Dietary nitrate supplementation in cardiovascular health: An ergogenic aid or exercise therapeutic?

Dietary Nitrate Supplementation in Cardiovascular Health: An Ergogenic Aid or Exercise Therapeutic?

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Abstract

Oral consumption of inorganic nitrate, which is abundant in green leafy vegetables and roots, has been shown to increase circulating plasma nitrite concentration, which can be converted to NO in low oxygen conditions. The associated beneficial physiological effects include a reduction in blood pressure, modification of platelet aggregation and increases in limb blood flow.

There have been numerous studies of nitrate supplementation in healthy recreational and competitive athletes, however, the ergogenic benefits are currently unclear due to a variety of factors including small sample sizes, different dosing regimens, variable nitrate conversion rates, the heterogeneity of participants' initial fitness levels and the types of exercise tests employed. In clinical populations, the study results seem more promising, particularly in patients with cardiovascular diseases who typically present with disruptions in the ability to transport oxygen from the atmosphere to working tissues and reduced exercise tolerance. Many of these disease-related, physiological maladaptations including, endothelial dysfunction, increased reactive oxygen species, reduced tissue perfusion and muscle mitochondrial dysfunction have been previously identified as potential targets for NO restorative effects.

This review is the first of its kind to outline the current evidence for inorganic nitrate supplementation as a therapeutic intervention to restore exercise tolerance and improve quality of life in patients with cardiovascular diseases. We summarise the factors that appear to limit or maximize its effectiveness and present a case for why it may be more effective in patients with CVD than as ergogenic aid in healthy populations.

Introduction

Nitric oxide (NO) is a diatomic, lipid-soluble gas, implicated in numerous physiological functions including neurotransmission, immune defence, blood flow regulation, among others. In the presence of oxygen, NO is produced by the vascular endothelium via the oxidation of L-arginine to NO and L-citrulline by endothelial NO-synthase (2). NO bioavailability is a balance between its rate of production and subsequent rate of consumption via various biological signaling pathways and chemical reactions. Vascular NO bioavailability has been shown to be essential for cardiovascular health and a reduction in the ability to produce NO by the vascular endothelium is an early event in the process of atherosclerotic lesion formation and is associated with cardiovascular risk factors (41, 42, 218), diabetes (50) and established cardiovascular disease (155). This dysfunctional endothelium limits eNOS-dependant therapeutic strategies to increase vascular NO bioavailability, and approaches utilizing NO-donor compounds have been limited in their clinical applications primarily due to their systemic vascular effects often resulting in hypotension.

The short half-life of NO makes it difficult to measure directly in vivo human models, but its expression has previously been shown to be directly proportional to plasma nitrite levels(4, 179), suggesting nitrite may be a measurable reflection of vascular NO bioavailability. Despite decades long knowledge that nitrite acts as a vasodilator at supra-physiological (micromolar) concentrations(70), it was regarded within biological systems as an inactive “NO-sink,” which was ultimately excreted by the kidneys. Recently, nitrite (along with S-nitrosothiols(213), N-nitroso proteins and iron-nitrosyl complexes(178)) have been shown to be reduced back to NO under hypoxic conditions (134). This indicates a discrete yet complimentary system to

oxygen-dependant eNOS production, which may enable vascular NO bioavailability across the oxygen gradient. Furthermore, it suggests conservation of NO and an endocrine-like function where delivery via plasma nitrite may target specific tissues with low oxygen concentrations. Consequently, mechanisms to increase plasma nitrite may be particularly useful in conditions associated with tissue ischemia, including some cardiovascular diseases pathologies and specifically during a physiological challenge requiring an upregulation in tissue perfusion such as exercise.

Inorganic Nitrate Supplementation to Increase Plasma Nitrite

Inorganic nitrate supplementation has been shown to be a simple, non-invasive means of exogenously increasing plasma nitrite concentration and, consequently, NO bioavailability(132, 133). Inorganic nitrate is found in relatively high concentrations, approximately 250mg per 100g, in green leafy vegetables such as kale, cabbage, lettuce, rocket, spinach, and beetroot(95). It is important to note that the exact NO_3^- content of these vegetable sources can vary depending on growth environment, geographical location and how they are treated(194).

Oral supplementation with inorganic nitrate works in a two-step process (Figure 1) whereby following consumption, nitrate is rapidly absorbed in the small intestine and enters circulation. While a majority (~75%) is subsequently excreted by the kidneys, approximately 25% becomes highly concentrated in the salivary glands (up to 10 times the plasma concentration)(211). When this nitrate is released from the salivary glands, commensal oral bacterial on the dorsal surface of the tongue reduces nitrate to nitrite(60). The nitrite is then swallowed and absorbed into circulation via the intestinal tract(23, 135). Due to this two-pass process, plasma

nitrite concentrations take approximately 2.5 to 3 hours to reach maximal levels (~200 to 400nM), following a single dose of inorganic nitrate. The half-life of nitrite appears to be approximately 6 hours(100, 143, 146, 233, 244). Chronic nitrate supplementation can maintain elevated nitrite levels continuously and helps to avoid the short-lived bolus effects of direct oral nitrite administration (228, 245).

The circulating plasma nitrite can then undergo one-electron reduction to NO by numerous nitrite-reductases including deoxyhemoglobin(54), deoxymyoglobin(204), mitochondrial enzymes(157) and chemical acidification(249). In this way, inorganic nitrate acts as a targeted supplement, whereby the resulting nitrite is reduced to NO in tissues with a low partial pressure of oxygen (PO_2) which may facilitate better overall distribution of the available blood flow and allow for greater oxygen extraction in those with cardiovascular disease (CVD) but also during exercise stress.

Inorganic Nitrate Supplementation in Cardiovascular Disease

Several pharmacological agents for CVD enhance NO signalling either via increasing bioavailability or inhibiting NO breakdown. The most obvious of these is organic nitrate (eg. glyceryl trinitrate) which acts via the rapid release of NO causing nonspecific arterial and venodilation and is subject to the development of tolerance. Another type is phosphodiesterase-5-inhibitors which are used in patients with erectile dysfunction and pulmonary hypertension(72). In addition, HMG-CoA reductase inhibitors (statins) and angiotensin-converting enzyme inhibitors/receptor blockers indirectly increase NO bioavailability(162).

Currently several countries recommend dietary interventions high in inorganic nitrate for patients with cardiovascular conditions. For example, the Dietary

Approaches to Stop Hypertension (DASH) dietary pattern(10, 192), which emphasizes fruits, vegetables and low-fat dairy foods, and includes whole grains, poultry, fish, and nuts can potentially contain up to 20mmol of inorganic nitrate per day(95). It is recommended by The National Heart, Lung and Blood Institute(196), The American Heart Association(11), the American Diabetes Association(21), and the Dietary Guidelines for Americans(227). High dietary inorganic nitrate intake has been shown to decrease blood pressure(71, 112), and lower the risk for heart disease(108) and stroke(107).

The most consistent applied clinical outcome from increased oral inorganic nitrate intake is a reduction in blood pressure. In 2006, 3 days of sodium nitrate administration (0.1mmol/day) was shown to reduce diastolic blood pressure (DBP) by 3.7mmHg in healthy volunteers(128). In 2008, Webb et al., demonstrated an acute dose of 22.5mmol inorganic nitrate via beetroot juice (500ml) reduced systolic blood pressure (SBP) and DBP by ≈10 and 8mmHg respectively(233). Furthermore, the drop in blood pressure was correlated to plasma nitrite concentrations and both changes could be abolished by interruption of the enterosalivary conversion of nitrate to nitrite. Since this study, similar benefits have been observed in studies of patients with hypertension(73). A double-blind placebo controlled study where 68 patients were given a 6mmol dose of inorganic nitrate, via 250ml beetroot juice, for 4 weeks, demonstrated significant reductions in clinic measured (≈8/2.5mmHg), 24-hour ambulatory (≈8/5mmHg), and home measured (≈8/4mmHg) blood pressures(112). These reductions are clinically significant when it is considered that a 1mmHg increase in SBP is estimated to increase cerebrovascular incident mortality by 2% and a 1 mmHg increase in DBP may increase stroke mortality by 3%(162, 165).

Other documented benefits for CVD include increased endothelial function, (86, 126), reduced tissue loss following an myocardial infarction(38, 209) reduced platelet aggregation(184, 233), and attenuation of pulmonary hypertension(96). Recently, Bondonno et al.(25), showed that, after adjusting for other cardiovascular risk factors and lifestyle components, a higher dietary vegetable nitrate intake over a period of 14 years was associated with a lower carotid artery intimal-medial thickness and a lower risk of an ischemic cerebrovascular disease events in 1226 elderly women. Excellent reviews of other benefits of increased dietary inorganic nitrate supplementation for cardiovascular and metabolic health have been published previously(162, 181, 234).

Inorganic Oral Nitrate Supplementation and Exercise

During resting conditions, peripheral skeletal muscle tissues are usually adequately perfused, however, during exercise stress the increased metabolic demands of skeletal muscles can outstrip the ability to supply blood flow and oxygen causing a decline in pH and inter-myocyte and microvascular oxygen tensions(67, 185, 219). Given that nitrite is reduced in low oxygen and acidic conditions, this environment may be ideal to liberate NO and contribute to optimal matching of perfusion to metabolic demands.

In support of this theory, intravascular consumption of nitrite during physiological stress in humans was first reported by Gladwin et al., in 2000. They showed artery to venous nitrite gradients in the forearm of healthy subjects during L-NMMA infusion coupled with handgrip exercise(75). Similarly, our data in subjects with peripheral arterial disease (PAD) and documented endothelial dysfunction showed a net loss of plasma nitrite stores following maximal exercise stress. This was in comparison to

healthier counterparts with a functioning endothelium(3, 7).These studies allow us to speculate that, in the setting of a depleted or inhibited endogenous source of vascular NO during exercise-induced tissue ischemia, there is the potential for significant decrease in the circulating nitrite/NO pool, potentially in an attempt to normalize blood flow and oxygen delivery to hypoxic tissues.

In addition to increasing tissue perfusion, NO has been shown to have a variety of potential physiological benefits in exercising skeletal muscle beds (as outlined below) which may contribute to increasing exercise performance. They also suggest the ergogenic benefit of consuming inorganic nitrate may be optimal under conditions where the cardiorespiratory and musculoskeletal systems are close to or exceed their maximal capacity to transport oxygen from the lungs to the working myocyte.

In this review, we will outline the evidence for inorganic nitrate supplementation as an ergogenic aid and summarise the factors that appear to limit or maximize its effectiveness. We will present evidence that suggests inorganic nitrate supplementation offers a greater opportunity as a therapeutic intervention to partially restore exercise tolerance and improve quality of life in patients with cardiovascular diseases than as an ergogenic aid in healthy populations.

Inorganic Nitrate Supplementation and Exercise Performance in Healthy Subjects

The main physiological parameters during exercise that are documented to be influenced by inorganic nitrate supplementation include mitochondrial function(110, 148, 204), skeletal muscle contractile efficiency(18, 48, 81), and tissue perfusion/oxygen delivery(19, 66, 67, 113, 141).

a) Changes in Mitochondrial Function

A period of intense interest in the role of dietary inorganic nitrate as a potential ergogenic aid was initiated in 2007 by Larsen and colleagues' discovery that 3 days of dietary sodium nitrate supplementation resulted in a reduction in oxygen cost during submaximal cycling(130). These changes were observed following a relatively small dose of nitrate (0.1mmol kg^{-1} bodyweight day^{-1}) likened to that which is readily available from everyday dietary sources (~150-250g of green leafy vegetables)(132).

Prior to Larsen's discovery, the prevailing dogma was that oxygen cost (ml/kg/min) during sub-maximal exercise at a particular workload was fixed, with responses being almost identical within and between subjects(174). While it was understood that individuals with a period of training could become mechanically more efficient, the subjects in Larsen's study had no differences in training status, heart rate, or blood lactate between tests. They appeared to have become more efficient via changes in mitochondrial function.

In a follow-up study, the group investigated the effects of nitrate supplementation on maximal aerobic exercise capacity (VO_{2max}) during combined upper and lower body exercise. The results showed that nitrate supplementation resulted in a lower VO_{2max} but an increased time to exhaustion(129). This occurred without changes in anaerobic energy consumption (measured by maximal ventilation), respiratory exchange ratio, blood lactate levels, or heart rate. They suggested that this may be due to not only improved muscular efficiency but a corresponding reduction in mitochondrial proton leakage(129). Further elucidating the potential mechanisms of dietary nitrate on exercise economy, Larsen showed

that reductions in whole body VO_2 occurred simultaneously with increased oxidative phosphorylation efficiency(127).

Others have shown that nitrite and NO signalling can affect mitochondrial function at several key steps in order to potentially match respiration to oxygen availability(22, 204-206). For example, during low oxygen conditions, nitrite has been shown to inhibit Complex I (NADH Coenzyme Q oxidoreductase) by S-nitrosylation leading to decreased mitochondrial reactive oxygen species (ROS) generation. Similarly, the reduction of nitrite to NO (potentially via deoxymyoglobin or xanthine oxidase) has been shown to specifically and reversibly inhibit cytochrome oxidase (complex IV)(34). In addition, peroxynitrite (ONOO-) may inhibit multiple respiratory complexes under specific conditions(34). When oxygen availability is restored, these inhibitory mechanisms are reversed (NO is oxidized to nitrite) to resume ATP production, while inhibition of complex I is prolonged to limit ROS production(206). These mechanisms have also been implicated in nitrite mediated cytoprotection following ischemia/reperfusion injury(87, 206, 232). Interestingly, studies that have employed an NO-blockade approach to measure its effects on changes in skeletal muscle mitochondrial function and oxygen uptake in humans have been mainly negative(195). This may be due to multiple integrated or redundant mechanisms employed in intact model physiology(226) or potentially multiple nitration and nitrosylation signalling pathways initiated by exogenous administration of NO species (as described above). It may even be a function of the technology used to take measurements. Recently Heinonen et al.(84), using positron emission tomography and radiolabelled water, showed that NO blockade enhanced resting oxygen uptake and when combined with cyclooxygenase (COX) inhibition muscle oxygen uptake also increased during exercise.

b) Changes in Skeletal muscle contractile efficiency

A second major area in which inorganic nitrate supplementation may increase exercise performance is via changes in neuromuscular contractile efficiency. In 2010, Bailey et al. demonstrated a reduced oxygen cost of exercise following dietary nitrate, which they attributed to a reduced ATP turnover in the contracting myocytes which can influence the stimulus for oxidative metabolism. Similarly, the sparing of PCr was associated with improved exercise tolerance in high intensity exercise(18). Others have shown increased maximal knee extensor speed and power in voluntary (48, 49, 187, 236) and stimulated muscle contractions(81). These benefits have been attributed to increases in NO led activation of sGC, cGMP and subsequent phosphorylation of myosin(139), although others showed no changes in redox status and calcium handling proteins(236).

c) Changes in Skeletal muscle tissue perfusion/oxygen delivery

A third major mechanism of action of inorganic nitrate supplementation is improving skeletal muscle tissue perfusion. Oxygen supply to myocytes is a balance between blood flow delivery and oxygen extraction. It is essential that perfusion is optimised to the muscle fibers that are actively contracting. Microvascular PO_2 represents the dynamic balance between oxygen supply and myocyte consumption. An increase in PO_2 suggests enhanced blood flow (supply) and potentially increased mitochondrial and contractile efficiency during exercise.

Infusion of the vasodilator ATP into the leg at near-maximal intensities of exercise has been shown to increase vascular conductance but not limb VO_2(37). This suggests a concomitant decrease in arterial-venous oxygen extraction which

may be caused by some of the increased blood flow directed to less-active fibers (that may normally be under a vasoconstrictive influence)(85). Given that nitrite is reduced to liberate NO in low oxygen and acidic conditions, this system may contribute to optimal matching of perfusion to metabolic demands and allow for greater oxygen extraction.

Neuronal-NOS (nNOS) is located beneath the sarcolemma of skeletal muscle fibers and is associated with the dystrophin-g1 ycoprotein complex. It has been suggested that the greater distribution of nNOS to type II fibers(177) may play a role in the differential fiber type responses. When healthy skeletal muscle is exercised nNOSμ-derived NO attenuates α-adrenergic vasoconstriction, thus optimizing perfusion(220). During high intensity exercise in rats, there are reductions in blood flow and vascular conductance and the greatest occur in type II fibers. However, no changes were observed during low-intensity running(52). Humans with Becker muscular dystrophy lack sarcolemma nNOS, and have been shown to have functional muscle ischemia which was relieved by a single dose of oral sodium nitrate. There was no effect on healthy controls(156). In addition, the lower levels of antioxidant enzymes in type II muscle fibers in comparison to type I fibers(102) suggest that during high intensity activity, exogenous NO bioavailability within the muscle may also benefit NO-mediated calcium signalling and mitochondrial function as outlined above.

In support of these ideas, in animal models, dietary inorganic nitrite supplementation (via beetroot juice) increased exercise skeletal muscle blood flow predominantly to type II fibers(65). Subsequent studies by the same group showed nitrate supplementation increased the microvascular and myocyte PO_2 only in type IIx/d fibers compared to control(67). In humans, the data is less clear. A recent

study employing NOS-inhibition and PET scanning, failed to show differences in blood flow between the different muscles that make up the quadriceps femoris; vastus intermedius (VI), rectus femoris (RF), vastus medialis (VM), and vastus lateralis (VL)(83). Similarly, Breese et al., using near infra-red spectroscopy saw no differences in the spatial variance of absolute deoxyhemoglobin+myoglobin kinetics across the RF, VL and VM muscles following the onset of heavy step cycling(32).

Differences between these human results and those of rat studies are likely attributable to the fact that only one exercise intensity was used and that human muscles have less spatial stratification of muscle fibre types than rodents. Future human studies may be best served by utilizing several different intensities of workload and investigating the musculature of the calf, which has more distinct fibre types in its muscle parts.

A further physiological mechanism to suggest benefits from dietary inorganic nitrate on fast twitch skeletal muscle fibers is increases in contractile force. While the process is not currently fully elucidated, it is clear NO plays a role in skeletal muscle calcium flux via S-nitrosylation of ryanodine receptor Ca_2^+ release channels in the sarcoplasmic reticulum membrane and that this occurs only at low physiological PO_2 levels. Following 7 days of inorganic nitrate supplementation in rats, Hernandez et al.(89), showed an increased rate of muscle force development in the predominantly fast twitch extensor digitorum longus muscle (but not the predominantly slow twitch soleus). This was accompanied by changes in protein concentrations of the voltage-sensing dihydropyridine receptor (voltage sensor for excitation coupling located in the transverse tubular membrane) and the calcium handling protein calsequestrin 1, found in sarcoplasmic reticulum of fast–twitch fibers. In humans, however, despite improvements in skeletal muscle contractile

function, there we no changes in calcium handling proteins(236). The muscle samples taken in this study were from the vastus lateralis, which is estimated to be composed of ~50% type I and ~50% type II fibres and may have contributed to a dilution of potential differences.

In terms of human exercise performance, the preferential effects of dietary inorganic nitrate on fast twitch muscle fibers suggests ergogenic effects may be most evident in activities of high intensity and short duration, such as sprint or interval training. During these short, high-intensity efforts (at greater than 75% VO_{2Max}) there is an increased activation of type II muscle fibers(231). Bailey et al., showed that short-term beetroot juice supplementation can increase muscle oxygenation, expedite the adjustment of oxidative metabolism, and enhance exercise tolerance in healthy recreationally active subjects when cycling at high-intensities(19, 20). There are also several examples that support the fiber-type specific responses in relation to better tissue muscular power/force generation in repeated sprint activities and team sports(15, 48, 81, 175, 187, 242, 243). Following an acute dose of inorganic nitrate supplementation (~11.1mmol) collegiate athletes were able to increase their maximum power output (pre-nitrate: 1160 ± 301W post-nitrate: 1229 ± 317 =W)(187). In 2016, Porcelli et al.(175), also showed that following 6 days of a high nitrate diet (~8.2mmol/day) in healthy males (VO_{2max} 41.2 ± 4.7 $ml/kg^{-1}/min^{-1}$) there was a significant improvement in peak power during repeated sprint ability test in the final 3 of 5 bouts when compared to a control diet. Improvements in mean power during repeated sprints have also been demonstrated in team sport athletes (VO_{2max} 58 ± 8 $ml/kg^{-1}/min^{-1}$) in short duration intervals 24 x 6s with short recovery, but not long 7 x 30s and 6 x 60s with an extended recovery(242).

Recently, Thompson et al.(222), sought to exploit the enhanced conversion of nitrite to NO in low oxygen conditions by combining sprint interval training with nitrate supplementation. They reported an increase in proportion of type I and type IIa muscle fibers (Pre:93 ± 8%, Post: 96 ± 6%), highlighting the potential of nitrate to influence training adaptations in a positive oxidative fiber-type switching manner. Roberts et al.(189), generated similar findings using an in vitro model, whereby nitrate increased the proportion of type I and IIa oxidative fibers. They also found in animals and humans that both nitrate and exercise training can stimulate PGC1α-mediated, γ-aminobutyric acid secretion from the muscle.

Administration and Variability of Inorganic Nitrate Supplementation

The use of inorganic nitrate supplementation to increase the bioavailability of NO in exercise studies has been achieved mainly through the use of concentrated beetroot juice (approximately 3/4 of studies)(144) . This supplementation allows for easy oral administration and a controlled dosage. To date the results of these studies have been mixed. While some studies focused on submaximal exercise variables as the primary outcome, including both acute and chronic supplementation regimens, have shown positive effects (20, 43, 125, 130, 152, 176, 223, 228, 245) many have also shown no significant benefit (25, 31, 115, 193). Similarly, in studies employing incremental exercise tests or time trial approaches (which require maximal efforts) the results are similarly mixed between positive effects (20, 43, 124, 125, 129, 168), and no significant benefit (24, 25, 45, 152, 167, 193, 238). Excellent reviews detailing the specifics of individual studies in detail have been published previously (17, 104, 105).

The reasons for divergent findings are not entirely clear, but it is evident that numerous factors may influence and regulate physiological responses to inorganic dietary nitrate. For example, several studies have shown that the extent of the increase in plasma nitrite correlates with improvements in parameters of exercise tolerance and performance(221, 238, 244). This suggests that factors which optimise conversion of an oral inorganic nitrate dose may be important.

As outlined earlier in this text, the function of oral commensal bacteria has been shown to be essential for conversion of nitrate to nitrite. This process occurs through the utilization of nitrate as a terminal respiratory electron acceptor by bacteria under anaerobic conditions. Oral nitrate reduction appears to occur mainly on the dorsal surface of the tongue and is predominantly mediated via two broad categories of bacteria; the strict anaerobes *Veillonella spp*, and the facultative anaerobes *Actinomyces spp*(58). In a subsequent study, which combined metagenomics and biochemical techniques, *Veillonella* was again the most abundant nitrate-reducing genus detected though *Prevotella*, *Neisseria*, and *Haemophilus* were found at a higher abundance than *Actinomyces*(97) Other bacteria have also been identified which may play supporting or inhibiting roles in these processes. The current literature limits our ability to draw far-reaching conclusions about the importance of the specific species and abundance of nitrate-reducing bacteria in the oral cavity on the conversion of inorganic nitrate to plasma nitrite. However, studies which have eradicated or inhibited these bacteria via the use of anti-septic and anti-bacterial mouthwash treatments have been shown to reduce salivary and plasma nitrite increases and lead to increases systemic blood pressure(77, 111, 240).

A second contributing factor in the variability of the plasma nitrite concentration responses following oral inorganic nitrate supplementation involves

differences in the vehicle of administration, nitrate dosage and the number of days of supplementation. A recent crossover study in 10 healthy males, showed that an acute dosage of 4.2, 8.4 and 16.8mmol inorganic nitrate (via beetroot juice) increased plasma nitrite in a dose-dependent manner with peak concentrations occurring at approximately 2-3 hours post consumption(244). Interestingly, the oxygen cost of moderate-intensity cycling was increased relative to dosage but there was no additional benefit to severe-intensity cycle exercise above 8mmol. Peak reductions in blood pressure also occurred at 8.4mmol dosage. This suggests a threshold of at least ~8.4mmol may be required to realise exercise benefits.

Comparisons between acute versus chronic dosing of inorganic nitrate suggest that chronic dosing (15 days) may help maintain exercise economy benefits(228) but can potentially have a greater effect on peak power output and time trial performance benefits(25, 228). A recent systematic review and meta-analysis on endurance exercise performance showed a positive trend toward improvements in time to exhaustion (TTE) when utilising chronic nitrate supplementation(144). It has also been reported that longer-term nitrate supplementation (5-7 days) can result in changes in mitochondrial(127) and contractile(89) proteins that would be expected to enhance skeletal muscle metabolic and mechanical efficiency. It would seem unlikely that these changes could be fully effected within a few hours of nitrate ingestion and therefore the duration of nitrate supplementation is likely to introduce variability into the potential efficacy of nitrate on the physiological responses to exercise. Overall, these findings suggest at least 5 days of supplementation may be optimal to realise exercise benefits.

A third contributor to outcome variability is the training status or fitness level of an individual (40, 106, 176). Among well trained subjects, there appears to be a lack of effect of nitrate supplementation (acute or chronic) on exercise performance and efficiency(25, 45, 123, 167, 238). Porcelli et al.,(176), found that 6 days sodium nitrate supplementation (~5.5mmol) resulted in a reduction in oxygen cost during sub-maximal exercise and improved 3km running time trial in individuals with low fitness level (VO_{2max}: ~38 mL/min/kg) but not a high fitness level (VO_{2max}: ~72 mL/min/kg). There was a strong correlation between changes in plasma nitrite and changes in exercise performance. Carriker et al.(40), found similar results when they compared the effects of 4 days of nitrate supplementation (~6.2mmol/day) on treadmill running at intensities of 45, 60, 70, 80, and 85% VO_{2max}. Low fitness individuals (VO_{2max}: 42.4 ± 3.2 mL/min/kg) showed a reduction in oxygen cost at intensities of 45 and 60% of maximal, but there was no difference for the high fitness subjects (VO_{2max}: 60.1 ± 4.6 mL/min/kg). The reasons for the potential ineffectiveness of inorganic nitrate supplementation in athletes could be several-fold. Perhaps they have specialized diets that already contain high levels of nitrate(123) . There may also be a high inter-subject variability in the conversion of nitrate to nitrite, or nitrite to various NO-signalling species. Another possibility is that eNOS activity is already maximized in athletes and endothelial NO production is strongly associated with exercise performance(180, 224).

In summary, the response to dietary nitrate supplementation on exercise parameters appears to be highly variable both between studies and between individual participants. The majority of the studies undertaken have small sample sizes (n<15), which may be a contributing factor to the sometimes-conflicting results.

Further studies are required with a focus on the sources and mechanisms by which this variability occurs and how it can be minimized.

Currently, it appears that nitrate supplementation in individuals of a high training status results in minimal positive benefits. Additionally, nitrate supplementation appears to have the greatest chance of benefit when given for a prolonged period of time (>5 days) at a dosage above 8mmol per day and the exercise is of a high intensity (relative to the individual), that relies predominantly on type II muscle fiber activation. These conditions may best lead to adequate plasma (and potentially tissue) nitrite concentrations coupled with low PO_2 and high H+ concentrations in the skeletal muscle, creating an ideal environment for the reduction of nitrite to NO. The effects of inorganic nitrate supplementation on long term training adaptations as part of a chronic exercise regimen is currently not known.

Inorganic Nitrate Supplementation and Exercise in Hypoxia

Given the reduction of nitrite to NO in hypoxic and acidic conditions, an innovative way to test the ergogenic effects of inorganic nitrate supplementation is by a reduction in the pulmonary oxygen supply. Interest in this area was stimulated by studies of humans indigenous to high-altitude environments. In 2007, Erzurum et al. (63), showed that native Tibetans who reside at 4,200m, offset physiological hypoxia and achieve normal tissue oxygen delivery by means of higher blood flow, enabled by higher levels of bioactive forms of NO. The authors suggested this was due to increased eNOS production, which has been shown to be impaired with increasing altitude in native lowlanders (59). Interestingly, circulating nitrogen species, including nitrate and nitrite, seem to increase as part of the altitude acclimatization process and those individuals with the highest levels of S-nitrosohemoglobin were

able to walk the furthest in a six-minute walk test(101). Subsequent studies then confirmed that dietary nitrate supplementation may hold promise as a prophylactic for acute altitude sickness(88).

In a laboratory setting, several studies have shown that dietary nitrate has the potential to minimize the ergolytic effect of hypoxia on exercise capacity(115, 141, 151, 229). In 2011, Vanhatalo et al.(229), demonstrated that an acute dose of dietary nitrate via beetroot juice (~9.3mmol) during the 24hour run up to testing improved time to exhaustion during maximal knee-extension exercise by ~21% while breathing reduced oxygen air (FiO_2 14.5%). These improvements were attributed to reduced muscle perturbations related to fatigue. At lower oxygen conditions (FiO_2 11%), Masschelein et al. showed that a chronic dose of beetroot juice (6 days ~5 mmol/day nitrate) improved exercise efficiency via lower VO_2 uptake during submaximal exercise (~45% VO_{2peak}) and increased overall exercise tolerance(141). This and a second recent study suggest improvements in skeletal muscle tissue oxygenation, measured via near-infrared spectroscopy, may be mediators of this benefit(141, 198). In more applied conditions, acute beetroot juice supplementation (~5mmol nitrate) reduced submaximal VO_2 and improved 16km cycle race time when performed breathing FiO2 of 15%(151).

Interestingly, similar to the data in normoxia, nitrate supplementation appears to be less effective for increasing exercise efficiency or performance in hypoxic conditions when ingested by well-trained athletes(13, 28, 136). For example, in well-trained individuals (VO_{2max}>65ml/kg/min) there were no changes in exercise economy or endurance in a simulated 10km cycling time trial following a single ~6.5mmol dose (beetroot juice) 2 hours before testing at FiO_2~15%(136). Similarly, despite having a longer supplementation period (3 days ~7mmol/day oral sodium

nitrate) there were no improvements in time to completion of a 15km cycle time trial at FiO_2~11% of the inspired air(28).

Overall, in low oxygen conditions, such as at altitude, inorganic nitrite supplementation appears to hold promise as prophylactic. In fact, it has even been suggested that hypoxic conditions may be optimal to reveal ergogenic benefits of dietary nitrate supplementation(115). However, nitrate's role in short term hypoxic exposures in highly trained athletes appears limited. This suggests nitrate supplementation is most effective in conditions of low tissue oxygenation when coupled with dysfunctional cellular metabolism, such as what is seen in patients with chronic cardiovascular disease.

Cardiovascular Disease and Exercise

Patients with CVD usually experience significant levels of disability due to a reduction in exercise capacity and a loss of physical function. This results in a lower quality of life and increased morbidity and mortality. In many populations with CVD, despite differences in disease aetiologies, exercise capacity, in the form VO_{2peak}, is a strong independent predictor of survival(158). For example, patients with PAD are primarily limited by leg claudication pain whereas those with chronic heart failure (CHF) suffer from dyspnoea and fatigue. In both cases, the end result is 30-55% lower VO_{2peak} than their healthy counterparts(14, 82).

Conversely, even modest improvements in exercise tolerance have been shown to lower all cause-mortality and morbidity in these individuals. For example, a ~6% improvement in VO_{2peak} reduced all-cause morbidity and mortality in CHF by 5%(53, 217). Additionally, data from a widely used six-minute walk test, which may

519 better represent a measure daily function(142), shows that an improvement of just
520 45 meters is deemed to be a clinically meaningful change in patients with CHF(207).
521 The relationship between exercise capacity and physical function and health
522 outcomes has led to a plethora of exercise based studies in clinical CVD
523 populations. However, the burden of exercise participation for individuals with CVD
524 may be increased due to numerous peripheral tissue maladaptations borne of
525 chronic under-perfusion and underuse. Peripheral tissue abnormalities common to
526 multiple chronic CVD disease states are shown in figure 2 and include endothelial
527 dysfunction/reduced NO bioavailability(199, 201), capillary density rarefaction(14,
528 119, 188), and skeletal muscle hypo-perfusion(78, 216), increased reactive oxygen
529 species(1, 191, 237) and inflammation(109), increased insulin resistance,
530 mitochondrial dysfunction(190), reduced aerobic enzyme activity(215), and a
531 preferential loss of type I oxidative fibers(119). Overall this results in patients
532 exhibiting a glycolytic phenotype which, in addition to any central cardiovascular
533 limitations, promotes the early onset of fatigue and exercise intolerance. In turn, this
534 may contribute to an increased burden of exercise participation for these individuals,
535 ultimately leading to higher recidivism rates in training regimens.
536 Inorganic nitrate supplementation has been shown to play a key role in
537 exercise capacity in numerous studies in healthy subjects (as previously illustrated).
538 The intent in this cohort is to use nitrate supplementation as an “ergogenic” to
539 augment “normal” levels of bioavailable NO in exercising tissues in order to enhance
540 physical performance, stamina or recovery. Supplementation within the clinical
541 cohort, however, takes a “therapeutic” approach with the aim of restoring deficient
542 NO bioavailability, correcting physiological dysfunctions, and recovering exercise
543 capacity/performance and health.

In this section, we will build on the data presented in healthy supplementation studies and focus on known physiological maladaptations that reduce exercise tolerance in individuals with PAD, CHF, and Type II Diabetes Mellitus (T2DM). We will highlight the potential mechanisms by which inorganic nitrate consumption, and the associated increase in circulating nitrite and NO bioavailability, may act as a therapeutic to attenuate these dysfunctions and increase exercise tolerance.

Inorganic Nitrate Supplementation and Exercise Performance in Peripheral Arterial Disease

Peripheral artery disease is caused by atherosclerotic plaque formation in the large arteries of the legs, resulting in reduced blood flow to the lower extremities(9). It is estimated that the worldwide prevalence of PAD has increased by 23.5% in the last decade and now affects 202 million people(68). Intermittent claudication (IC) is the major clinical manifestation of PAD and occurs when arterial occlusive disease reduces blood flow to the peripheral vasculature during exercise. Among subjects with intermittent claudication from PAD, 1/3rd have pain during light activity at home and an additional 1/3rd have pain walking a short distance (one block)(91). These patients suffer from a markedly impaired quality of life and a high perception of disability(161). Increased pain free walking capacity is a primary goal of therapy for patients with PAD.

Although measures of conduit vessel and gross limb blood flow, such as ankle brachial systolic blood pressure index (ABI), are used to diagnose PAD, they show a poor relationship with functional capacity(29, 92, 93, 138, 171, 248). Additionally, surgical revascularization, which improves blood flow, does not normalize exercise

performance(183) and conversely exercise performance can be increased without changes in conduit vessel hemodynamics(153, 154, 210).

It appears that the key to increasing functionality in patients with IC may lie at the resistance arteries, arterioles and capillaries that serve the skeletal muscle tissue distal to the site of stenosis. These are the vessels which are responsible for much of the oxygen delivery(225) and become hypoxic during the increased demands for perfusion accompanying physical exertion. Therefore, inorganic nitrate supplementation may be a novel intervention to improve oxygenation to these areas of skeletal muscle ischemia and increase physical function. This would be a significant step forward in the treatment of PAD.

In 2010, our group(7) demonstrated increases in time to claudication onset pain (66%) and peak walking time (52%) in subjects with PAD following three months of supervised exercise training. The strongest independent predictor of these changes was the ability to increase plasma nitrite concentrations during maximal exercise, which was most likely as a result of an increase in endothelial NO production. In a follow-up repeated measures crossover study, we orally administered 500ml of beetroot juice containing 9mmol nitrate (compared to an orange juice placebo) in 8 subjects (4 male, 4 female) age 67+13years with IC (ABI in the incident leg of 0.64+0.2). The results of a maximal graded treadmill test (Gardner protocol) showed an increase in average exercise time before the subject reported the onset of claudication pain (COT) of 18% (32sec), and an increase in maximal walking time of 17% (65sec) respectively(116). This is a clinically meaningful and statistically significant increase for a disease state characterized by reduced physical function and quality of life(170, 207). Additionally, there were no changes in ABI or endothelial function, suggesting no increase in endogenous

vascular NO production. The increases in performance were accompanied by a reduction in fractional oxygen extraction at the working tissues, measured by near infra-red spectroscopy (NIRS) suggesting increased perfusion to working tissues.

Currently, there are two clinical trials listed as in progress on clinical trials.gov investigating the effects supplementation of either beetroot juice (NCT02553733) or Neo 40 (a tablet containing beetroot powder, L-citrulline and sodium nitrite) (NCT02934438) on walking performance in PAD, but there are no other results that we are aware of at the time of submission.

Studies in animal models of PAD are also promising with a dose dependent relationship between nitrite dose (via intraperitoneal injection twice daily for 7 days) and improved tissue perfusion via angiogenesis in a murine model with permanent femoral artery ligation of the hind limb(121). Co-administration of the NO scavenger carboxy-PTIO with the nitrite completely abrogated the increase in perfusion suggesting the mechanism of effect is NO mediated.

While it is premature to speculate on overall clinical utility of a nitrate based therapy for peripheral artery disease, the early data appears encouraging. Additional large clinical trials and basic science studies are required to determine important molecular mediators conveying beneficial effects of nitrite therapy during specific disease states.

<u>Inorganic Nitrate Supplementation and Exercise Performance in Chronic Heart Failure</u>

Chronic heart failure is characterised by the inability of the heart to pump sufficient blood to meet the body's metabolic needs. It affects approximately 23 million people worldwide with a direct cost of $36 billion per year in the U.S.

alone(131). While there are unique aetiologies associated with the development of CHF, the hallmark symptom experienced by patients is exercise intolerance. In comparison to healthy controls, patients with CHF have significantly lower VO_{2peak} (~50% reduction) with accompanying reductions in cardiac output by 52-53% during maximal exercise(57, 82, 214). As exercise capacity (and in particular VO_{2peak}) is a strong independent predictor of mortality and morbidity in patients with CHF, targeting this deficit is of clinical importance(12, 137). Endothelial dysfunction and reduced NO bioavailability have been linked to both the initiation and progression of CHF(140). More specifically, imbalances in the production and utilization of NO contribute to the elevated cardiac filling pressures, symptoms of dyspneoa, the severity of the disease, and the functional capacity of the patient(145, 200)

It was historically assumed that this inability to augment cardiac output during exercise (central dysfunction) was the main contributor to the exercise intolerance experienced by patients with CHF(173). However, more recently, maladaptation's within the peripheral tissues (secondary to the initial central dysfunction) have been highlighted as crucial limiters in exercise capacity. Chronic peripheral tissue under perfusion (due to reduced cardiac output) results in capillary density rarefaction, decreased mitochondrial function and a preferential loss of type I oxidative fibres, which cumulatively shift individuals with CHF to a more glycolytic phenotype(47, 61, 172, 215, 216, 239). These conditions are ideal for inorganic nitrate targeted therapeutics.

CHF is not a single uniform state, but rather a multifarious syndrome that presents generally as one of two classifications depending on whether the patient has a preserved ejection fraction (HFpEF) or a reduced ejection fraction

(HFrEF)(36). There are key etiological characteristics that differentiate the two classes. HFrEF often has a sudden onset following a myocardial infarction whereas patients with HFpEF are typically older, more commonly female, and usually have multiple comorbidities associated with a slower onset(122). Patients with HFrEF characteristically present with reduced cardiac output (Q) at rest and during exercise. Patients with HFpEF usually have a normal resting Q but exhibit increased left ventricular (LV) filling pressures which become pronounced under stress(182), and are associated with exertional dyspnoea and reduced exercise cardiac output (16, 57). Despite the heterogeneity of the two classes of CHF, the growing body of literature suggests that nitrate supplementation remains potentially efficacious in both syndromes.

a) HFpEF studies

Interestingly studies of inorganic nitrate supplementation in patients with HFpEF have shown more positive outcomes than those in HFrEF(62, 246, 247). There are two potential explanations for these findings. First, peripheral under-perfusion and an inability to extract oxygen at the tissue level has been found to be more significant in patients with HFpEF, as evidenced by significantly lower a-vO_{2diff} during exercise than both HFrEF and controls(57). Second, a recent study by Borlaug et al.(27) has demonstrated that a sodium nitrite infusion in patients with HFpEF significantly reduced LV filling pressures during exercise. While the focus of this review is on natural product supplementation, these mechanistic benefits from nitrate/nitrite products lend promise to the use of similar more natural options.

In 2015, Zamani et al.(246), used a single dose of beetroot juice (12.9mmol nitrate), in 17 patients with HFpEF. They showed significant improvements in VO_{2peak} and time to exhaustion (TTE) during a maximal exercise test. The authors postulated that the beneficial changes in exercise capacity were due to an accompanying decrease in systemic vascular resistance, thus reducing afterload and increasing Q. Surprisingly, they showed no improvements to exercise efficiency, suggesting nitrate may have differential effects on the mitochondrial function in aging/diseased populations when compared to healthy individuals (as described previously).

Similarly, in 2016, Eggebeen et al.(62), used beetroot juice to examine the effects of both a single dose (6.1mmol) and 1 week dosing (6.1mmol/day) to determine the effects of nitrate supplementation in HFpEF during a submaximal cycling endurance exercise bout (at 75% of measure maximal power). They found no significant benefits in exercise performance with acute supplementation, but the chronic dosing elicited a 24% increase in TTE. Their data also suggested that the improvements were likely due to decreases in systemic vascular resistance (SVR) (62). To complement these findings, other mechanistic studies utilizing infusions or nebulized inorganic sodium nitrite have demonstrated improvements in SVR (26, 27). Significantly, Borlaug et al.(27), noted that the improvements in cardiac function following nitrite ingestion were actually more pronounced during exercise, again supporting nitrite's preferential effects in low oxygen environments and its potential utility as a targeted approach to treating HFpEF.

A second, more recent study by Zamani et al.(247), utilizing a high chronic dose of potassium nitrate (6mmol/day for 1 week, increasing to 18mmol per day for the second week) also found significant improvements in TTE as well as decreases

in CHF symptoms (via the Kansas City Cardiomyopathy Questionnaire). While they did not assess muscle fibre composition or recruitment, the authors suggested that the maximal exercise approach employed during testing may provide preferential conditions to optimise the benefits of inorganic nitrate supplementation (hypoxia and greater type II fibre recruitment).

In an effort to discover if nitrate supplementation may have an additive beneficial effect on physical function when consumed in conjunction with exercise training, Shaltout et al.(197), recently gave beetroot juice (6.1mmol nitrate) plus exercise training for 3 days per week for 4 weeks versus exercise alone. While, as expected, they saw significant improvements in aerobic capacity in both groups, the nitrate did not have a significant additive benefit. However, given the sample size was small for a study using an exercise comparison group (exercise alone elicits relative large benefit), a short treatment period, and a low dosage regimen, this additive approach may be worth of greater exploration.

b) HFrEF studies

Patients with HFrEF usually demonstrate reductions in Q at both rest and during exercise(57, 64) and chronotropic incompetence (the inability to sufficiently augment HR during exercise) substantially contributes to the reductions in VO_{2peak}. Interestingly (and in contrast to HFpEF) peripheral oxygen extraction during exercise (a-VO_2diff) appears to remain similar to that of healthy cohorts(35, 57). However, they still demonstrate skeletal muscle abnormalities that contribute to exercise intolerance(46, 118, 230). The potential therapeutic benefits of nitrate/nitrite interventions were highlighted by a recent study in HFrEF rats. Glean et al.(76), demonstrated that a single dose of sodium nitrate lead to significant

elevation (10%) in vascular conductance within the hind limb skeletal muscles. Moreover, the hind limb skeletal muscles that showed increases in vascular conductance and blood flow following dosing were primarily comprised of (63%) type IIb + IId/x fast twitch fibers. This further supports nitrate/nitrite's potential as particularly effective intervention for those individuals known to be more type II fiber dominant, as is the case for patients with CHF.

Unfortunately, to date there is only one study of exercise capacity in individuals with HFrEF, following nitrate supplementation. In an elegantly designed cross-over study, Hirai et al., found that 9 days of beetroot juice supplementation (12.1mmol/day) did not result in any improvements in exercise tolerance (TTE or VO_{2peak}). They also saw no significant changes in central hemodynamics, skeletal muscle oxygenation, or the oxygen cost of exercise(94). The authors suggest the negative findings could be due to the aforementioned relatively normal peripheral oxygen extraction in comparison to HFpEF. However, future studies in this cohort are warranted and should aim to optimize both the dosing amount and duration. There has been a second study in patients with HFrEF but this examined isokinetic knee extensor power in isolation(49). They showed that a single dose of inorganic nitrate (11.2mmol) via beetroot juice, improved maximal power output by 13%, which is much larger than the 6% increase observed in healthy controls following nitrate supplementation. They proposed the response was mediated by NO's known stimulation of guanyl cyclase which increases c-GMP levels. As activation of c-GMP increases max power, especially in type II fibers, this type of intervention could be particularly efficacious in CHF where the fast-twitch fibres are more readily recruited (234).

It is clear that inorganic nitrate supplementation holds a good deal of promise in patients with CHF. To date, results are predominantly in support of an exercise benefit in patients with HFpEF, which is logical given our understanding of nitrates mechanism of action in the peripheral tissues and the greater deficits in a-vO_2diff in HFpEF. However, patients with HFrEF are currently understudied and as of yet there is no direct comparison of HFpEF and HFrEF in the same study design to provide an accurate assessment of any differential benefits of inorganic nitrate supplementation between the two classifications.

Inorganic Nitrate Supplementation and Exercise Performance in Diabetes Mellitus

The incidence of diabetes mellitus has quadrupled since 1980, from 108 to 422 million people(241). Despite medical treatment diabetics die approximately 5-10 years earlier than non-diabetics, with approximately 50% of deaths being attributed to cardiovascular disease(69, 150). Regular participation in physical activity (along with diet and pharmacotherapy) is a cornerstone of the treatment forT2DM(51, 98). Exercise has been shown to increase insulin sensitivity, glucose uptake, and reduce cardiovascular morbidity. However, the burden of exercise participation for individuals with T2DM appears to be increased due to several skeletal muscle tissue maladaptations(78, 114, 159, 163, 164). The function of skeletal muscle is of particular importance for individuals with T2DM given that it is responsible for approximately 80% of whole body glucose uptake following hyperinsulinemia and exercise(55). The increase in glucose uptake is correlated closely with increase in blood flow (approx. nine-fold) in the exercising muscle(55).

Individuals with T2DM appear to have several defects in NO production and transport that could contribute to exercise intolerance and to a decline in cardiovascular health. One study showed that impaired endothelial production of NO

during acute exercise stress in subjects with T2DM was the strongest predictor of exercise intolerance, in a multivariate regression model(3). The ability to conserve and transport NO via the plasma and red blood cells (RBC) (as described in an earlier section) may be dysfunctional in individuals with diabetes(99, 147, 212). One mechanism outlined for this deficiency is the preferential binding of NO to glycosylated RBC's and decrease in disassociation with changes in PO_2. Ultimately this results in decreased NO bioavailability in the microvasculature as well as reductions in NO and O_2 delivery to peripheral tissues. Furthermore, individuals with T2DM have a number of other pathologies that may cause inactivation of NO, for instance, an increase in superoxide production which interacts with NO to produce peroxynitrite(80, 149).

Compared to patients with PAD, those with concomitant T2DM failed to increase endogenous vascular NO production and exercise capacity following 3 months of supervised exercise training(8). This suggests the possibility that they are less able to increase endogenous endothelial NO production which may be reflected in reduced plasma nitrite concentration following exercise and reduced hyperaemic response following ischemic stimuli(5, 120).

Patients with T2DM present several potential therapeutic opportunities for dietary nitrate supplementation to improve their metabolic and cardiovascular health. In animal models, it has been demonstrated that NO bioavailability influences several aspects of glucose-insulin homeostasis including regulation of mitochondrial function, insulin secretion, glucose uptake and blood flow(39, 90, 103, 160, 169). The seminal work by Carlstrom and colleagues(39), demonstrated that eNOS deficient mice with several of key features of diabetes, benefitted from chronic nitrate supplementation. Restoring NO bioavailability resulted in improvements in glucose

tolerance, glycosylated haemoglobin, fasting glucose, and circulating triglycerides. These findings have subsequently been reproduced and further investigated by several others(103, 160, 169, 208). From a mechanistic perspective, nitrate or nitrite supplementation results in an increase in glucose uptake by increased GLUT4 translocation via AMPK pathway(56, 103), similar to the pathways activated by exercise(186). Collectively, these animal models provide an in-depth investigation into the promising metabolic benefits of nitrate or nitrite supplementation for metabolic conditions, in particular T2DM. However, to date, no studies in animal models have assessed the effects of nitrate supplementation on exercise in T2DM.

Unfortunately, positive metabolic findings from animal models have failed to translate into humans with T2DM. This is despite acute and chronic nitrate supplementation studies producing significant increases in plasma nitrite(44, 74, 202). Cermak et al(44). showed no differences in an oral glucose tolerance test, following single dose sodium nitrate (~10.5mmol) and Shepherd et al.(202), failed to observe changes in the oxygen cost of exercise or exercise tolerance following 4 days of beetroot juice (6.43mmol/day). In a longer period of supplementation, two weeks of nitrate (7.5mmol/day) where the median plasma nitrite reached 390 nM, Gilchrist and colleagues(74), found no effects on endothelial function or insulin sensitivity.

Possible explanations for the lack of physiological changes following nitrate supplementation in humans include the aforementioned defects in NO production and transport. Additionally, the duration of diabetes may be much longer in human patients compared to in animal studies and possibly most significantly, there could be interference effects from diabetic medications. For instance, metformin, the most prescribed first-line medication for diabetics (used to lower blood glucose), may

interfere with beneficial effects of dietary nitrate on aspects of exercise related parameters. A mechanism of action for increased glucose uptake via metformin involves the non-competitive inhibition of the skeletal muscle mitochondrial electron transport chain at complex 1. This causes a decrease in mitochondrial respiration, mitochondrial dysfunction and a decreased ATP production(33, 235), which although beneficial for glucose uptake, produces a negative effect on muscle function and a reduction in exercise capacity(30, 166). This is in contrast to the role that nitrite alone may play on the efficiency of mitochondrial respiration in both human whole body and isolated muscle fiber experiments (as described earlier). Additionally, nitrite exhibits beneficial effects in normoxia for glucose uptake via mitochondrial fusion activation of protein kinase A(110, 117). For further information on this area see: (79, 203). This mechanism may be especially pertinent in T2DM where tissue perfusion is reduced during exercise and a glycolytic phenotype dominates in the skeletal muscle.

Given that only one study has assessed this (and only at a relatively low exercise intensity using the 6-minute walk), future studies may wish to further examine the effects of longer term supplementation on exercise. These studies should also aim to target individuals who are newly diagnosed or who have prediabetes.

Conclusion

In summary, over the last 10 years there has been tremendous growth of interest in the role of inorganic nitrate supplementation, especially in the form of beetroot juice, on exercise performance. The majority of the studies have been

841 focused on healthy populations with mixed results. Much of the variation may be
842 attributed to small sample sizes and differences in dosing regimens.
843 It appears that a chronic dosing strategy, consisting of ~8mmol per day, for at
844 least 5 days provides the greatest likelihood of achieving plasma nitrite
845 concentrations greater than 400nM and a subsequent ergogenic benefit. However,
846 at this time there is demonstrated within and between subject variability in the
847 conversion of nitrate to nitrite, as well as in the physical function benefits following
848 treatment. This has led to the potential of individuals being classed as “responders”
849 or “non-responders” within an otherwise homogeneous sample. This is a current
850 area of intense research, with investigations into the role of the oral and gut
851 microbiome or particular interest.
852 It appears that nitrate supplementation in individuals with a high training
853 status in lower intensity aerobic-type activities, has a low chance of positive results.
854 Elite athletes are well adapted to maintain adequate microvascular perfusion and
855 match oxygen delivery to the increased requirements of the working muscle during
856 the majority of exercise conditions. Thus, it is logical that there would be mixed
857 results following nitrate supplementation when we consider that nitrite is
858 preferentially reduced to NO in conditions of low PO_2 and low pH. It also provides a
859 potential explanation for why high-intensity activities that rely predominantly on fast –
860 twitch muscle fibers have shown the greatest potential for an ergogenic benefit in
861 healthy, trained individuals.
862 Along the same lines, patients with CVD develop multiple peripheral tissue
863 abnormalities, often as a maladaptation to chronic under perfusion, which result in an
864 overall glycolytic phenotype. This, coupled with endothelial dysfunction (an inability
865 to endogenously upregulate NO) and increased NO scavenging, make nitrate

866 supplementation a particularly promising intervention for patients with CVD. This
867 theory is supported by encouraging data showing restorative effects on time to
868 claudication pain onset and peak walk times in PAD as well as muscle contractile
869 function and exercise performance in patients with CHF. Interestingly, to date no
870 benefits in exercise performance following inorganic nitrate supplementation have
871 been shown in patients with T2DM, although the role of metformin in mitochondrial
872 function may be a mitigating factor to be further investigated.
873 In summary, inorganic nitrate supplementation within the CVD cohort shows
874 promise as a potential “therapeutic” with the aim of restoring deficient NO
875 bioavailability, correcting physiological dysfunctions and recovering exercise
876 capacity/performance and health. Given the well documented relationship between
877 reduced exercise capacity with morbidity and mortality it may be an intervention
878 which provides significant functional and clinical benefits to patients with CVD.
879

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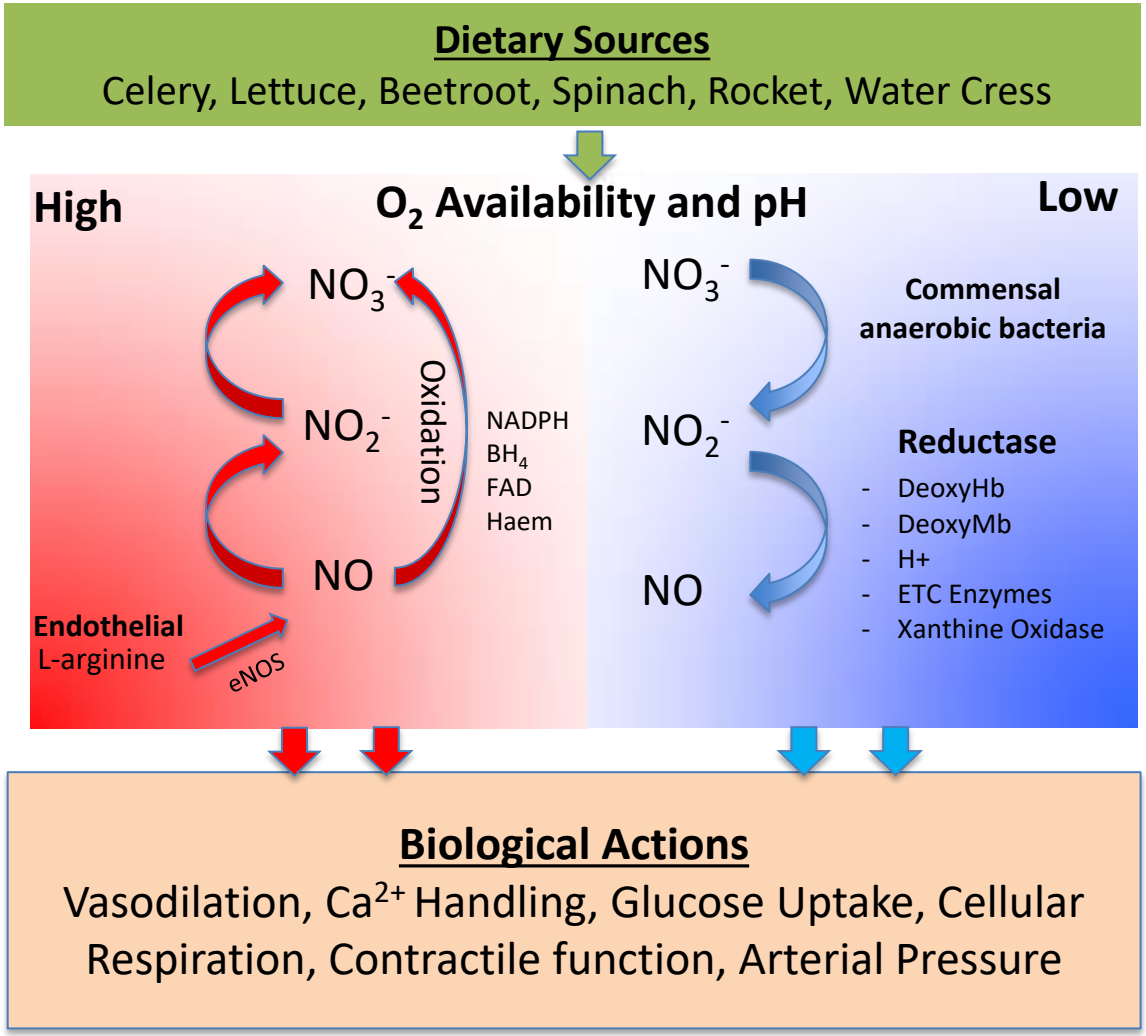
Figure Legends

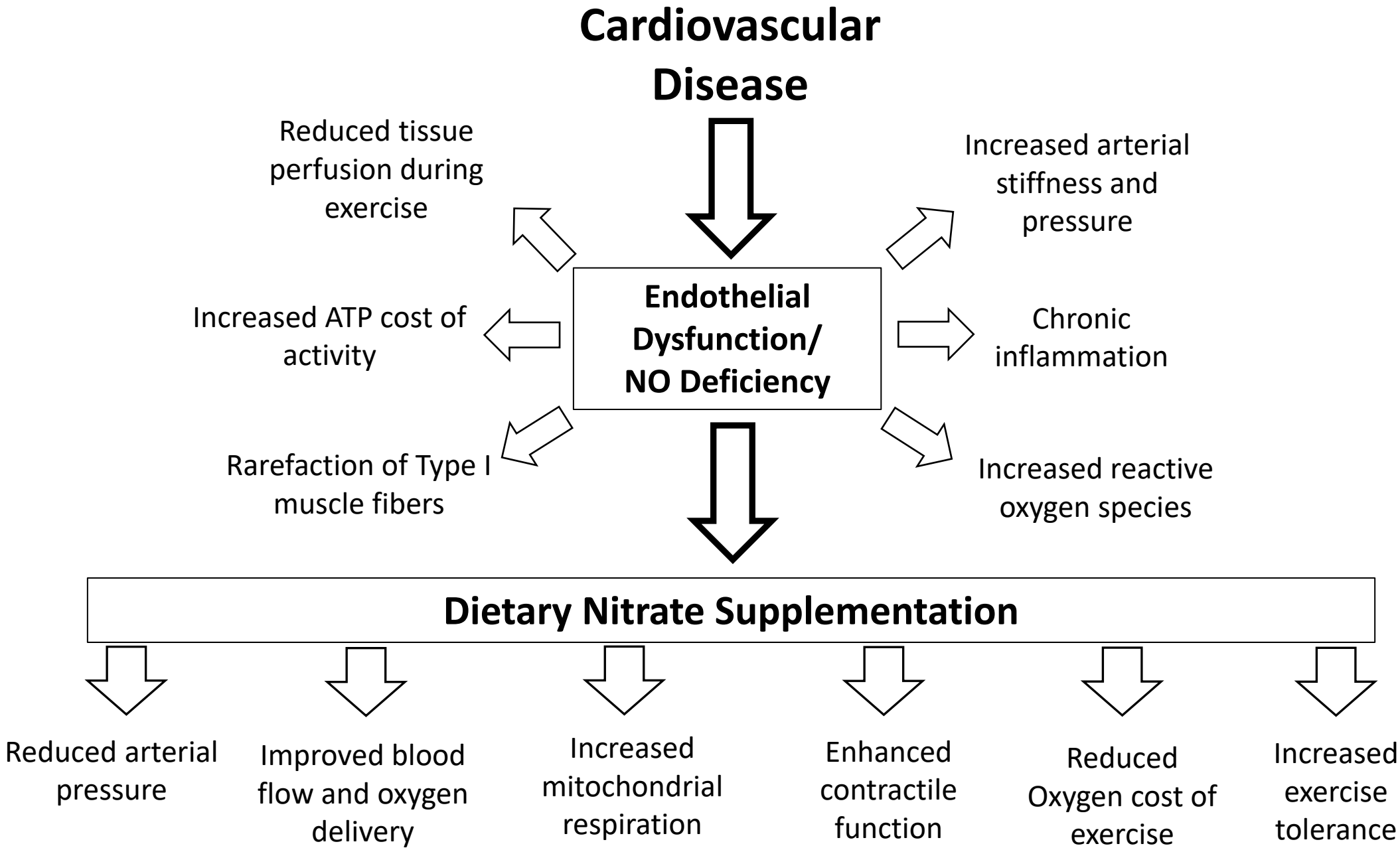
Figure 1: Nitrate-Nitrite-Nitric Oxide Formation/Recycle Pathways.

In the presence of oxygen endothelial nitric oxide synthase (eNOS) catalyzes the oxidation L-arginine to NO. NO may also be rapidly oxidized to nitrite (NO_2-) and nitrate (NO_3-). A secondary source of vascular NO is via diet. Consumption of food stuffs high in inorganic nitrate (green leafy vegetables, beetroot) have been shown to increase plasma nitrate which can be secreted in saliva and reduced to nitrite by commensal bacteria in the mouth. Nitrite can then be further reduced to NO (and other biologically active nitrogen oxides) via several mechanisms which are expedited under hypoxic conditions. Hence, although some of the circulating nitrate and nitrite are excreted in the kidneys they are also able to be recycled back to NO

Adapted from (6)

Figure 2: Peripheral Tissue Maladaptation's in Cardiovascular Disease Populations and Potential Therapeutic benefits of Inorganic Nitrate Supplementation


Dietary Sources
Celery, Lettuce, Beetroot, Spinach, Rocket, Water Cress
High
O2 Availability and pH
Low
NO3-
NO2-
NO
Oxidation
NADPH
BH4
FAD
Haem
Endothelial
L-arginine
eNOS
NO3-
NO2-
NO
Commensal anaerobic bacteria
Reductase
- DeoxyHb
- DeoxyMb
- H+
- ETC Enzymes
- Xanthine Oxidase
Biological Actions
Vasodilation, Ca2+ Handling, Glucose Uptake, Cellular Respiration, Contractile function, Arterial Pressure


Cardiovascular Disease
Reduced tissue perfusion during exercise
Increased arterial stiffness and pressure
Endothelial Dysfunction/ NO Deficiency
Increased ATP cost of activity
Chronic inflammation
Rarefaction of Type I muscle fibers
Increased reactive oxygen species
Dietary Nitrate Supplementation
Reduced arterial pressure
Improved blood flow and oxygen delivery
Increased mitochondrial respiration
Enhanced contractile function
Reduced Oxygen cost of exercise
Increased exercise tolerance

CHF type	Author	N	Duration	Design	Dose/Administration	Exercise Outcomes
HFpEF	Zamani, 2015	17	Acute	Double-blind, randomized, crossover	Beetroot Juice-12.9mmol Nitrate	No change in maximal exercise efficiency Increase in VO2peak (p=0.005) Increase in time to exhaustion (p=0.02)
	Eggebeen, 2016	18	Acute	A: Cross-over design	Beetroot Juice-6.1mmol Nitrate	No change in sub-max time to exhaustion
			Chronic	B: All treated	Beetroot Juice 7 days-6.1mmol Nitrate	Increase in sub-maximal time to exhaustion (p=0.02)
	Zamani, 2017	12	Chronic	Single Blind	Potassium Nitrate 7days 12mmol *followed by* Potassium Nitrate 7days 18mmol	No change in VO2peak Increase in Time to exhaustion: (p=0.002)
HFrEF	Hirai, 2017	10	Chronic	Double-blind, randomized crossover	Beetroot Juice 9 days-12.9mmol Nitrate	No change in exercise performance measures