

No longer beeting around the bush: a review of potential sex differences with dietary nitrate supplementation¹

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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.

Novelty

- 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.

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

Résumé : Depuis une dizaine d'années, les avantages pour la santé et les performances sportives associés à la supplémentation alimentaire ponctuelle et à long terme en nitrate (« NO_3^- ») suscitent un vif intérêt. Le NO_3^- alimentaire, généralement présent dans les légumes verts feuillus et les légumes-racines, subit une réduction séquentielle en nitrite et en oxyde nitrique (« NO ») via la circulation entérosalivaire. Fait important, le NO engendre, documentation à l'appui, un certain nombre d'effets biologiques allant de la réduction de la pression artérielle à l'amélioration de l'économie de l'exercice et de la performance sportive. Cependant, on dénote souvent en recherche biologique l'absence de participantes attribuables à la complexité des fluctuations hormonales au cours du cycle menstruel. Nonobstant l'accumulation de données significatives incontournables au sujet des différences anthropométriques, métaboliques et physiologiques importantes entre les sexes, ce problème s'étend au domaine de la supplémentation en NO_3^- alimentaire où les femmes sont sous-représentées en tant que participantes à la recherche. Cette analyse documentaire examine la recherche existante sur la supplémentation en NO_3^- alimentaire en ce qui concerne la pharmacocinétique du NO_3^- alimentaire, la tension artérielle au repos, l'économie de l'exercice, la performance sportive et les mécanismes d'action. Cette analyse procure également des données probantes et une justification des différences potentielles entre les sexes en réponse à la supplémentation en NO_3^- alimentaire et des orientations futures pour ce domaine de recherche. [Traduit par la Rédaction]

Les nouveautés

- La supplémentation en NO_3^- alimentaire a, documentation à l'appui, des effets positifs sur la santé et les performances sportives chez des populations généralement masculines. Cependant, les femmes sont sous-représentées dans la recherche sur la supplémentation en NO_3^- alimentaire.
- Les données probantes actuelles suggèrent qu'il existe des différences entre les sexes en réponse à la supplémentation en NO_3^- alimentaire et cette analyse met en évidence les pistes de recherche futures.

Mots-clés : nitrate alimentaire, différences entre les sexes, jus de betterave, oxyde nitrique, pression artérielle, économie de l'exercice, performance, couplage excitation-contraction.

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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^- concentration ($[\text{NO}_3^-]$) and NO_2^- concentration ($[\text{NO}_2^-]$) (Kapil et al. 2010; Larsen et al. 2010; Wylie et al. 2013).

Addressing the sex gap

It was not 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 Institutes 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) highlights 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).

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 (i) examine selected areas of dietary NO_3^- supplementation research that have been performed using males and females; (ii) identify existing sex differences; and (iii) highlight future research questions and directions for this field.

Supplemental NO_3^- dose-response and 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 2 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 $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ following administration of 4.2, 8.4, and 16.8 mmol dietary NO_3^- administered as BRJ in healthy males. They found that peak plasma $[\text{NO}_3^-]$ occurred 1 h after ingestion of BRJ and peak plasma $[\text{NO}_2^-]$ occurred ~2–2.5 h after ingestion. Kapil et al. (2010) also reported that plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ increased in a dose-dependent manner following the administration of 4, 12, and 24 mmol KNO_3 . Plasma $[\text{NO}_3^-]$ rose 30 min after ingestion, peaked at 3 h, and remained elevated for 24 h. Plasma $[\text{NO}_2^-]$ rose significantly 1.5 h after ingestion, peaked at 2.5 h, and remained elevated above baseline at 24 h. Interestingly, females had significantly greater baseline plasma $[\text{NO}_2^-]$ compared with men, despite having similar baseline plasma $[\text{NO}_3^-]$. Absolute plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ rose significantly higher in females compared with 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 with males, females have an enhanced ability to reduce NO_3^- to NO_2^- at baseline and following NO_3^- supplementation because of 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 with 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

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

Sex difference	Males	Females
Anthropometrics	<ul style="list-style-type: none"> • 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) 	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • Greater hemoglobin levels • Greater red blood cell mass • Greater oxygen carrying capacity (Murphy 2014) 	<ul style="list-style-type: none"> • Lower hemoglobin levels • Lower red blood cell mass • Lower oxygen carrying capacity (Murphy 2014)
Skeletal muscle fibre type	<ul style="list-style-type: none"> • Greater relative proportion of type II glycolytic skeletal muscle fibres (Haizlip et al. 2015) • Lower skeletal muscle capillarization (Roepstorff et al. 2006) 	<ul style="list-style-type: none"> • Greater relative proportion of type I oxidative skeletal muscle fibres (Haizlip et al. 2015) • Greater skeletal muscle capillarization (Roepstorff et al. 2006)
Fuel stores and utilization	<ul style="list-style-type: none"> • Greater glycolytic enzyme activity (Green et al. 1984) • Greater reliance on carbohydrate during submaximal exercise (Tarnopolsky 2000) 	<ul style="list-style-type: none"> • 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)

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 $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ increase in a dose-dependent manner following dietary NO_3^- supplementation in men and women.
- Following an acute dose of dietary NO_3^- , plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ remained significantly elevated above baseline for up to 24 h.
- Women appear to have elevated baseline plasma $[\text{NO}_2^-]$ and experience greater relative increases in plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ following dietary NO_3^- supplementation than men. However, this may reflect larger doses of dietary $\text{NO}_3^-/\text{kg BM}$.

Blood pressure (BP)

High 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 BP (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 mm Hg in SBP and ~ 3 mm Hg in DBP following dietary NO_3^- supplementation in healthy individuals (Siero 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 $\text{KNO}_3/\text{kg BM}$ and a greater subsequent rise in absolute and relative $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ 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 with 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 $[\text{NO}_3^-]$ or $[\text{NO}_2^-]$. Finally, 2 studies measured the BP response to dietary NO_3^- in exclusively female participants. Collofello et al. (2014) demonstrated an ~ 5 -mm Hg reduction in SBP and nonsignificant 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 with females. It is unclear if this is due to differences in baseline plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$, 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 $[\text{NO}_3^-]$, $[\text{NO}_2^-]$, 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 mm Hg) 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 mm Hg). 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 \cdot min)) compared with the studies where no effects were seen (~ 62 mL O_2 /(kg \cdot 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 mm Hg in SBP and ~ 3 mm Hg in DBP following acute and chronic dietary NO_3^- supplementation.
- Women appear to have lower resting SBP and DBP compared with 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 under-represented 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 $\sim 3\%$ – 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 /day for 3 days decreased O_2 uptake by 3%–5% during submaximal cycling exercise between 45%–80% peak oxygen uptake ($\dot{V}\text{O}_{2\text{peak}}$). However, there was no improvement in the ability to cycle to exhaustion at $\dot{V}\text{O}_{2\text{peak}}$.

Subsequent work from the laboratory of Dr. Andrew Jones demonstrated that BRJ ingestion containing 5.5 mmol NO_3^- /day for 6 days in 8 recreationally active males decreased end-exercise submaximal oxygen uptake ($\dot{V}\text{O}_2$) compared with placebo (PL) (1.45 vs. 1.52 L/min) by $\sim 5\%$ 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) versus 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 days of supplementation (5.2 mmol NO_3^- /day) 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 with a PL after 6 days 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%) versus 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; de Castro 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 an $\sim 5\%$ reduction in the O_2 cost of moderate-intensity exercise in 5 male and 3 female recreationally active subjects following 2.5 h, 5 days, and 15 days 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 days of supplementation (Wylie et al. 2016), suggesting that BRJ was effective in the women.

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 et al. (2014) explored the effects of acute BRJ supplementation on submaximal $\dot{V}\text{O}_2$ in 12 sedentary women ($\dot{V}\text{O}_{2\text{peak}}$, 26.1 ± 3.3 mL/(kg \cdot 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% $\dot{V}\text{O}_{2\text{peak}}$. BRJ had no effect on $\dot{V}\text{O}_2$ at rest, but reduced $\dot{V}\text{O}_2$ by $\sim 15\%$ at 40%, 60%, and 80% $\dot{V}\text{O}_{2\text{peak}}$. 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 ($\dot{V}\text{O}_{2\text{peak}}$, 36.1 ± 4.7 mL/(kg \cdot min), no control for menstrual cycle). The subjects ingested 12.9 mmol dietary NO_3^- at 2.5 h prior to cycling and $\dot{V}\text{O}_2$ 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 $\dot{V}\text{O}_2$. However, $\dot{V}\text{O}_2$ 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 $\dot{V}\text{O}_2$ and TT performance in 12 recreationally active females using hormonal contraceptives. The subjects supplemented acutely (2.5 h prior) and chronically (8 days) with 280 mL BRJ/day (~ 26 mmol NO_3^-) or a NO_3^- -free PL. On days 1 and 8, participants cycled for 10 min at 50% and 70% $\dot{V}\text{O}_{2\text{peak}}$ and completed a 4-kJ/kg BM TT. Plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ increased significantly following acute and chronic BRJ but $\dot{V}\text{O}_2$ at 50% or 70% $\dot{V}\text{O}_{2\text{peak}}$ and TT performance were unaffected. These results were surprising given the large doses of NO_3^- used in this study.

The sparse existing literature that examines 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 with 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;

Table 2. Summary of the studies that have examined the effects of dietary nitrate supplementation on physiological and performance parameters using exclusively female participants.

Author	Participants	Dose of dietary NO ₃ ⁻	Primary outcome(s)	Main finding(s)	Limitations/considerations
Collofello et al. 2014	<ul style="list-style-type: none"> 12 healthy females Age: 18 to 30 y 	<ul style="list-style-type: none"> Acute: ~5.0 mmol 	<ul style="list-style-type: none"> Blood pressure Maximal static apnea 	<ul style="list-style-type: none"> Dietary NO₃⁻ reduced resting SBP Dietary NO₃⁻ did not improve maximal static apnea 	<ul style="list-style-type: none"> Small dose of dietary NO₃⁻ Did not control for menstrual cycle phase No measure of plasma [NO₃⁻] or [NO₂⁻]
Bond et al. 2014	<ul style="list-style-type: none"> 12 overweight, sedentary females Age: 20.7 ± 0.8 y 	<ul style="list-style-type: none"> Acute: ~12.0 mmol 	<ul style="list-style-type: none"> Blood pressure Submaximal $\dot{V}O_2$ 	<ul style="list-style-type: none"> Dietary NO₃⁻ reduced resting and exercise SBP Dietary NO₃⁻ reduced submaximal $\dot{V}O_2$ at 40%, 60% and 80% $\dot{V}O_{2peak}$ 	<ul style="list-style-type: none"> Did not use a NO₃⁻-free placebo
Lane et al. 2014	<ul style="list-style-type: none"> 12 female competitive cyclists or triathletes Age: 28 ± 6 y 	<ul style="list-style-type: none"> Acute: ~8.4 mmol (2 doses; 8–12 h and 130 min prior) 	<ul style="list-style-type: none"> 29.35-km cycling time trial 	<ul style="list-style-type: none"> Dietary NO₃⁻ had no effect on time trial performance 	<ul style="list-style-type: none"> Did not control for menstrual cycle phase
Rienks et al. 2015	<ul style="list-style-type: none"> 10 recreationally active females Age: 25 ± 3 y 	<ul style="list-style-type: none"> Acute: ~13 mmol 	<ul style="list-style-type: none"> Blood pressure 20-min RPE clamp protocol Submaximal $\dot{V}O_2$ (5 min cooldown) 	<ul style="list-style-type: none"> Dietary NO₃⁻ reduced resting SBP Dietary NO₃⁻ did not change performance Dietary NO₃⁻ reduced submaximal $\dot{V}O_2$ (cooldown) 	<ul style="list-style-type: none"> Did not control for menstrual cycle phase No measure of plasma [NO₃⁻] or [NO₂⁻]
Glaister et al. 2015	<ul style="list-style-type: none"> 14 competitive female cyclists Age: 31 ± 7 y 	<ul style="list-style-type: none"> Acute: ~7.3 mmol 	<ul style="list-style-type: none"> 20 km cycling time trial 	<ul style="list-style-type: none"> Dietary NO₃⁻ had no effect on time trial performance 	<ul style="list-style-type: none"> Small dose of dietary NO₃⁻ Did not control for menstrual cycle phase
Buck et al. 2015	<ul style="list-style-type: none"> 13 female amateur team-sport athletes Age: 25.5 ± 1.9 y 	<ul style="list-style-type: none"> Acute: ~6.0 mmol 	<ul style="list-style-type: none"> Performance on a simulated team-game circuit 	<ul style="list-style-type: none"> Dietary NO₃⁻ had no effect on simulated team-game circuit performance 	<ul style="list-style-type: none"> Small dose of dietary NO₃⁻
Pospieszna et al. 2016	<ul style="list-style-type: none"> 11 university-level female swimmers Age: 20.9 ± 1.3 y 	<ul style="list-style-type: none"> Chronic: ~10.0 mmol/(d·8 d) 	<ul style="list-style-type: none"> Blood pressure Anaerobic swimming performance (6 × 50 m) Aerobic swimming performance (800 m) 	<ul style="list-style-type: none"> No difference in resting blood pressure Dietary NO₃⁻ improved anaerobic swimming in the final 3 of 6 sprint efforts Dietary NO₃⁻ improved aerobic swimming performance 	<ul style="list-style-type: none"> Did not control for menstrual cycle phase Did not use a NO₃⁻-free placebo No measure of plasma [NO₃⁻] or [NO₂⁻]
Wickham et al. 2019	<ul style="list-style-type: none"> 12 recreationally active females Age: 23 ± 1 y 	<ul style="list-style-type: none"> Acute: ~13.0 mmol Chronic: ~26.0 mmol/(day·8 d) 	<ul style="list-style-type: none"> Submaximal $\dot{V}O_2$ 4 kJ/kg time-trial performance 	<ul style="list-style-type: none"> Dietary NO₃⁻ did not reduce submaximal $\dot{V}O_2$ Dietary NO₃⁻ did not improve time trial performance 	<ul style="list-style-type: none"> Recruited females using hormonal contraceptives to control for menstrual cycle

Note: NO₂⁻, nitrite; NO₃⁻, nitrate; RPE, rating of perceived exertion; SBP, systolic blood pressure; $\dot{V}O_2$, oxygen uptake.

Wilkerson et al. 2012; Christensen et al. 2013; Glaister et al. 2015; McQuillan et al. 2017; 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 days of NaNO₃ (5.5 mmol/day) supplementation on plasma [NO₃⁻] and [NO₂⁻], the O₂ 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 [NO₃⁻] compared with individuals with low or moderate aerobic fitness, but the less fit subjects demonstrated the greatest increases in both relative and absolute plasma [NO₃⁻] and [NO₂⁻]. The individuals with low and moderate fitness also experienced 10% and 7% reductions in the O₂ cost of submaximal exercise and a significant improvement in 3-km running TT performance. However, there was no difference in the O₂ 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 O₂ cost and performance benefits with dietary NO₃⁻ due to the dramatic elevation in plasma [NO₂⁻] following supplementation compared with individuals with high aerobic fitness. Similar results were obtained by Carriker et al. (2016) who found a reduced O₂ cost of exercise at 45% and 60% $\dot{V}O_{2max}$ 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 with trained individuals is unclear. Future work should determine whether untrained individuals become as efficient or more efficient than trained individuals following dietary NO₃⁻ 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 NO₃⁻) compared with a PL trial. Lane et al. (2014) reported that an acute dose of 8.4 mmol NO₃⁻ 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 NO₃⁻, 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 NO₃⁻ 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-day supplementation periods where they received 0.5 L of BRJ and chokeberry juice or 0.5 L of carrot juice with added KNO₃. Each supplement delivered 10.5 mmol of dietary NO₃⁻/day. The dietary NO₃⁻ 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 NO₃⁻) on repeated sprint swimming performance in 10 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 O₂ 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 $\dot{V}O_2$ during submaximal exercise and control for menstrual status to determine the effects of dietary NO₃⁻ 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 NO₃⁻ supplementation on submaximal exercise economy and athletic performance differ acutely and chronically in trained female populations.

Key points

- BRJ supplementation (>5 mmol NO₃⁻) decreased the O₂ 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 O₂ 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 O₂ economy during submaximal exercise and TTE and TT performance. More work with trained female athletes is needed.

Mechanisms of action

Dietary NO₃⁻ supplementation has been shown to elicit a number of biological effects because of its sequential reduction to NO₂⁻ 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 NO₃⁻ 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 NO₃⁻ supplementation in females is severely lacking.

BP regulation

BP is regulated through many complex pathways and signalling cascades. It is imperative to understand the relationship between NO and BP regulation, specifically how NO interacts with these pathways and signalling 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 (Ca²⁺) entry into the cell, ultimately decreasing intracellular Ca²⁺ 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 NO₃⁻ supplementation (Bode-Böger et al. 1999, Larsen et al. 2010; Kapil et al. 2010) and 2 studies also reported significantly reduced BP (Bode-Böger et al. 1999; Kapil et al. 2010). However, no studies have compared cGMP levels following dietary NO₃⁻ supplementation in men and women. This information would highlight potential sex differences that may exist in dietary NO₃⁻ conversion through the NO₃⁻–NO₂⁻–NO pathway, and the potential for BP reduction.

Reduction in the oxygen cost of exercise

Following acute and chronic NO₃⁻ supplementation, there is a consistent 3%–5% reduction in the O₂ cost of steady-state submaximal exercise in recreationally active males (Bailey et al. 2012). Originally, it was proposed that there may be 3 potential explanations for the reduced O₂ cost of submaximal exercise following dietary NO₃⁻ supplementation: (i) inhibition of mitochondrial adenosine triphosphate (ATP) production requiring increased anaerobic energy production from the glycolytic and phosphocreatine pathways; (ii) improved mitochondrial phosphate/oxygen (P/O) ratio suggesting a decrease in the O₂ required to produce the

same amount of ATP; and (iii) 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; Coggan et al. 2015; Hoon 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) comprises 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 adenosine diphosphate 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 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 1 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 with 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 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 with a NO_3^- -free placebo in males, but later found no effect of 7 days 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

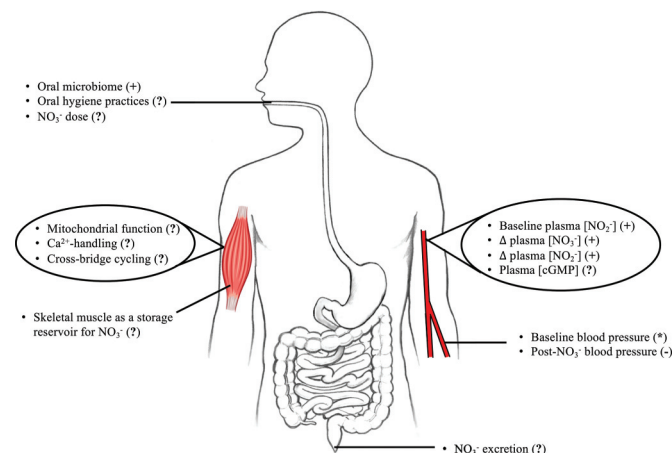
The major ATP cost of skeletal muscle contraction is related to sarcoendoplasmic reticulum calcium ATPase (SERCA) and myosin ATPase activities, which account for ~25%–30% and 70% of the total ATP cost of contraction (Barclay et al. 2007). Due to the contributions of these proteins to the ATP cost of 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 $\dot{V}\text{O}_2$ 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 $\dot{V}\text{O}_2$ 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 with females. These parameters are used as proxy measures for calcium release and reuptake during skeletal muscle contraction (Baylor and Hollingworth 2012). This suggests that

Fig. 1. Potential sex differences associated with dietary nitrate (NO_3^-) supplementation. +, response more robust in females; –, response less robust in females; *, lower in females; ?, unknown. [Colour online.]



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 fibre-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.

Finally, 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.

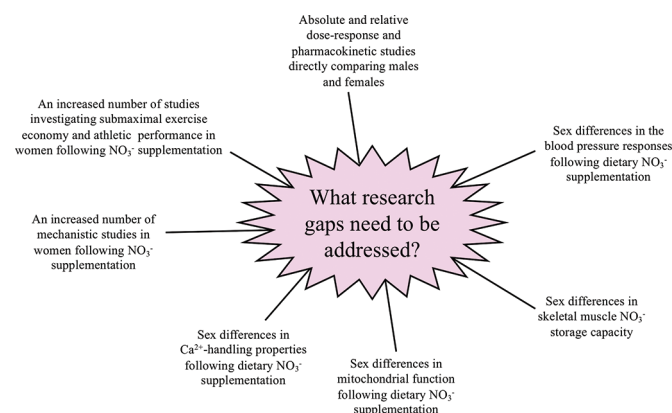
Key points

- NO is a potent signalling 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).

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

Fig. 2. Current sex-specific research gaps in the field of dietary nitrate (NO_3^-) supplementation. Ca^{2+} , calcium. [Colour online.]



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.

Conflict of interest statement

The authors have no conflicts of interest to report.

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