochondrial function either through the inhibition of mitochondrial ATP production or improving the mitochondrial P/O ratio (Bailey et al. 2010). There are reports that NO reversibly inhibits complex IV of the ETC by competing with O_2 at its binding site which would ultimately inhibit oxidative phosphorylation, but this would result in increased anaerobic fuel provision to meet the energy requirements of exercise (Bailey et al. 2010). However, human studies have reported no change in resting O_2 consumption and no shift in fuel utilization during submaximal exercise following dietary NO_3^- supplementation (Bailey et al. 2010). Dietary NO_3^- supplementation may also improve the mitochondrial P/O ratio with a decrease in the O_2 required to produce a constant amount of ATP. However, only one group has demonstrated improvements in mitochondrial coupling following dietary NO_3^- supplementation in 14 healthy adults (11 males, 3 females) (Larsen et al. 2011).

Dietary NO_3^- supplementation may improve mitochondrial coupling and there may be sex-based differences in the mitochondrial responses of men and women, although this is not clear. There is evidence suggesting that the livers and brains of female rodents produce less mitochondrial H_2O_2 than males (Borrás et al. 2003). Although speculative, this may suggest that female rodents have decreased electron slippage, which may improve mitochondrial coupling compared to males. Interestingly, it appears that this relationship is estrogen-dependent, as Borrás et al. (2003) found increased mitochondrial H_2O_2 emissions in ovariectomized rats and subsequent estrogen replacement therapy returned H_2O_2 production to basal levels. Although these findings have not been investigated in skeletal muscle, other groups have recently demonstrated sex differences in skeletal muscle mitochondrial function (Miotto et al. 2018) making this is an interesting avenue for future work. Furthermore, following 7 days of BRJ

supplementation, Whitfield et al. (2016) found significant increases in mitochondrial H_2O_2 emissions with BRJ supplementation in human skeletal muscle compared to a NO_3^--free placebo in males, but later found no effect of 7 d of BRJ supplementation on cellular redox stress (Whitfield et al. 2017). Future work could use advanced techniques, such as proteomics, to investigate cellular redox balance in skeletal muscle following dietary NO_3^- supplementation in men and women.

Although improvements in mitochondrial efficiency is an intriguing hypothesis to explain the positive effects of dietary NO_3^- supplementation, Whitfield et al. (2016) demonstrated no effect of dietary NO_3^- supplementation on mitochondrial efficiency in healthy males. Given the equivocal support for the mitochondrial efficiency hypothesis the most likely explanation for the reduced O_2 cost of submaximal exercise following dietary NO_3^- supplementation may be improved contractile efficiency.

ii) Contractile Efficiency

The major ATP cost of skeletal muscle contraction is related to sarcoendplasmic reticulum calcium ATPase (SERCA) and myosin ATPase activities which account for ~25-30 and 70% of the total ATP cost of contraction (Barclay et al. 2007). Due to the contributions of these proteins to the ATP cost of exercise, they may be key players behind the effects of dietary NO_3^- supplementation. Accordingly, the largest body of work exploring the mechanisms of action associated with dietary NO_3^- supplementation is related to improved contractile efficiency, specifically SERCA and myosin ATPase activities.

Cell culture work in muscle cell lines utilizing NO has demonstrated slowing of cross-bridge cycling by myosin ATPase resulting in greater force production per ATP hydrolyzed (Nogueira et al. 2009; Evangelista et al. 2010). These authors suggested that myosin ATPase

undergoes post-translational modifications on multiple cysteine thiols via nitrosylation by NO. Furthermore, dietary NO_3^- supplementation in animal models demonstrated improvements in skeletal muscle force production, attributed to altered calcium-handling protein function (Ishii et al. 1998) and increased calcium-handling protein content (Hernández et al. 2012). However, the positive effects of dietary NO_3^- are seen with both acute and chronic supplementation, suggesting that the mechanism of action is at least partially independent of changes in protein content, as this does not change acutely.

Interestingly, studies in humans have demonstrated increased force production following dietary NO_3^- supplementation (Bailey et al. 2010; Haider and Folland 2014; Coggan et al. 2015; Hoon et al. 2015; Whitfield et al. 2017). This has been reported in the absence of changes in calcium-handling protein content following chronic dietary NO_3^- supplementation, suggesting a role of post-translational modifications on skeletal muscle contractile proteins (Whitfield et al. 2017). Most of this work has been performed using male subjects. However, recent work from our laboratory demonstrated an improvement in skeletal muscle torque production following both acute and chronic dietary NO_3^- supplementation in recreationally active females (Wickham et al. 2019). This suggests that dietary NO_3^- can influence skeletal muscle contractile properties in both sexes, however the changes may not be large enough to translate into whole-body reductions in VO_2 or increases in performance in recreationally active females. Due to the lack of mechanistic work in females regarding dietary NO_3^- supplementation, it is unclear if sex-differences in skeletal muscle contractile properties may explain the divergent effects of dietary NO_3^- supplementation on the whole-body VO_2 response.

Sarwar et al. (1996) demonstrated differences in skeletal muscle torque production throughout the menstrual cycle, suggesting that estrogen may influence the activity of skeletal

muscle contractile proteins. In addition, other authors have shown that time to peak tension and rate of relaxation are faster in human males compared to females. These parameters are used as proxy measures for calcium release and reuptake during skeletal muscle contraction (Baylor and Hollingworth 2012). This suggests that men have an enhanced ability to release and requester calcium during skeletal muscle contractions, which may affect the skeletal muscle contractile properties following dietary NO_3^- supplementation between the sexes. However, these differences may also be attributed to significant differences in skeletal muscle fiber type distribution between the sexes (Haizlip et al. 2015). Future dietary NO_3^- studies should make direct comparisons between men and women with regards to calcium-handling protein content and function and should determine how these properties influence submaximal exercise economy.

Lastly, it has recently been shown that skeletal muscle is a storage reservoir for NO_3^- and NO_2^- in both healthy and type 2 diabetic men (Nyakayiru et al. 2017b). Since there are major anthropometric sex differences in absolute and relative skeletal muscle mass and skeletal muscle mass distribution (Table 1), this suggests there are differences in the ability to store and utilize dietary NO_3^- between the sexes which may influence dietary NO_3^- retention and excretion. Future research should utilize skeletal muscle biopsies to determine sex differences in NO_3^- storage following both absolute and relative doses of dietary NO_3^-. Furthermore, this research should be performed in conjunction with 24 h urinary analysis for NO_3^- excretion. Together, this work will advance our understanding of sex differences in dietary NO_3^- storage and excretion.

Key Points

- NO is a potent signaling molecule that elicits many biological effects, and therefore likely has a number of mechanisms of action that may work simultaneously.

- There is little evidence to suggest that NO_3^- supplementation improves mitochondrial efficiency to reduce the O_2 cost of submaximal exercise.
- The current leading hypothesis suggests dietary NO_3^- supplementation improves excitation-contraction coupling to reduce the O_2 cost of submaximal exercise.
- Women are heavily underrepresented in this mechanistic work and there are many potential areas where men and women may respond differently to NO_3^- supplementation (Fig. 1).

Fig. 1 about here.

Conclusion

Dietary NO_3^- supplementation has the potential to elicit profound biological effects within the body including lowering resting BP, reducing the O_2 cost of submaximal exercise and improving athletic performance. Due to the widespread effects of NO, the benefits of dietary NO_3^- supplementation are likely a product of several mechanisms of action working together. However, despite significant anthropometric, metabolic, and physiological differences between the sexes, it is abundantly clear that women are underrepresented in dietary NO_3^- supplementation research. There are many areas of research that may influence the sex-specific responses to dietary NO_3^- supplementation (Fig. 2). Ultimately, addressing this gap will help target nutrition-based health initiatives aimed at lowering blood pressure and enhancing athletic performance.

Fig. 2 about here.

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Conflicts of Interest

The authors have no conflicts of interest to report.

References

Abe, T., Kearns, C.F., and Fukunaga, T. 2003. Sex differences in whole body skeletal muscle mass measured by magnetic resonance imaging and its distribution in young Japanese adults. Br. J. Sports Med. **37**: 436 – 40. doi: 10.1136/bjsm.37.5.436.

Affourtit, C., Bailey, S.J., Jones, A.M., Smallwood, M.J., and Winyard, P.G. 2015. On the mechanism by which dietary nitrate improves human skeletal muscle function. Front. Physiol. **6**(211): 1 – 8. doi: 10.3389/fphys.2015.00211.

Archer, S.L., Huang, J.M., Hampl, V., Nelson, D.P., Shultz, P.J., and Weir, E.K. 1994. Nitric oxide and cGMP cause vasorelaxation by activation of charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. Proc. Natl. Acad. Sci. **91**(16): 7583 – 7. doi: https://doi.org/10.1073/pnas.91.16.7583.

Ashor, A.W., Lara, J., and Siervo, M. 2017. Medium-term effects of dietary nitrate supplementation on systolic and diastolic blood pressure in adults: a systematic review and meta-analysis. J. Hypertens. **35**: 1353 – 9. doi: 10.1097/HJH.0000000000001305.

Bailey, S.J., Winyard, P., Vanhatalo, A., Blackwell, J.R., Dimenna, F.J., Wilkerson, D.P., et al. 2009. Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. J. Appl. Physiol. **107**: 1144 - 55. doi:10.1152/japplphysiol.00722.2009.

Bailey, S.J., Fulford, J., Vanhatalo, A., Winyard, P.G., Blackwell, J.R., DiMenna, F.J., et al. 2010. Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. J. Appl. Physiol. **109**: 135 – 48. doi: 10.1152/japplphysiol.00046.2010

Bailey, S.J., Vanhatalo, A., Winyard, P.G., and Jones, A.M. 2012. The nitrate-nitrite-nitric oxide pathway: Its role in human exercise physiology. Eur. J. Sport Sci. **12**(4): 309 – 20. doi: https://doi.org/10.1080/17461391.2011.635705.

Barclay, C.J., Woledge, R.C., and Curtin, N.A. 2007. Energy turnover for Ca^{2+} cycling in skeletal muscle. J. Muscle Res. Cell Motil. **28**(4-5): 259– 74. doi:10.1007/s10974-007-9116-7.

Baylor, S.M., and Hollingworth, S. 2012. Intracellular calcium movements during excitation-contraction coupling in mammalian slow-twitch and fast-twitch muscle fibers. J. Gen. Physiol. **139**(4): 261 – 72. doi: 10.1085/jgp.201210773.

Blatter, L.A., and Wier, W.G. 1994. Nitric oxide decreases [Ca2+]i in vascular smooth muscle by inhibition of the calcium current. Cell Calcium, **15**(2): 122 – 31. doi: https://doi.org/10.1016/0143-4160(94)90051-5.

Bode-Böger, S.M., Böger, R.H., Löffler, M., Tsikas, D., Brabant, G., and Frölich, J.C. 1999. L-arginine stimulates NO-dependent vasodilation in healthy humans – effect of somatostatin pretreatment. J. Invest. Med. **47**(1): 43 – 50.

Bond, H., Morton, L., and Braakhuis, A.J. 2012. Dietary nitrate supplementation improves rowing performance in well-trained rowers. Int. J. Sport Nutr. Exerc. Metab. **22**: 251 – 6.

Bond Jr., V., Curry, B.H., Adams, R.G., Millis, R.M., and Haddad, G.E. 2014. Cardiorespiratory function associated with dietary nitrate supplementation. Appl. Physiol. Nutr. Metab. **39**(2): 168 – 72. doi: 10.1139/apnm-2013-0263.

Boorsma, R.K., Whitfield, J., and Spriet, L.L. 2014. Beetroot juice supplementation does not improve performance of elite 1500-m runners. Med. Sci. Sports Exerc. **46**(12): 2326 – 2334. doi: 10.1249/MSS.0000000000000364.

Borrás, C., Sastre, J., García-Sala, D., Lloret, A., Pallardó, F.V., and Viña, J. 2003. Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. Free Radic. Biol. Med. **34**(5): 546 – 52. doi: 10.1016/S0891-5849(02)01356-4.

Brown, M. 2008. Skeletal muscle and bone: effect of sex steroids and aging. Adv. Physiol. Educ. **32**: 120 – 6. doi: https://doi.org/10.1152/advan.90111.2008.

Bruinvels, G., Burden, R.J., McGregor, A.J., Ackerman, K.E., Dooley, M., Richards, T., et al. 2017. Sport, exercise and the menstrual cycle: where is the research? Br. J. Sports Med. **51**(6): 487 – 8. doi: http://dx.doi.org/10.1136/bjsports-2016-096279.

Buck, C.L., Henry, T., Guelfi, K., Dawson, B., McNaughton, L.R., and Wallman, K. 2015. Effects of sodium phosphate and beetroot juice supplementation on repeated-sprint ability in females. Eur. J. Appl. Physiol. **115**: 2205 – 13. doi: 10.1007/s00421-015-3201-1.

Carriker, C.R., Vaughan, R.A., VanDusseldorp, T.A., Johnson, K.E., Beltz, N.M., McCormick, et al. 2016. Nitrate-containing beetroot juice reduces oxygen consumption during submaximal exercise in low but not high aerobically fit male runners. J. Exerc. Nutr. Biochem. **20**(4): 27 – 34. doi: http://dx.doi.org/10.20463/jenb.2016.0029.

deCastro, T.F., Manoel, F.A., Figueiredo, D.H., Figueiredo, D.H. and Machado, F.A. 2019. Effect of beetroot juice supplementation on 10-km performance in recreational runners. Appl. Physiol. Nutr. Metab. **44**(1): 90 - 94. doi: 10.1139/apnm-2018-0277.

Cermak, N.M., Gibala, M.J., and van Loon, L.J.C. 2012a. Nitrate supplementation's improvement of 10-km time-trial performance in trained cyclists. Int. J. Sport Nutr. Exerc. Metab. **22**: 64 – 71.

Cermak, N.M., Res, P., Stinkens, R., Lundberg, J.O., Gibala, M.J., and van Loon, L.J.C. (2012b). No improvement in endurance performance after a single dose of beetroot juice. Int. J. Sport Nutr. Exerc. Metab. **22**: 470 - 478.

Christensen, P.M., Nyberg, M., and Bangsbo, J. 2013. Influence of nitrate supplementation on VO_2 kinetics and endurance of elite cyclists. Scand. J. Med. Sci. Sports **23**: 21 – 31. doi: 10.1111/sms.12005.

Coggan, A.R., Leibowitz, J.L., Kadkhodayan, A., Thomas, D.P., Ramamurthy, S., Spearie, C.A., et al. 2015. Effect of acute dietary nitrate intake on maximal knee extensor speed and power in healthy men and women. Nitric Oxide, **48**:16–21. doi: https://doi.org/10.1016/j.niox.2014.08.014.

Coggan, A.R., and Peterson, L.R. 2018. Dietary nitrate enhances the contractile properties of human skeletal muscle. Exerc. Sport Sci. Rev. **46**(4): 254 – 61. doi: 10.1249/JES.0000000000000167.

Coles, L.T., and Clifton, P.M. 2012. Effect of beetroot juice on lowering blood pressure in free-living, disease-free adults: a randomized, placebo-controlled trial. Nutr. J. **11**: 106 – 112. doi: http://www.nutritionj.com/content/11/1/106.

Collofello, B., Moskalik, B., and Essick, E. 2014. Acute dietary nitrate supplementation decreases systolic blood pressure and increases dry static apnea performance in females. J. Exerc. Physiol. **17**(4): 15 – 26.

Cornelissen, V.A., and Smart, N.A. 2013. Exercise training for blood pressure: A systematic review and meta-analysis. J. Am. Heart Assoc. **2**(1): e004473. doi: 10.1161/JAHA.112.004473.

Costello, J.T., Bieuzen, F., and Bleakley, and C.M. 2014. Where are all the female participants in Sports and Exercise Medicine research? Eur J Sport Sci. **14**(8): 847 – 51. doi: https://doi.org/10.1080/17461391.2014.911354.

Etter, E.F., Eto, M., Wardle, R.L., Brautigan, D.L., and Murphy, R.A. 2001. Activation of myosin light chain phosphatase in intact arterial smooth muscle during nitric oxide-induced relaxation. J. Biol. Chem. **276**(37): 34681 – 5. doi: 10.1074/jbc.M104737200.

Evangelista, A.M., Rao, V.S., Filo, A.R., Marozkina, N.V., Jones, D.R., Gaston, B., and Guilford, W.H. 2010. Direct regulation of striated muscle myosins by nitric oxide and endogenous nitrosothiols. PLoS ONE, **5**(6): 11209 – 16. doi: 10.1371/journal.pone.0011209.

Gilchrist, M., Winyard, P.G., and Benjamin, N. 2010. Dietary nitrate – good or bad? Nitric Oxide, **22**(2): 104 – 9. doi: 10.1016/j.niox.2009.10.005.

Glaister, M., Pattison, J.R., Muniz-Pumares, D., Patterson, S.D., and Foley, P. 2015. Effects of dietary nitrate, caffeine, and their combination on 20-km cycling time trial performance. J. Strength Cond. Res. **29**(1): 165 – 74. doi: 10.1519/JSC.0000000000000596.

Green, H.J., Fraser, I.G., and Ranney, D.A. 1984. Male and female differences in enzyme activities of energy metabolism in vastus lateralis muscle. J. Neurol. Sci. **65**(3): 323 – 31. doi: https://doi.org/10.1016/0022-510X(84)90095-9.

Haider, G., and Folland, J.P. 2014. Nitrate supplementation enhances the contractile properties of human skeletal muscle. Med. Sci. Sports Exerc. **46**(12): 2234 – 43. doi: 10.1249/MSS.0000000000000351.

Haizlip, K.M., Harrison, B.C., and Leinwand, L.A. 2015. Sex-based differences in skeletal muscle kinetics and fiber-type composition. Physiol. **30**: 30 – 9. doi: 10.1152/physiol.00024.2014.

Hernández, A., Schiffer, T.A., Ivarsson, N., Cheng, A.J., Bruton, J.D., Lundberg, J.O., et al. 2012. Dietary nitrate increases tetanic $[Ca^{2+}]_i$ and contractile force in mouse fast-twitch muscle. J. Physiol. **590**(15): 3575 – 83. doi: 10.1113/jphysiol.2012.232777.

Holloway, G.P. 2017. Nutrition and training influences on the regulation of mitochondrial adenosine diphosphate sensitivity and bioenergetics. Sports Med. **47**(1): 13 – 21. doi: 10.1007/s40279-017-0693-3.

Hoon, M.W., Fornusek, C., Chapman, P.G., and Johnson, N.A. 2015. The effect of nitrate supplementation on muscle contraction in healthy adults. Eur. J. Sport Sci. **15**: 712 – 9. doi: 10.1080/17461391.2015.1053418.

Hord, N.G., Tang, Y., and Bryan, N.S. 2009. Food sources of nitrates and nitrites: the physiologic context for potential health benefits. Am. J. Clin. Nutr. **90**(1): 1 – 10. doi: 10.3945/ajcn.2008.27131.

Ishii, T., Sunami, O., Saitoh, N., Nishio, H., Takeuchi, T., and Hata, F. 1998. Inhibition of skeletal muscle sarcoplasmic reticulum Ca2+-ATPase by nitric oxide. FEBS Lett. **440**(1-2): 218 – 22.

Jones, A.M., Thompson, C., Wylie, L.J., and Vanhatalo, A. 2018. Dietary nitrate and physical performance. Annu. Rev. Nutr. **38**: 303 - 328. doi: 10.1146/annurev-nutr-082117-051622.

Jonvik, K.L., Nyakayiru, J., van Dijk, J., Wardenaar, F.C., van Loon, L.J.C., and Verdijk, L.B. 2016. Habitual dietary nitrate intake in highly trained athletes. Int. J. Sport Nutr. Exerc. Metab. **27**: 148 – 57. doi: 10.1123/ijsnem.2016-0239.

Kapil, V., Milson, A.B., Okorie, M., Maleki-Toyserkani, S., Akram, F., Rehman, F., et al. 2010. Inorganic nitrate supplementation lowers blood pressure in humans. Hypertension, **56**: 274 – 81. doi: 10.1161/HYPERTENSIONAHA.110.153536.

Kapil, V., Rathod, K.S., Khambata, R.S., Bahra, M., Velmurugan, S., Purba, A., et al. 2018. Sex differences in the nitrate-nitrite-NO pathway: role of oral nitrate-reducing bacteria. Free Rad Biol Med. **126**: 113 – 21. doi: 10.1016/j.freeradbiomed.2018.07.010.

Katan, M.B. 2009. Nitrate in foods: harmful or healthy? Am. J. Clin. Nutr. **90**(1): 11 – 12. doi: https://doi.org/10.3945/ajcn.2009.28014.

Lane, S.C., Hawley, J.A., Desbrow, B., Jones, A.M., Blackwell, J.R., Ross, M.L., et al. 2014. Single and combined effects of beetroot juice and caffeine supplementation on cycling time trial performance. Appl. Physiol. Nutr. Metab. **39**: 1050 – 1057. doi: dx.doi.org/10.1139/apnm-2013-0336.

Lansley K.E., Winyard, P.G., Fulford, J., Vanhatalo, A., Bailey, S.J., Blackwell, J.R., et al. (2011a). Dietary nitrate supplementation reduces the O2 cost of walking and running: a placebo-controlled study. J. Appl. Physiol. **110:** 591 - 600. doi:10.1152/japplphysiol.01070.2010.

Lansley, K.E., Winyard, P.G., Bailey, S.J., Vanhatalo, A., Wilkerson, D.P., Blackwell, J.R., et al. 2011b. Acute dietary nitrate supplementation improves cycling time trial performance. Med. Sci. Sport Exerc. **43**(6): 1125 – 31. doi: 10.1249/MSS.0b013e31821597b4.

Larsen, F.J., Weitzberg, E., Lundberg, J.O., and Ekblom, B. 2007. Effects of dietary nitrate on oxygen cost during exercise. Acta Physiol. **191**(1): 59 – 66. doi: 10.1111/j.1748-1716.2007.01713.x.

Larsen, F.J., Weitzberg, E., Lundberg, J.O., Ekblom, B. 2010. Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise. Free Radic. Biol. Med. **48**(2): 342 – 47. doi: 10.1016/j.freeradbiomed.2009.11.006.

Larsen, F.J., Schiffer, T.A., Borniquel, S., Sahlin, K., Ekblom, B., Lundberg, J.O., et al. 2011. Dietary inorganic nitrate improves mitochondrial efficiency in humans. Cell Metab. **13**(2): 149 – 59. doi: 10.1016/j.cmet.2011.01.004

Lawes, C.M.M., Vander Hoorn, S., and Rodgers, A. 2008. Global burden of blood-pressure-related disease, 2001. Lancet, **371**(9623): 3 – 9. doi: https://doi.org/10.1016/S0140-6736(08)60655-8.

Logan-Sprenger, H.M., and Logan, S.L. 2016. Acute dose of beet root juice does not improve endurance performance in elite triathletes. Sports Nutr. Ther. **1**: 1000108. doi: 10.4172/2473-6449.

Lowings, S., Shannon, O.M., Deighton, K., Matu, J., and Barlow, M.J. 2017. Effect of dietary nitrate supplementation on swimming performance in trained swimmers. Int J Sport Nutr Exerc Metab. **27**(4): 377 – 84. doi: 10.1123/ijsnem.2016-0251.

Maréchal, G., and Gailly, P. 1999. Effects of nitric oxide on the contraction of skeletal muscle. Cell. Mol. Life Sci. **55**(8-9): 1088. doi:10.1007/s000180050359.

Mazure, C.M., and Jones, D.P. 2015. Twenty years and still counting: including women as participants and studying sex and gender in biomedical research. BMC Womens Health, **15**: 94. doi: 10.1186/s12905-015-0251-9.

McNally, B., Griffin, J.L., and Roberts, L.D. 2016. Dietary inorganic nitrate: From villain to hero in metabolic disease? Mol. Nutr. Food Res. **60**(1): 67 – 78. doi: 10.1002/mnfr.201500153.

McQuillan, J.A., Dulson, D.K., Laursen, P.B., and Kilding, A.E. 2017. Dietary nitrate fails to improve 1 and 4 km cycling performance in highly-trained cyclists. Int. J. Sports Nutr. Exerc. Metab. **27**: 255 – 63. doi: https://doi.org/10.1123/ijsnem.2016-0212.

Miotto, P.M., McGlory, C., Holloway, T.M., Phillips, S.M., and Holloway, G.P. 2018. Sex differences in mitochondrial respiratory function in human skeletal muscle. Am. J. Physiol. **314**(6): 909 – 15. doi: 10.1152/ajpregu.00025.2018.

Murphy, W.G. 2014. The sex difference in haemoglobin levels in adults – mechanisms, causes, and consequences. Blood Rev. **28**: 41 – 7. doi: 10.1016/j.blre.2013.12.003.

Nogueira, L., Figueiredo-Freitas, C., Casimiro-Lopes, G., Magdesian, M.H., Assreuy, J., and Sorenson, M.M. 2009. Myosin is reversibly inhibited by S-nitrosylation. Biochem. J. **424**: 221 – 31. doi: 10.1042/BJ20091144.

Nyakayiru, J.M., Jonvik, K.L., Pinckaers, P.J., Senden, J., van Loon, L.J., and Verdijk, L.B. 2017a. No effect of acute and 6-day nitrate supplementation on VO_2 and time-trial performance in highly trained cyclists. Int. J. Sport Nutr. Exerc. Metab. **27**(1): 11 – 7. doi: 10.1123/ijsnem.2016-0034.

Nyakayiru, J., Kouw, I.W.K., Cermak, N.M., Senden, J.M., van Loon, L.J.C., and Verdijk, L.B. 2017b. Sodium nitrate ingestion increases skeletal muscle nitrate content in humans. J. Appl. Physiol. **123**(3): 637 – 44. doi: 10.1152/japplphysiol.01036.2016.

Peacock, O., Tjonna, A.E., James, P., Wisløff, U., Welde, B., Böhlke, N., et al. 2012. Dietary nitrate does not enhance running performance in elite cross-country skiers. Med. Sci. Sports Exerc. **44**(11): 2213 – 2219. doi: 10.1249/MSS.0b013e3182640f48.

Poole, D.C., and Richardson, R.S. 1997. Determinants of oxygen uptake implications for exercise testing. Sports Med. **24**(5): 308 – 320. doi: 10.2165/00007256-199724050-00003.

Pinto, E. 2007. Blood pressure and ageing. Postgrad Med. J. **83**(976): 109 – 114. doi: 10.1136/pgmj.2006.048371.

Porcelli, S., Ramaglia, M., Bellistri, G., Pavei, G., Pugliese, L., Montorsi, M., et al. (2015). Aerobic fitness affects the exercise performance responses to nitrate supplementation. Med. Sci. Sports Exerc. **47**(8): 1643 – 1651. doi: 10.1249/MSS.0000000000000577.

Pospieszna, B., Wochna, K., Jerszyński, D., Gościnna, K., and Czapski, J. 2016. Ergogenic effects of dietary nitrates in female swimmers. Trends Sport Sci. **1**(23): 13 – 20.

Regitz-Zagrosek, V. 2012. Sex and gender aspects in clinical medicine. Berlin, Germany: Springer Science and Business Media.

Rienks, J.N., Vanderwoude, A.A., Maas, E., Blea, Z.M., and Subudhi, A.W. 2015. Effect of beetroot juice on moderate-intensity exercise at a constant rating of perceived exertion. Int. J. Exerc. Sci. **8**(3): 277 – 86.

Roepstorff, C., Thiele, M., Hillig, T., Pilegaard, H., Richter, E.A., Wojtaszewski, J.F.P., et al. 2006. Higher skeletal muscle α_2AMPK activation and lower energy charge and fat oxidation in men than in women during submaximal exercise. J. Physiol. **574**:125–38. doi: https://doi.org/10.1113/jphysiol.2006.108720.

Sarwar, R., Niclos, B., and Rutherford, O.M. 1996. Changes in muscle strength, relaxation rate and fatiguability during the human menstrual cycle. J. Appl. Physiol. **493**(1): 267 – 72. doi: 10.1113/jphysiol.1996.sp021381.

Siervo, M., Lara, J., Ogbonmwan, I., and Mathers, J.C. 2013. Inorganic nitrate and beetroot juice supplementation reduces blood pressure in adults: A systematic review and meta-analysis. J. Nutr. **143**: 8181 – 26. doi: 10.3945/jn.112.170233.

Tarnopolsky, M.A. 2000. Gender differences in substrate metabolism during endurance exercise. Can. J. Appl. Physiol. **25**(4): 312 – 27.

Tarnopolsky, M.A., Rennie, C.D., Robertshaw, H.A., Fedak-Tarnopolsky, S.N., Devries, M.C., and Hamadeh, M.J. 2007. Influence of endurance exercise training and sex on intramyocellular lipid and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. Am. J. Physiol. **292**: 1271 – 8. doi: 10.1152/ajpregu.00472.2006.

Vanhatalo, A., Bailey, S.J., Blackwell, J.R., DiMenna, F.J., Pavey, T.G., Wilkerson, D.P., et al. 2010. Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. Am. J. Physiol. **299**: 1121 – 31. doi: 10.1152/ajpregu.00206.2010.

Vasconcellos, J., Henrique Silvestre, D., Dos Santos Baião, D., Werneck-de-Castro, J.P., Silveira Alvares, T., et al. 2017. A single dose of beetroot gel rich in nitrate does not improve performance but lowers blood glucose in physically active individuals. J. Nutr. Metab. Epub 2017 Jan 24. doi: https://doi.org/10.1155/2017/7853034.

Webb, A.J., Patel, N., Loukogeorgakis, S., Okorie, M., Aboud, Z., Misra, S., et al. 2008. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. Hypertension, **51**: 784 – 90. doi: 10.1161/HYPERTENSIONAHA.107.103523.

Wells, J.C.K. 2007. Sexual dimorphism of body composition. Best Pract. Res. Clin. Endocrinol. Metab. **21**(3): 415 – 30. doi: https://doi.org/10.1016/j.beem.2007.04.007.

Whitfield, J., Ludzki, A., Heigenhauser, G.J.F., Senden, J.M.G., Verdijk, L.B., van Loon, L.J.C., et al. 2016. Beetroot juice supplementation reduces whole body oxygen consumption but does not improve indices of mitochondrial efficiency in human skeletal muscle. J. Physiol. **594**(2): 421 – 35. doi: 10.1113/JP270844.

Whitfield, J., Gamu, D., Heigenhauser, G.J.F., van Loon, L.J.C., Spriet, L.L., Tupling, R.A., et al. 2017. Beetroot juice increases human muscle force without changing Ca^{2+}-handling proteins. Med. Sci. Sports Exerc. **49**(10): 2016 – 24. doi: 10.1249/MSS.0000000000001321.

Wickham, K.A., McCarthy, D.G., Pereira, J.M., Cervone, D.T., Verdijk, L.B., van Loon, L.J.C., et al. 2019. No effect of beetroot juice supplementation on exercise economy and performance in recreationally active females despite increased torque production. Physiol. Rep. **7**(2): e13982. doi: 10.14814/phy2.13982.

Wilkerson, D.P., Hayward, G.M., Bailey, S.J., Vanhatalo, A., Blackwell, J.R., and Jones, A.M. 2012. Influence of acute dietary nitrate supplementation on 50 mile time trial performance in well-trained cyclists. Eur J Appl Physiol. **112**: 4127 – 34. doi: 10.1007/s00421-012-2397-6.

Wylie, L.J., Kelly, J., Bailey, S.J., Blackwell, J.R., Skiba, P.F., Winyard, P.G., et al. 2013. Beetroot juice and exercise: pharmacodynamic and dose-response relationships. J. Appl. Physiol. **115**: 325 – 36. doi: 10.1152/japplphysiol.00372.2013.

Wylie, L.J. deZevallos, O., Isidore, T., Nyman, L., Vanhatalo, A., Bailey, S.J., et al. 2016. Dose-dependent effects of dietary nitrate on the oxygen cost of moderate-intensity exercise: Acute vs. chronic supplementation. Nitric Oxide, **57**: 30 – 39. doi: http://dx.doi.org/10.1016/j.niox.2016.04.004.

Table 1. Summary of key sex differences in human anthropometry, physiology and metabolism.

Sex Difference	Males	Females
Anthropometrics	• Larger in stature (Wells 2007) • Greater total mass (Wells 2007) • Greater relative lean mass (Wells 2007) • Greater proportion of lean mass in the shoulders, chest, lower gluteal region (Abe et al. 2003)	• Smaller in stature (Wells 2007) • Lower total mass (Wells 2007) • Greater relative fat mass (Wells 2007) • Greater proportion of lean mass in the thighs (Abe et al. 2003)
[Hemoglobin] and red blood cell mass	• Greater hemoglobin levels • Greater red blood cell mass • Greater oxygen carrying capacity (Murphy 2014)	• Lower hemoglobin levels • Lower red blood cell mass • Lower oxygen carrying capacity (Murphy 2014)
Skeletal muscle fiber type	• Greater relative proportion of type II glycolytic skeletal muscle fibers (Haizlip et al. 2015) • Lower skeletal muscle capillarization (Roepstorff et al. 2006)	• Greater relative proportion of type I oxidative skeletal muscle fibers (Haizlip et al. 2015) • Greater skeletal muscle capillarization (Roepstorff et al. 2006)
Fuel stores and utilization	• Greater glycolytic enzyme activity (Green et al. 1984) • Greater reliance on carbohydrate during submaximal exercise (Tarnopolsky 2000)	• Greater intramuscular triglyceride stores (Tarnopolsky et al. 2007) • Greater capacity to mobilize intramuscular triglyceride stores (Tarnopolsky et al. 2007) • Greater reliance on fat during submaximal exercise (Tarnopolsky 2000)

Table 2. Summary of the studies that have examined the effects of dietary nitrate supplementation on physiological and performance parameters using exclusively female participants. Yrs, years; NO_3^-, nitrate; NO_2^-, nitrite; SBP, systolic blood pressure; RPE, rating of perceived exertion.

Author	Participants	Dose of Dietary NO_3^-	Primary Outcome(s)	Main Finding(s)	Limitations/Considerations
Collofello et al. (2014)	• 12 healthy females • Age: 18 to 30 yrs	• Acute: ~5.0 mmol	• Blood pressure • Maximal static apnea	• Dietary NO_3^- reduced resting SBP • Dietary NO_3^- did not improve maximal static apnea	• Small dose of dietary NO_3^- • Did not control for menstrual cycle phase • No measure of plasma $[NO_3^-]$ or $[NO_2^-]$
Bond Jr. et al. (2014)	• 12 overweight, sedentary females • Age: 20.7 ± 0.8 yrs	• Acute: ~12.0 mmol	• Blood pressure • Submaximal VO_2	• Dietary NO_3^- reduced resting and exercise SBP • Dietary NO_3^- reduced submaximal VO_2 at 40, 60 and 80% VO_{2peak}	• Did not use a NO_3^--free placebo
Lane et al. (2014)	• 12 female competitive cyclists or triathletes • Age: 28 ± 6 yrs	• Acute: ~8.4 mmol (2 doses; 8-12 h and 130 min prior)	• 29.35 km cycling time trial	• Dietary NO_3^- had no effect on time trial performance	• Did not control for menstrual cycle phase
Rienks et al. (2015)	• 10 recreationally active females • Age: 25 ± 3 yrs	• Acute: ~13 mmol	• Blood pressure • 20 min RPE clamp protocol • Submaximal VO_2 (5 min cooldown)	• Dietary NO_3^- reduced resting SBP • Dietary NO_3^- did not change performance • Dietary NO_3^- reduced submaximal VO_2 (cooldown)	• Did not control for menstrual cycle phase • No measure of plasma $[NO_3^-]$ or $[NO_2^-]$
Glaister et al. (2015)	• 14 competitive female cyclists • Age: 31 ± 7 yrs	• Acute: ~7.3 mmol	• 20 km cycling time trial	• Dietary NO_3^- had no effect on time trial performance	• Small dose of dietary NO_3^- • Did not control for menstrual cycle phase
Buck et al. (2015)	• 13 female amateur team-sport athletes • Age: 25.5 ± 1.9 yrs	• Acute: ~ 6.0 mmol	• Performance on a simulated team-game circuit	• Dietary NO_3^- had no effect on simulated team-game circuit performance	• Small dose of dietary NO_3^-
Pospieszna et al. (2016)	• 11 university level female swimmers • Age: 20.9 ± 1.3 yrs	• Chronic: ~ 10.0 mmol/d/8d	• Blood pressure • Anaerobic swimming performance (6 x 50 m) • Aerobic swimming performance (800 m)	• No difference in resting blood pressure • Dietary NO_3^- improved anaerobic swimming in the final 3 of 6 sprint efforts • Dietary NO_3^- improved aerobic swimming performance	• Did not control for menstrual cycle phase • Did not use a NO_3^--free placebo • No measure of plasma $[NO_3^-]$ or $[NO_2^-]$
Wickham et al. (2019)	• 12 recreationally active females • Age: 23 ± 1 yrs	• Acute: ~13.0 mmol • Chronic: ~ 26.0 mmol/d/8d	• Submaximal VO_2 • 4 kJ/kg time trial performance	• Dietary NO_3^- did not reduce submaximal VO_2 • Dietary NO_3^- did not improve time trial performance	• Recruited females using hormonal contraceptives to control for menstrual cycle

Figure Captions

Fig. 1. Potential sex differences associated with dietary NO_3^- supplementation. +, response more robust in females; **-**, response less robust in females; *****; lower in females; **?**, unknown.

Fig. 2. Current sex-specific research gaps in the field of dietary NO_3^- supplementation.

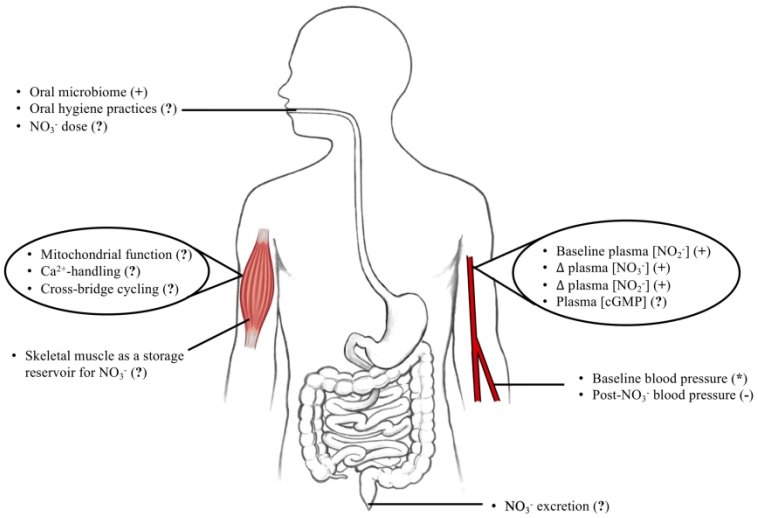

Fig. 1. Potential sex differences associated with dietary NO3- supplementation. +, response more robust in females; -, response less robust in females; *; lower in females; ?, unknown.

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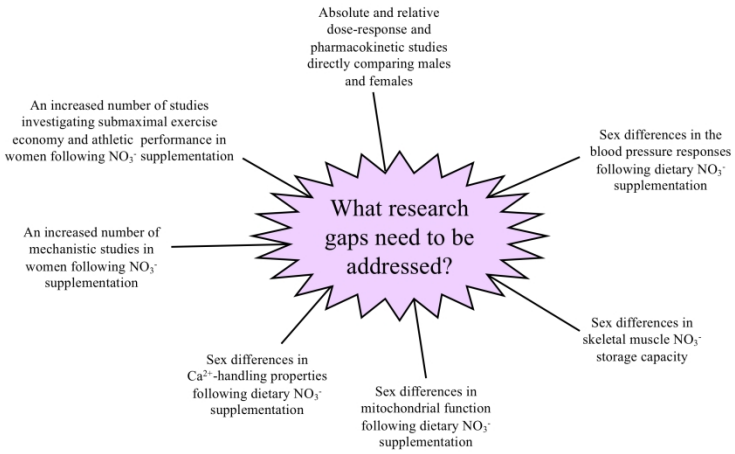

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