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DIETARY NITRATE AND EXERCISE PERFORMANCE: NEW STRINGS TO THE BEETROOT BOW

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KEY POINTS

- Nitric oxide (NO) is a gaseous molecule that is essential to life itself. It must be continuously produced to support a wide variety of physiological processes, including those relating to muscle contraction, metabolism, and blood flow.
- There are two complementary pathways for NO production: one is enzymatic and requires the amino acid arginine and oxygen; the other – the nitrate-nitrite-NO pathway – depends on the availability of nitrate. Nitrate is produced endogenously but can also enter our bodies via the diet to augment our nitrate stores.
- When nitrate-rich foodstuffs (such as green leafy vegetables) or supplements are consumed, the ingested nitrate is primarily converted to bioactive nitrite by the bacteria residing in the mouth. This nitrite enters the circulation and can be easily reduced to NO where required, such as in areas where oxygen availability is low, including in contracting skeletal muscle.
- Early studies indicated that dietary nitrate supplementation reduced the oxygen cost of submaximal exercise and improved endurance exercise performance. However, more recent studies suggest that these effects may only be evident in non-elite endurance athletes.
- More recently, it has been shown that dietary nitrate may be ergogenic during activities requiring high muscle power output and during both single and repeated sprints. The enhancement of muscle contractile function by nitrate may be related to effects on muscle calcium handling.
- There is evidence that skeletal muscle may serve as a storage site for nitrate and that muscle nitrate levels are sensitive to diet and exercise.
- Nitrate supplementation may have applications in both low- and high-intensity exercise and in a variety of team sports. However, the efficacy of nitrate supplementation depends upon several important factors, including the dose administered and the timing of ingestion relative to the start of exercise.

INTRODUCTION

Athletes, both elite and recreational, are naturally interested in improving their exercise performance. While the consumption of a balanced diet is important for the maintenance of general health and to fuel and recover from training, many athletes also use dietary supplements with the aim of enhancing performance during competition. Examples of these supplements include caffeine, creatine, sodium bicarbonate, beta alanine and, most recently, inorganic nitrate (NO_3-) (Maughan et al., 2011). The evidence base for the efficacy of nutritional ergogenic aids is notoriously mixed, and nitrate is no exception. However, recent meta-analyses do support the suggestion that dietary nitrate, which is normally ingested by athletes in the form of beetroot juice, has the potential to enhance performance in several types of sport and exercise activities (McMahon et al., 2017; Pawlak-Chaouch et al., 2016; Senefeld et al., 2020). Moreover, unlike other supplements that are mainly of value to athletes, nitrate supplementation may also provide cardiovascular health benefits to the general (non-athletic) public via its well-described effects on reducing resting blood pressure.

The purpose of this Sports Science Exchange article is to highlight recent advances in our understanding of the effects of dietary nitrate supplementation on physical performance. The article will focus on the role of the oral microbiome in activating ingested nitrate, discuss the possibility that skeletal muscle is an important site for nitrate storage, and review new information on the potential situations in which nitrate supplementation may be ergogenic. In particular, while initial observations that nitrate could reduce the oxygen cost of exercise (i.e., improve exercise economy) pointed to applications in endurance sports, more recent findings suggest a potentially important role for nitrate in sprint and multiple-sprint sports and in activities requiring high muscle power generation.

BASIC BIOLOGY OF NITRATE, NITRITE AND NITRIC OXIDE

Nitric oxide (NO) is a gaseous signaling molecule that regulates an array of physiological functions essential for maintaining metabolic, neurological, and cardiovascular integrity. The effects of NO were first described in the vasculature, and several decades of research have confirmed that NO plays an essential role in vasodilation and therefore the control of blood pressure and tissue blood flow (Kapil et al., 2013). However, it is now known that NO has many other physiological effects, including, for example, in processes as diverse as neurotransmission, immune defense, mitochondrial respiration, and skeletal muscle contractile function. Given that NO has an extremely short half-life, of perhaps only a few milliseconds in biological tissues, it is essential that it is produced continuously to support these physiological processes.

Most tissues contain one or more isoforms of the nitric oxide synthase (NOS) enzyme, which catalyzes NO production through the conversion of the semi-essential amino acid L-arginine to L-citrulline. This reaction

requires the presence of oxygen, and the NO that is produced may subsequently be oxidized to form nitrate. When oxygen availability is limited, NOS-derived NO generation may be inhibited or impaired. Relatively recently, it was discovered that, rather than being an inert product of NO oxidation, nitrate can be reduced under appropriate physiological circumstances back to nitrite (NO_2-) and then NO (Cosby et al., 2003). Importantly, this complementary nitrate-nitrite-NO generation pathway does not require the presence of oxygen and is in fact facilitated by a more acidic pH and lower oxygen tension. In this way, NO may be produced, and vasodilation and other NO effects may be sustained, across a wide range of tissue oxygenation states. Indeed, given the prevailing physiological conditions, the nitrate-nitrite-NO pathway may be the favored mechanism for NO production in skeletal muscle.

Continuous NO generation is essential for the maintenance of cellular function and overall health. This has led to efforts to enhance NO availability through the diet. While oral L-arginine supplementation has not convincingly improved NO bioavailability or bioactivity, at least in healthy humans, dietary inorganic nitrate supplementation appears to be much more promising (Jones et al., 2018; Lundberg et al., 2010). Indeed the substrate for the nitrate-nitrite-NO pathway includes not only the nitrate generated from the endogenous oxidation of NO produced via NOS, as described above, but also exogenous inorganic nitrate from the diet, particularly that derived through the ingestion of green leafy vegetables such as rocket, kale, lettuce, and spinach, as well as some root vegetables like beetroot (Hord et al., 2009). These vegetables typically contain over 250 mg (or ~4 mmol) of nitrate per 100 g fresh weight.

Following ingestion, dietary nitrate is absorbed by the upper gastrointestinal tract into the bloodstream. Approximately 25% of this circulating nitrate is then absorbed by the salivary gland and concentrated in the saliva. In the mouth, resident bacteria reduce some of the salivary nitrate to nitrite (Duncan et al., 1995). When subsequently swallowed, a portion of this nitrite is reduced to NO in the acidic environment of the stomach but some enters the systemic circulation and is distributed in blood and stored in various tissues, where it can undergo a one-electron reduction to yield NO. Following the acute ingestion of a nitrate bolus, the peak plasma nitrate and nitrite concentrations are reached after about 1 h and 2-3 h, respectively, with a clear dose-response relationship between the quantity of nitrate ingested and the magnitude of the subsequent peak plasma nitrate and nitrite concentrations (Wylie et al., 2013a). The delayed peak in plasma nitrite, compared with nitrate, highlights the importance of the bacteria in the mouth, the so-called oral microbiome, in the activation of dietary nitrate.

THE IMPORTANT ROLE OF THE ORAL MICROBIOTA IN NITRIC OXIDE GENERATION

The metabolic activity of the microbial community that inhabits the human alimentary canal can have far-reaching effects on host physiology. Epidemiological studies have shown that a perturbed oral microbiota and poor oral health are associated with a variety of disease conditions, several of which have also been linked to NO insufficiency (Kumar, 2017). Inorganic nitrate is a natural micronutrient and is abundant in a vegetable-rich diet, but human cells have limited ability to activate biologically inert nitrate. Instead, humans depend to a large extent upon the symbiotic bacteria residing in the mouth, and to a lesser extent the alimentary canal, to reduce ingested nitrate to bioactive nitrite.

The importance of the oral microbiota in eliciting NO-mediated effects via the diet is illustrated by studies in which the use of antibacterial mouthwash blunted both the increase in plasma nitrite concentration and the decrease in blood pressure following the ingestion of a standardized nitrate dose (Kapil et al., 2013). There are marked differences between individuals in physiological responsiveness to nitrate supplementation and some of this variability likely stems from differences in oral nitrate-reduction capacity. Recent studies have begun to identify the key nitrate-reducing bacterial species in humans and to investigate the influence of factors such as diet, age and fitness on their prevalence and activity. For example, 7 to 10 days of dietary nitrate supplementation has been shown to radically alter the constitution of the oral microbiota, with proportional increases in Neisseria and Rothia and decreases in Prevotella and Veillonella being reported (Burleigh et al., 2019; Vanhatalo et al., 2018). In a recent study, a systems-level bacterial co-occurrence network analysis identified two distinct microbiome modules that were sensitive to nitrate supplementation and were related to indices of cardiovascular (Rothia-Streptococcus) and cognitive (Neisseria-Haemophilus) health in older people (Vanhatalo et al., 2021). The substantial interindividual variability in, and the remarkable plasticity of, the oral microbiota to dietary and other interventions suggest that prebiotic and/or probiotic modification of the oral microbiome might have potential in the future to enhance human health.

SKELETAL MUSCLE AS A NITRATE RESERVOIR

The components of the nitrate-nitrite-NO cycle, described earlier, have traditionally been considered to be “transient” – that is, easily absorbed from the diet and excreted with a half-life in the order of hours. Such a situation, which does not involve longer-term storage of any of the biochemical entities, might be considered inefficient if nitrate/nitrite-derived NO is important for maintaining normal physiological function.

Recently, studies have begun to investigate nitrate and nitrite levels in skeletal muscle. Intriguingly, nitrate has been reported to be significantly higher in rat skeletal muscle compared with other organs and blood, with nitrite distribution showing a smaller variation between tissues (Piknova et al., 2015). The discovery of significantly elevated nitrate levels in muscle and the presence of a muscle-to-blood nitrate gradient have led to the suggestion that muscle serves as an endogenous nitrate reservoir (Jones et al., 2021). Skeletal muscle has the necessary machinery for nitrate generation including high expression of neuronal NOS (nNOS), one of the three isoforms of NOS. Therefore, it is possible that NO produced by nNOS within the skeletal muscle cell is oxidized in

situ into nitrate by oxymyoglobin. However, in addition to the endogenous production of nitrate within skeletal muscle, recent studies also indicate that nitrate can be transported into muscle from exogenous, dietary sources. When rats were fed low or high nitrate diets, the amount of nitrate present in muscle tissue decreased and increased, respectively (Gilliard et al., 2018). This diet-derived nitrate is sequestered from the bloodstream and is likely to be transported into muscle cells by anion transporters and, perhaps, diffusion.

The sensitivity of skeletal muscle to nitrate availability via the diet is emphasized by the results of a study in which rats were first fed a low nitrate diet for 7 days to deplete the muscle nitrate reservoir (nitrate starvation) before being switched to a high nitrate diet for 7 days (Gilliard et al., 2018). The reintroduction of nitrate to the diet effectively restored muscle nitrate levels to baseline levels within 3 days. However, after 7 days of access to the high nitrate diet, muscle nitrate levels greatly exceeded not only baseline levels but also the levels measured in a separate group that consumed a high nitrate diet without initial nitrate deprivation. These data suggest that muscle nitrate homeostasis is tightly regulated, with a period of nitrate deprivation triggering muscle nitrate "supercompensation" when reintroduced to the diet. This appears to be akin to the effects reported on muscle glycogen levels when dietary carbohydrate is initially restricted and then reintroduced (Ahlborg et al., 1967).

These reports of relatively high nitrate levels in skeletal muscle may be important in understanding the mechanisms by which dietary nitrate could enhance exercise performance, with the existence of a local nitrate store being available to support NO-related contractile, metabolic, and vascular processes. Indeed, rather than simply acting as a passive reservoir for the supply of nitrate to other organs, such as the liver via the bloodstream, muscle tissue appears to be able to draw on its nitrate reservoir in situ. When rats were exercised, nitrate levels in skeletal muscle decreased and nitrite levels increased (Piknova et al., 2016), suggesting that nitrate stored in skeletal muscle is an important source of NO generated during exercise. Moreover, homogenates of skeletal muscle are able to reduce nitrate into nitrite and nitrite into NO, with this process being more efficient at low pH compared with that at neutral pH (Srihirun et al., 2020). An early study in humans also demonstrated a gradient for nitrate between arterial and venous blood (Cosby et al., 2003), suggesting that contracting muscle might utilize nitrate to support its activity, with deoxyhemoglobin and xanthine oxidoreductase potentially acting as nitrate and/or nitrite reductases.

It is important to emphasize that the majority of the work done to date in this area derives from rat and mouse models. However, preliminary data in humans are promising and appear to be largely consistent with these early findings (Nyakayiru et al., 2017a; Wylie et al., 2019). For example, Wylie et al. (2019) reported that: (1) baseline nitrate and nitrite concentrations were appreciably higher in muscle samples (from biopsy of m. vastus lateralis) than in plasma, (2) ingestion of 13 mmol dietary nitrate significantly elevated muscle nitrate levels, and (3) in the supplemented condition, muscle nitrate levels were decreased by exercise. These results reinforce the notion that skeletal muscle is sensitive to both nitrate supply and demand. It should be recognized, however, that these investigations are at an early stage, and significant further research is required to confirm and expand upon these results. The extent to which exercise performance may be related to muscle nitrate or nitrite content is also an important topic for future research.

OLDER AND NEWER APPLICATIONS OF DIETARY NITRATE SUPPLEMENTATION

In a seminal study, Larsen et al. (2007) reported that sodium nitrate supplementation reduced the oxygen cost of submaximal cycling. Similar findings were confirmed with nitrate-rich beetroot juice (Bailey et al., 2009), where a ~3-5% reduction in oxygen uptake at a fixed submaximal power output was reported. These results imply that dietary nitrate permits more muscular work to be performed per unit time for the same energy cost (i.e., that the efficiency of skeletal muscle contraction is enhanced) (see Pawlak-Chaouch et al., 2016, for meta-analysis). Exercise efficiency or economy is well known to be an important physiological factor influencing performance in endurance sports. Commensurate with this notion, several studies have indeed reported that dietary nitrate supplementation can, under certain circumstances, enhance endurance exercise performance (Cermak et al., 2012; Kelly et al., 2013; Lansley et al., 2011; Rokkedal-Lausch et al., 2019). However, studies on nitrate supplementation and endurance sport performance have shown variable results, and it is clear that nitrate supplementation is not beneficial in all instances (Jones et al., 2018). In particular, ergogenic effects of nitrate supplementation are much less commonly reported in athletes who are highly trained for endurance sports (i.e., $\dot{V}O_{2peak}$ > 65 ml/kg/min) (Porcelli et al., 2015; Senefeld et al., 2020).

In recent years, attention has shifted to the potential benefits of nitrate supplementation in high-power, sprint and multiple-sprint sports (Jones et al., 2018). The nitrate-nitrite-NO pathway is particularly favored under conditions of low pH and low oxygen availability, and therefore nitrate supplementation has the potential to be more effective during high-intensity continuous and intermittent exercise when anaerobic glycolysis makes a significant contribution to energy turnover. The relatively low oxygen tension surrounding type II (fast-twitch) muscle fibers may create optimal circumstances for the reduction of nitrite to NO, and animal studies suggest that nitrate may elicit beneficial effects on contractile function and blood flow in type II muscle (see Jones et al., 2016, for review). Well-trained athletes competing in high-intensity sports, such as sprinting, track cycling, speed skating and team sports like basketball, soccer and rugby, likely have a high proportion of type II muscle fibers, providing a rationale for the efficacy of nitrate supplementation in these sports.

Several recent studies indicate that nitrate may enhance skeletal muscle contractility, power generation, and sprint and repeated sprint performance (Coggan et al., 2015; Porcelli et al., 2016; Rimer et al., 2016; Wylie et al., 2013b; and see Coggan & Peterson, 2018, and Jones et al., 2018, for reviews). In particular, there is convincing evidence that repeated-sprint performance in moderately to well-

trained subjects can be improved by nitrate supplementation (Nyakayiru et al., 2017b; Thompson et al., 2016; Wylie et al., 2013b). Moreover, in a protocol designed to simulate the physiological demands of team sports (i.e., 2 x 40 min halves involving repeated sprints and simultaneous cognitive function tests), Thompson et al. (2015) reported that nitrate supplementation increased total work done and also resulted in better maintenance of the speed and accuracy of decision making in the second half of the test compared with the placebo condition. These findings suggest that nitrate supplementation may be beneficial to performance in sports involving repeated bouts of high-intensity exercise, such as soccer, basketball, hockey and rugby.

Wylie et al. (2016) compared the effects of nitrate on different types of intermittent exercise performance in recreational team sport athletes. These authors reported no improvement in power during repeated 30-s sprints but found that the performance of shorter sprints was improved following nitrate supplementation. These findings are consistent with evidence that nitrate supplementation may enhance force production during the initial phase of muscle contraction (Haider & Folland, 2014) and enhance muscle speed and power during maximal contractions (Coggan et al., 2015) and all-out sprint efforts (Thompson et al., 2016; Rimer et al., 2017). Jonvik et al. (2018) reported that the time to reach peak power output during repeated 30-s sprints was shorter following nitrate supplementation and that the effect was similar in recreational, competitive, and elite sprint athletes. This suggests that in high-intensity sports where rapid acceleration is crucial, nitrate supplementation could result in reaching top speed faster and improving actual sports performance even at the elite level. It is possible that these effects are related to the influence of nitrate supplementation on muscle calcium handling and/or sensitivity (Bailey et al., 2019; Coggan & Peterson, 2018; Hernandez et al., 2012). In humans, however, Whitfield et al. (2017) found that beetroot juice supplementation increased force production at low-stimulation frequencies but without altering the expression of muscle proteins associated with calcium handling.

UPDATED PRACTICAL RECOMMENDATIONS

Based on the available evidence and supported by a recent meta-analysis (Senefeld et al., 2020), the following recommendations for nitrate supplementation for athletes can be made.

- The acute or daily dose of nitrate supplementation should be > 370 mg (> 6 mmol), but there appears to be no additional benefit in consuming more than 740 mg (12 mmol).
- Both acute and multiple-day (typically 3-7 days) supplementation protocols can be effective.
- The final dose of nitrate should be ingested at least 90 min prior to the event.
- Consuming nitrate naturally via vegetable intake appears to be more effective than nitrate salts. However, while positive cardiovascular and ergogenic effects of nitrate can be achieved via meal ingestion, consuming a beetroot juice concentrate would likely be a more practical strategy for athletes.
- Nitrate supplementation may be of limited benefit to elite endurance athletes, while elite athletes competing in very high-intensity exercise tasks may still benefit from nitrate.
- The recent discovery that skeletal muscle may serve as a nitrate reservoir opens up the possibility that sufficient nitrate loading could occur on the days leading up to a competition (with no necessity to top up shortly before the event). However, because the dose, duration, timing, and type of athlete has varied substantially in published studies, additional research is required to determine the optimal supplementation strategy for nitrate to enhance performance in specific sports.
- As is the case for many other aspects of sports nutrition, women have been underrepresented in studies on nitrate, and potential sex-based differences in responses to nitrate supplementation require elucidation (Wickham & Spriet, 2019).

SUMMARY

Dietary nitrate is perhaps the newest putative nutritional ergogenic aid, and our understanding of the role of nitrate in the physiological responses to exercise and in sports performance is still developing. While nitrate and nitrite are continuously produced endogenously as products of NOS-mediated NO production, the body's nitrate and nitrite stores may be augmented exogenously via the diet and can be used to generate NO in situations where NOS function is impaired or when tissue oxygen availability is limited.

Several recent observations indicate that nitrate may be essential to skeletal muscle, and perhaps wider biological function. For example, (1) during exercise, muscle nitrate stores are decreased, and (2) a high nitrate diet increases the muscle nitrate store, whereas a low nitrate diet reduces it. This sensitivity of skeletal muscle to nitrate availability and the dynamic changes in nitrate and nitrite during exercise suggests an important role for muscle in the maintenance of whole-body nitrate and NO homeostasis. If nitrate and/or nitrite are indeed essential for normal muscle function, then the extent to which muscle performance might be enhanced by increasing muscle nitrate stores via dietary nitrate supplementation becomes an important question. It is feasible that supplementation may be beneficial when muscle stores are low relative to demand. However, there may be a ceiling both in terms of muscle nitrate storage capacity and in relation to functional outcomes.

It is clear that many factors influence the potential for nitrate supplementation to enhance exercise performance, including the age, health, sex, aerobic fitness, and training status of the individual, as well as the intensity, duration, and nature of the sport or activity. However, it is also clear that the consumption of natural nitrate-containing foodstuffs such as green leafy vegetables is likely to be beneficial for our general health and that, at least for some people in some situations, nitrate supplementation can be a convenient and practical way to enhance performance in a variety of sports.

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