EFFECT OF BEETROOT SUPPLEMENTATION ON CONDUIT ARTERY BLOOD FLOW AND MUSCLE OXYGENATION DURING HANDGRIP EXERCISE

by

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Abstract

Dietary nitrate supplementation via beetroot juice (BR) has been shown to have positive effects on mitochondrial and muscle efficiency during large muscle mass exercise in humans, and more recently on locomotory muscle blood flow ($\dot{Q}$) in rats. To date, an integrated measure of these effects has not been performed in humans. Therefore, we assessed the influence of BR on $\dot{Q}$ and muscle oxygenation characteristics during moderate and severe intensity handgrip exercise. Seven healthy men (age: 25 ± 3 yrs; height: 179 ± 4 cm; weight: 82 ± 9 kg) completed four constant-power exercise tests randomly assigned to condition (BR or placebo (PL)) and intensity (moderate (40% peak) or severe (85% peak)). Resting mean arterial pressure was significantly lower after BR compared to PL (79.3 ± 5.8 vs 86.8 ± 6.7 mmHg; $p < 0.01$). All subjects were able to sustain 10 min of exercise at moderate intensity in both conditions. BR had no significant effect on exercise tolerance during severe (342 ± 83 vs 382 ± 138 s, $p = 0.382$). Brachial artery $\dot{Q}$ was not significantly different after BR at rest or any time during exercise in either intensity. Deoxygenated-[hemoglobin + myoglobin] was elevated at min 2 & 3 for moderate ($p < 0.05$) and throughout severe exercise ($p = 0.03$) after BR. The estimated metabolic cost ($\dot{V}O_2$) was not significantly different during either intensity after BR. These findings support the notion that an acute dose of BR may be valuable to reduce blood pressure in young adults, but revealed that it does not augment $\dot{Q}$ or $\dot{V}O_2$ during small muscle mass handgrip exercise.

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Dedication

This project is dedicated to my father, Jack E. Craig. We lost you entirely too soon. You did not get to see me finish, but you were able to see me start and I know that you would have been proud of what I have accomplished so far. I think about you every day. Love you dad!

Chapter 1 - Introduction

Dietary nitrate supplementation (including beetroot juice (BR)) is well documented to have positive effects during large muscle mass exercise in man (7, 61, 72, 100, 105). These effects include lowering the O_2 cost, or oxygen consumption ($\dot{V}O_2$) (7, 72) and/or reducing the ATP cost of work (5, 39) during submaximal exercise, which may translate to the enhanced exercise tolerance found during severe intensity exercise. The precise mechanism(s) for these effects still remains uncertain, but they are facilitated through the reduction of the dietary nitrate (NO_3^-) to nitrite (NO_2^-) in the mouth (75). Once absorbed into the circulatory system, NO_2^- is readily converted to nitric oxide (NO) in hypoxic (27, 95) and acidic (77) environments, which may be present at the exercising muscle.

A fundamental role of NO is that of a potent vasodilator (32, 35, 89); as such it has been demonstrated that BR supplementation augments blood flow ($\dot{Q}$) to the working muscle. This was first experimentally investigated in rats during submaximal treadmill running (37). These authors found that BR supplementation resulted in an increased $\dot{Q}$ to the hindlimb, despite a lower exercising mean arterial pressure (MAP). These findings demonstrate that BR may change the regulation of $\dot{Q}$ relative to $\dot{V}O_2$, as $\dot{V}O_2$ and $\dot{Q}$ generally increase in proportion to one another across a range of exercise intensities (1, 84). In a follow up study, Ferguson and colleagues (36) repeated the previous experiment using both a low (0.3 $mmol \cdot kg^{-1} \cdot day^{-1}$) and high (1 $mmol \cdot kg^{-1} \cdot day^{-1}$) dose of BR and found no difference in $\dot{Q}$ or microvascular partial pressure of O_2 ($P_{mv}O_2$) with the low dose. Importantly, the high dose yielded similar findings to the original study with an increased $\dot{Q}$, while further providing evidence of increased $P_{mv}O_2$, supporting that BR increased $\dot{Q}$ relative to $\dot{V}O_2$. Recently the effect on $\dot{Q}$ was investigated in human subjects (19, 62), but no change in brachial artery blood flow ($\dot{Q}_{BA}$) was found in healthy, young men and

women during moderate intensity handgrip exercise, consistent with the findings of low dose BR in rats.

These data in humans may provide valuable insights into the efficacy of BR as a therapy for patient populations, because handgrip exercise (with duty-cycles ≥ 50%) provides an opportunity to measure the effects of BR in an exercise model in which the O_2 delivery is inadequate to support the $\dot{V}O_2$ requirements due to mechanical impediments to $\dot{Q}$ (15, 99). This condition of reduced O_2 delivery is experienced in several patient populations (e.g., chronic heart failure (CHF), chronic obstructive pulmonary disease (COPD), and diabetes). Additionally, handgrip exercise may prove useful for investigation of these patient populations directly because of the reduced dependence on central cardiorespiratory adjustments, specifically of cardiac output.

Importantly, previous studies in humans using BR provided no measure of $\dot{V}O_2$, $P_{mv}O_2$, or fractional O_2 extraction (which can be estimated noninvasively via deoxygenated-[hemoglobin + myoglobin] (deoxy-[Hb + Mb]) and used to estimate $\dot{V}O_2$) (15, 29, 31, 66). Moreover, when measurements were made, it was after fixed durations of moderate intensity submaximal work, leaving the effects of BR on exercise tolerance and the resulting end-exercise variables unknown. These latter findings carry important implications for patient populations, such as CHF, as accumulating evidence suggests BR may be effective for enhancing quality of life through improvements in exercise and/or daily activity tolerance (106).

Therefore, the purpose of this investigation was to resolve whether or not BR supplementation provided beneficial effects in small muscle mass exercise at both moderate and severe exercise intensities. Specifically, we tested the hypotheses that with BR supplementation,

1) $\dot{Q}_{BA}$ would not be significantly different during exercise; 2) $\dot{V}O_2$ would be lower during exercise; and 3) tolerance of exercise (T_{lim}) would be increased during severe intensity exercise.

Chapter 2 - Review of Literature

Theory of measurements

Doppler ultrasound

Doppler ultrasound is a powerful tool to noninvasively and precisely measure vascular hemodynamics across a variety of vessels, exercise modes, exercise intensities, and importantly across the crucial rest-to-exercise transition. Instantaneously and continuously measuring $\dot{Q}$ for kinetic analysis and without stopping exercise was a significant advance of methodology for exercise physiologists over the previous standard, venous occlusion plethysmography. The value for $\dot{Q}$ is calculated using the variables directly measured by the ultrasound, vessel radius (r) and mean blood velocity (V_{mean}) in the formula: $\dot{Q} = \pi r^2 V_{mean}$. The measurement of r is taken in B-mode, or 2D mode, which utilizes the transducers of the ultrasound probe to scan a plane through the interrogated tissue. This mode allows for accurate measurement of vessel diameters, which are subsequently converted to r by dividing the diameter in half.

The measurement of V_{mean} is taken in pulsed wave Doppler mode. This mode takes advantage of the Doppler Effect and the interaction between the emitted sound waves and red blood cells (RBCs). Briefly, the frequency of the waves will be altered based on the direction of travel and velocity of the RBCs. If the RBCs are moving toward the probe, the frequency will increase with further rise compounded by their velocity. If the RBCs are moving away from the probe, the frequency will decrease and this drop will be further exacerbated by their velocity. This allows real time measure of the pulsatile nature of $\dot{Q}$ and gives researchers the ability to distinguish between antegrade and retrograde flow. The strength of these signals are maximized by controlling the angle of insonation (< 60°) as the above phenomenon requires movement toward or away from the probe.

Near infrared spectroscopy

Near infrared spectroscopy (NIRS) provides a measure of microvascular oxygenation characteristics with great accuracy and temporal resolution. This is accomplished by interrogating the tissue of interest with light composed of visible and near infrared (NIR) wavelengths and measuring the light that is able to traverse the tissue. The range of viable wavelengths has been defined at the upper limit of approximately 1000 nm due to the overriding absorption by water and at the lower limit of about 650 nm due to the vast absorption by hemoglobin (Hb). Many substances present in human tissue have well documented absorption spectra at NIR wavelengths and importantly have variable concentrations during exercise, of note to this investigation are oxygenated hemoglobin and myoglobin (Mb) (oxy-[Hb + Mb]), deoxygenated hemoglobin and myoglobin (deoxy-[Hb + Mb]), and the summation of these variables in total hemoglobin and myoglobin (total-[Hb + Mb]). The presence of oxygen alters these absorption characteristics and allows the technique to estimate the oxygenation characteristics of the tissue. Sampling at a rate of 50 Hz, the OxiplexTS (ISS, Champaign, IL, USA) used in the current study, allows for accurate kinetic analysis of NIRS signals.

The NIRS technique is not without limitations as it must deal with unresolved questions regarding the effect of adipose tissue thickness (ATT) on the signals, the contribution of Mb during exercise, and the effect of scattering within the tissue (38). Scientist have become aware of the negative effects of ATT on NIRS signal strength and some have proposed corrective formulas (12), but there are still questions concerning the anatomical and physiological make-up of the tissues underlying the NIRS probe. The deleterious effects of ATT have been addressed in the current study by selecting handgrip exercise and observing the oxygenation status of the flexor digitorum superficialis (FDS), which regularly has a low ATT compared to sites in the legs and allows for complete description of the active muscle.

While NIRS is unable to differentiate the contribution of Hb from Mb to oxygenation characteristics, studies have made effort to reveal their influence on deoxy-[Hb + Mb] and total-[Hb + Mb]. These studies suggest that while Mb may contribute 80% of the NIRS signal, it does restrict the interpretation of NIRS measurements. The time-course change of deoxy-[Hb + Mb] is similar to that of the microvascular partial pressure of oxygen (Pm_{VO2}) and remains a viable tool to index local O_2 extraction (66, 96). The increase in total-[Hb + Mb] with exercise can be used as an indication of microvascular hematocrit (28) because the volume of Mb remains unchanged and appears to match the increase of microvascular hematocrit of *in vivo* models (65).

Scattering of photons is a limitation of the NIRS measurement if the equipment used does not account for this effect. Briefly, scatter is the chaotic deflection of light particles as they pass through and interact with substances in the environment. An increase in scatter results in an increase in the path length of the photons, leading to artificial increases in the absorption of the photons. The largest source of scatter in this study is the melanin of the skin and to a lesser extent bone tissue. The NIRS system used in the current study corrects for this scatter by use of a frequency-domain multi-distance technique that alters the frequency and intensity of the emitted light as well as the distance between the emitters and sensors to develop a real-time scattering coefficient (μ_s'). This coefficient allows correction of the scattering and absolute quantification of the measured variables in micro-molar (μM) concentration.

Non-invasive blood pressure

The non-invasive blood pressure (NIBP) measurement used in the current investigation makes use of the oscillometric measuring principle. To accomplish this, the cuff is inflated to a pressure that exceeds systolic blood pressure (SBP) and is slowly released while measuring the pressures associated with oscillations in the cuff produced by the pulsating artery. The first and

last oscillations will approximate the Korotkoff sounds that designate the SBP and diastolic blood pressure (DBP), respectively. Using these values the mean arterial pressure (MAP) can be calculated as 1/3 (SBP – DBP) + DBP. The two-handed ergometer used in the current investigation precluded the use of the brachial artery and necessitated the use of the posterior tibial artery. To account for the increased hydrostatic pressure in the ankle while seated, a correction factor was used based on the distance between the heart and ankle which equated to subtracting 76 mmHg per meter difference (42). Pilot work in our laboratory validated the correction factor with measurements taken from the ankle and arm at heart level while the subjects were seated at the ergometer.

Exercise: Stress, parameters, and kinetics

The majority of physiological systems in humans have evolved with the function to maintain homeostasis in the face of a wide array of stressors. Exercise is a common, highly taxing stress imposed on the body that requires the systems to sustain energy supply and ATP concentration (14), regulate acid-base balance (54), and regulate core body temperature (86). Commensurate with the first contraction, ATP demand increases instantaneously and is supplied by the immediate and anaerobic energy pathways. These pathways are limited in capacity and thus the ATP must be supplied by alternative pathways as exercise continues, specifically oxidative phosphorylation. Oxygen uptake ($\dot{V}O_2$) measured at the mouth is a vital tool for the interpretation of whole body and synergistic muscle group performance during exercise as it has been shown to represent oxygen uptake at the muscle ($\dot{V}O_{2m}$) (9).

Central to the understanding of $\dot{V}O_2$ is the Fick principle, which describes $\dot{V}O_2$ as the product of cardiac output (CO) and the arteriovenous difference (a-$\bar{v}O_2$). As exercise continues or increases in intensity, the parameters of the Fick principle adjust accordingly to match the

metabolic demands. Any given increase in $\dot{V}O_2$ will be the result of an increase in CO, increase in a-$\bar{v}O_2$, or a combination of the two. The increase in CO is accomplished via central regulation with increases in heart rate (HR), stroke volume (SV), or a combination of both. The increase in a-$\bar{v}O_2$ is accomplished via a maintained arterial O_2 content (in most instances) and a decrease in venous O_2 content, indicative of an increased O_2 extraction by the active muscle. Small adjustments to the parameters allow characterization of $\dot{V}O_{2m}$ as the product of blood flow ($\dot{Q}$) and a-$\bar{v}O_2$. These muscle parameters are regulated in the same fashion as whole body $\dot{V}O_2$, with the further emphasis of resistance (R) or vascular conductance (VC) influencing $\dot{Q}$, which will be described in further detail below in the control of blood flow section.

Intensities below critical power (CP) (57) allow variables including $\dot{V}O_2$, $\dot{Q}$, lactate ([La^-]), pH, and phosphocreatine (PCr) to reach steady-state values before precipitous increase or decrease to levels that induce failure (58, 83). Exercise performed at intensities below CP will result in kinetic responses to steady-state lasting from seconds to minutes depending on the variable measured (45), the intensity of exercise (56), and the training status of the individual (10). Investigation of these variables in exercising forearms (53) revealed similar kinetic responses as Grassi *et al*. (45) found in lower body exercise.

Heterogeneity of $\dot{Q}$ during active hyperemia

During the transition from rest to maximal intensity exercise CO increases 5- to 6-fold that of resting values. This augmented $\dot{Q}$ during exercise, or active hyperemia, is not evenly distributed within the body. Areas of high demand (the active, exercising muscle) receive a substantial proportion of the CO (approximately 75-85%), increasing $\dot{Q}$ up to 100-fold that of resting values in individual areas and muscles. Even within the exercising muscle there exists important heterogeneity between the recruited regions and different muscle fiber types (68). By

prioritizing the distribution of $\dot{Q}$ to actively working muscle, the body ensures adequate O_2 delivery and the ability to sustain the effort (48, 49). Acknowledging this understanding, when the system is observed as whole body or limb exercise, the heterogeneity appears homogenous with $\dot{Q}$ and $\dot{V}O_2$ expressing a linear relationship from rest through increasing exercise intensity (1, 41, 84). Therefore, the next sections will describe the main factors influencing the control of $\dot{Q}$ at the onset of contraction and during continuous exercise with a universal view of the system. At the onset of exercise $\dot{Q}$ shows a biphasic increase with an initial rapid portion that plateaus at approximately 10 seconds (phase 1) and a second increase that begins around 20 seconds and progresses to the steady-state or maximal value at a slower rate lasting up to 2 minutes (phase 2).

Rapid increase of $\dot{Q}$ at onset of exercise

$\dot{Q}$ is defined using a variation of Ohm's law, stating $\dot{Q}$ is equal to the pressure gradient across a segment (ΔP) multiplied by VC. As a liquid, blood conforms to the laws of physics and will flow from an area of high pressure to one of low pressure. These areas are typically measured as the arterial (P_a) and venous (P_v) pressure on either side of a muscle bed. Mathematically VC is defined as the inverse of R (1/R) and in theory is the ease of flow through the vasculature. Therefore, $\dot{Q}$ can be augmented by increasing ΔP, increasing VC, or a combination of the two.

There are two prevailing hypotheses on the mechanism for phase 1 including the muscle pump and rapid vasodilation. The muscle pump theory suggests that with muscle contraction the increase in intramuscular pressure leads to compression of the veins consequently shunting the blood out and toward the heart. This venous emptying reduces P_v and increases the ΔP, thus augmenting $\dot{Q}$ (8, 82). This mechanism is particularly valuable during upright exercise as standing posture increases the hydrostatic column on venous blood returning to the heart. It has

been suggested that the pumping action of the muscles contributes more than 30% of the energy needed to circulate blood in humans (92) and 60% in rats (73). The hydrostatic pressure appears to be required to elicit any increase of $\dot{Q}$ as shown by the removal of an apparent muscle pump effect when the limb is elevated above heart level both in the leg (103) and the forearm (90, 98). Muscle contraction frequency is thought to have a more prominent effect on the muscle pump than the intensity of the contraction (73, 87), although contractions of sufficiently high intensity have been shown to impede $\dot{Q}$ during exercise (76). The muscle pump is not without controversy as studies have shown no muscle pump effect on $\dot{Q}$ when the vasculature is previously maximally dilated (47) or prevented from dilating during contractions (46).

The rapid vasodilation theory suggests that $\dot{Q}$ is increased at exercise onset due to a rapid increase in VC stimulated by mechanical factors, vasodilatory products of muscle contraction, or some combination of the two. These mechanical factors include myogenic response to vessel compression and lengthening. Muscle induced vessel deformation elicits endothelium-dependent and -independent dilation (25, 78) which may be crucial to the rapid $\dot{Q}$ increase at exercise onset, both independently via the increased VC and in combination with the muscle pump as suggested by the two studies of Hamann *et al*., (46, 47). Clifford *et al*., (25) suggest this compression induced dilation, rather than the artificial muscle pump, may account for the increased $\dot{Q}$ shown by Tschakovsky *et al*., (98) even in the presence of ideal muscle pump circumstances. These *in vitro* studies (25, 78) suggest a time-course of 5-7 s for the mechanically induced dilation, but when studied in the human forearm this dilation was found to occur rapidly within 1-2 cardiac cycles (64, 97).

There are many vasodilatory substances that have been shown to impart effects during the onset of skeletal muscle contraction with varying time-courses and effectiveness. These

include adenosine and ATP (18, 34), potassium (K^+) (33, 46), nitric oxide (NO) (18, 20), and prostaglandins (PG) (11, 79). Similar to the mechanically induced rapid vasodilation, the metabolically induced vasodilation has opposition because of an occasional delay that could negate the rapid augmentation of $\dot{Q}$. A study that used direct application of vasodilators (104) has suggested a time delay of 5-12 s in animal vessels. While it is easy to discount this hypothesis based on this finding, it is important to consider the complexity of the system in which these vasodilatory substances exist and that a metabolic demand or change in oxygen pressure (P_{O2}) is likely elemental to the dilatory process. This is emphasized by the largely attenuated $\dot{Q}$ following a single contraction when K^+ flow across a membrane is blocked or concentration is altered (2, 46).

Rise of $\dot{Q}$ to steady-state or peak

The second phase of $\dot{Q}$ augmentation is a matching phase during which $\dot{Q}$ is regulated in an attempt to match the metabolic demand. This matching is critical to the health of cells as hyperoxic and hypoxic environments have been shown to increase the rate of reactive oxygen species (ROS) production (22). Phase 2 has a slower rate of change relative to phase 1, but can see much greater absolute changes being influenced by the mass of the active muscle, exercise intensity, and environmental factors. Phase 2 is thought to be largely driven by the presence of vasoactive substances released locally consequential to metabolic increases and mechanical activation. Importantly, these vasodilators act to attenuate the local sympathetic vasoconstriction caused by the increased systemic sympathetic nerve activity which accompanies physical activity and exertion. There are many vasodilatory substances that have been shown to impart effects during skeletal muscle contraction with varying time-courses and efficacies including adenosine and ATP (18, 34), potassium (K^+) (33), nitric oxide (NO) (11, 26), and prostaglandins (PG) (11,

79). The predominant determinant of this control remains controversial and is currently under heavy investigation, but a general consensus supports redundancy between the dilators. However, the vasodilator of interest to the present study is NO and will be discussed further below.

Sources of NO

Endogenous production

Skeletal muscles in all mammals contain enzymes whose function is to produce NO. Called nitric oxide synthases (NOS), these enzymes make use of L-arginine and oxygen to produce NO (81). There are three forms of NOS that have been identified, with the most abundant and active being neuronal NOS (nNOS) (91). The function of NOS is greatly attenuated when oxygen availability is reduced, which may explain the lower NOS function shown in CHF and advanced age. Once produced, if not utilized quickly, NO is rapidly scavenged by hemoglobin, myoglobin, and free radicals. This suggests NO is a powerful, but local signaling molecule.

Nitrite to NO

For many years, nitrite (NO_2^-) was considered an inactive ion. Recently, it was discovered that NO_2^- is converted to NO when proper environmental conditions are present. This conversion occurs rapidly in hypoxic (27, 95) and acidic (77) conditions. Hemoglobin (26, 27, 52) and myoglobin (52, 95), the primary O_2 transporting molecules in mammals, facilitate this process. These considerations make the contracting muscle a prime location for this conversion to take place. It is possible that this pathway evolved to offset the reduced NO availability (via reduced NOS function) imparted by exercise.

Nitrate – NO_2^- – NO

Nitrate (NO_3^-) is present in substantial concentrations in many green leafy and root vegetables (e.g. spinach, arugula, lettuce, and beets) (74). Unfortunately, mammals lack the pathways to directly make use of this higher form of NO. Commensal anaerobic bacteria present in the oral cavity, however, maintain these functions. When NO_3^- is consumed, some will be converted to NO_2^- while in the mouth, but a majority will be swallowed and proceed to the stomach. Once in the digestive tract, NO_3^- is readily absorbed into the blood stream and begins circulating the body. The salivary glands attract and concentrate the NO_3^- allowing the bacteria sufficient time to facilitate the conversion to NO_2^- (75). The discovery of this pathway introduced the possibility of dietary nitrate supplementation to be used as a therapeutic and ergogenic agent.

Actions of NO_2^- in the body

The discovery of NO and its critical importance as a signaling molecule in the cardiovascular system led to the award of the 1998 Nobel Prize in physiology or medicine to Louis Ignarro, Ferid Murad, and Robert F. Furchgott. NO has many targeted areas of effect within the body and these have been reviewed in detail elsewhere (see (91)). The effects of particular interest to this study are NO's actions as a potent vasodilator and modulator of metabolic efficiency. With NO_2^- used to increase the opportunity for NO formation, many studies have investigated these vasodilatory and efficiency effects *in vitro* and *in vivo*.

One of the first studies to investigate the vasodilatory properties of NO_2^- supplementation was performed by Modin and colleagues, (77). This study utilized isolated rat aortas exposed to physiological levels of NO_2^- in both neutral and acidic pH conditions. The relaxation was augmented by the lower pH conditions and was correlated with the NO release. Two years later, Cosby *et al*., (26) investigated the effects of NO_2^- *in vivo* with direct infusion into the brachial

arteries of human subjects at rest and during handgrip exercise. $\dot{Q}$ was significantly elevated during both rest and during exercise, and this augmentation remained when NOS function was inhibited by L-NMMA.

In addition to the vasoactive properties of NO_2^-, accumulating evidence suggests that mitochondrial and muscle functions are impacted. The direct impact of NO on mitochondrial respiration has been observed since the early 1990s, and appears that NO inhibits the O_2 binding sites of cytochrome c oxidase by directly competing with O_2. The net outcome of this competition is an enhanced efficiency resulting from reduced O_2 consumption of the mitochondria and maintained ATP production (24). These findings have been further supported with the addition of NO_2^- and deoxygenated myoglobin to the mitochondrial solution (88). These findings inspired the work of Larsen and colleagues (72), which tested the effects of a dietary NO_3^- supplement on whole body O_2 consumption during cycling exercise. This study was the first to reveal that a dietary supplement could reduce the $\dot{V}O_2$ associated with a given work rate in humans. Subsequent investigation by Larsen *et al*., (71) revealed this increase in mitochondrial efficiency was attributable to a reduced proton leak across the inner mitochondrial membrane. In 2012, Hernandez and colleagues (50) showed that dietary NO_3^- supplementation increased fast-twitch muscle force production through effects on calcium handling and sensitivity.

Effects of beetroot supplementation

Control of $\dot{Q}$

A huge burst of investigations utilizing beetroot (BR) supplementation has happened over the last decade. This "hot topic" of research was triggered by the work of Larsen and colleagues (72) paired with the high nitrate concentration found in BR (74). It has been demonstrated that

BR supplementation augments $\dot{Q}$ to the working muscle. This was first experimentally investigated in rats during submaximal treadmill running (37). These authors found that BR supplementation resulted in an increased $\dot{Q}$ to the hindlimb, despite a lower exercising mean arterial pressure (MAP). These findings conflict with the reduced $\dot{V}O_2$ shown with BR supplementation in other studies, as $\dot{V}O_2$ and $\dot{Q}$ generally increase in proportion to one another across a range of exercise intensities (1, 84), and may reveal a dissociation of this relationship induced by BR. In a follow up study, Ferguson and colleagues (36) repeated the previous experiment using both a low (0.3 $mmol \cdot kg^{-1} \cdot day^{-1}$) and high (1 $mmol \cdot kg^{-1} \cdot day^{-1}$) dose of BR and found no difference in $\dot{Q}$ or microvascular partial pressure of O_2 ($P_{mv}O_2$) with the low dose. Importantly, the high dose yielded similar findings to the original study with an increased $\dot{Q}$, while further providing evidence of increased $P_{mv}O_2$. Recently the effect on $\dot{Q}$ was investigated in human subjects (19, 62), but no change in brachial artery blood flow ($\dot{Q}_{BA}$) was found in healthy, young men and women during moderate intensity single handed handgrip exercise, consistent with the findings of low dose BR in rats.

O_2 cost during exercise

It has been demonstrated that BR supplementation will reduce $\dot{V}O_2$ in a manner not unlike that shown by Larsen *et al*., (72). Bailey and colleagues (7) were the first to show this. These authors showed that BR supplementation reduced the $\dot{V}O_2$ during moderate intensity exercise, but this reduction was not apparent when exercise was performed in the severe domain. Subsequent studies utilizing BR supplementation have yielded mixed results across a variety of exercise modalities, with some showing modest reductions in $\dot{V}O_2$ (4, 70, 100, 105), and others no change (13, 21, 59, 60) after supplementation. Initial evidence appeared to suggest that BR had the greatest effect during moderate intensity exercise and/or in untrained subjects (4, 7, 105),

but again this finding has not been universally observed (69, 70, 102). NIRS-derived variables have been measured concurrently with $\dot{V}O_2$ in two studies to date (7, 13). Deoxy-[Hb + Mb], which represents the microvascular matching of $\dot{V}O_2$ to $\dot{Q}$, paralleled the change in $\dot{V}O_2$ when it occurred (7, 13).

The findings covered in this review highlight the value of BR supplementation as both an ergogenic and therapeutic aid. However, the studies performed over the last decade have shown variance in outcomes, which highlights the need for a greater understanding of the underlying physiological effects of BR. This supplement can be prescribed with greater efficacy once the specific physiological, environmental, and exercise conditions that provoke the greatest effect of the supplement are better understood.

Chapter 3 - Methods

Subjects

Seven healthy, recreationally active men volunteered for this investigation (mean ± SD: age: 25 ± 3 yrs; height: 179 ± 4 cm; body mass: 82 ± 9 kg). All experimental procedures in the present study were approved by the Institutional Review Board at Kansas State University and conformed to the standards set forth by the *Declaration of Helsinki*. Prior to participation in the study, all subjects were informed of the protocol, any possible health risks, as well as the probable benefits of the study. All subjects provided written informed consent to participate and completed a medical health history questionnaire to ensure absence of any known cardiovascular or metabolic diseases which would preclude them from the study.

Experimental Protocol

All testing sessions were performed on a custom-built, two-handed handgrip ergometer previously described by Broxterman *et al*. (15). Briefly, the subjects were seated in an upright position at arm's length from the ergometer with the hands at heart level and directly in front of their torso. All sessions were performed utilizing a 50% duty-cycle (1.5 s contraction, 1.5 s relaxation) that was maintained via audio cues. All subjects were familiarized with the exercise, audio cues, and duty-cycle prior to the first testing session. During the first visit, subjects performed an incremental test for the determination of peak power (P_{peak}) starting at 1 Watt (W) and increasing at 0.5 $W \cdot min^{-1}$. The test was performed until volitional exhaustion or after three consecutive contraction cycles in which the subject was unable to maintain the correct tempo or complete full contractions. P_{peak} was recorded as the highest power obtained in which the subjects completed at least 30 s of the stage.

The four subsequent visits were randomly assigned to 40 or 85 $\%P_{peak}$ and performed for 10 min or until exhaustion, respectively. All testing sessions were separated by at least 48 h and subjects were asked to abstain from vigorous activity, food, and caffeine prior to testing for 12, 3, and 2 h, respectively. Upon arrival to the laboratory, the subjects sat quietly for 10 min, after which resting blood pressure measurements and subsequent plasma samples were obtained. All exercise tests were performed at approximately the same time of day (± 1.5 h for each subject) between 1100 and 1500 hours.

Supplementation

The four exercise testing sessions were randomly assigned to either BR or placebo (PL) supplementation conditions, creating a randomized, double-blind, crossover study design. In each condition, the subjects consumed BR concentrate (2 x 70 ml providing ~13 mmol NO_3^-) or nitrate-depleted beetroot juice concentrate PL (2 x 70 ml providing ~0.006 mmol NO_3^-; both from Beet it, James White Drinks, Ipswich, UK). Subjects consumed the shots ~2.5 h before testing began to allow for maximal expression of plasma NO_3^- concentrations ($[NO_3^-]$) (101). During the study, subjects were asked to abstain from using mouthwash (44) and toothpaste or chewing gum that contained triclosan, as these products serve to reduce the oral bacteria needed to facilitate the conversion of NO_3^- to NO_2^-. Each exercise testing session was separated from the others by at least 48 h to allow plasma $[NO_3^-]$ adequate time to return to pre-supplementation concentrations (105). Subjects were asked to maintain their normal diet with the exception of limiting foods high in NO_3^-, such as spinach and arugula (74). No subjects reported taking any multivitamins or anti-oxidant supplements.

Measurements

Venous blood samples (5-6 ml) were separated into 1.5 ml Eppendorf tubes containing 5 µl heparin (concentration 1000U/ml) and centrifuged at 3250 rpm at 4 °C for 5 min. Plasma samples were removed into separate tubes, flash frozen in liquid nitrogen, and stored at -80 °C until later analysis.

The measurements of plasma NO_3^- and NO_2^- were performed within 30 min of thawing via chemiluminescence with a NO analyzer (NOA 280i, Sievers Instruments, Boulder, CO, USA). In order to obtain plasma NO_2^- levels and to avoid potential reduction of NO_3^-, potassium iodide in acetic acid was used as a reductant. This reductant has the ability to reduce NO_2^- to NO but is incapable of reducing higher oxides of nitrogen (i.e., NO_3^-), thus increasing the specificity for NO_2^-. Plasma NO_3^- concentrations were obtained using the same apparatus with the stronger reductant vanadium chloride in hydrochloric acid at a temperature of 95 °C. This stronger reductant reduces the sum of all nitrogen oxides with an oxidation state of +2 or higher, which is predominately NO_3^- (µM), but also includes both NO_2^- (nM) and nitrosothiols (nM).

Blood pressure was measured in the left ankle using an automated patient monitor (S/5 Light Monitor type F-LM1-03, Datex-Ohmeda General Electric, Findland) which makes use of the oscillometric technique. To increase accuracy, the machine utilizes a 3-lead ECG to monitor heart rate (HR). During the measurement, subjects were asked remain still and allow their leg to relax. A correction factor (pressure = measured pressure – (distance between the heart and ankle in meters x 76 mm Hg) was used to adjust for the increased hydrostatic pressure present between the ankle and heart (42). Pilot work performed in our lab validated the correction factor with measurements taken from the ankle and arm at heart level while the subjects sat at the ergometer.

The raw blood velocity profiles were measured in the right brachial artery using Doppler ultrasound (Vivid 3, GE Medical Systems, Milwaukee, WI, USA) operating in pulse wave mode

at a Doppler frequency of 4.0 MHz with a phased linear array transducer probe operating at an imaging frequency of 6.7 MHz, and were stored for *post-hoc* analysis. For all testing sessions the Doppler gate was set to the full width of the brachial artery to ensure complete insonation and all Doppler velocity measurements were corrected for the angle of insonation, which was adjusted to be less than 60°. Measurements were made at least 3 cm above the antecubital fossa to avoid bifurcation of the brachial artery. Brachial artery diameters were measured in the transverse axis using two-dimensional sonography.

Muscle and microvascular oxygenation status were measured noninvasively using a frequency-domain multi-distance near infrared spectroscopy (NIRS) system (OxiplexTS, ISS, Champaign, IL, USA) positioned over the belly of the left *flexor digitorum superficialis* (FDS). Details of this technique have been described previously (15, 17). Briefly, this device consists of one detector fiber bundle and eight light-emitting diodes (LED) operating at wavelengths of 690 and 830 nm (four LEDs per wavelength). The LED-detector fiber bundle separation distances are 2.0, 2.5, 3.0, and 3.5 cm. This NIRS device measures and incorporates the reduced scattering coefficient (μ_s'), measured dynamically, to provide absolute concentrations (μM) for deoxy-[Hb + Mb] and total-[Hb + Mb]. The NIRS probe was calibrated prior to each test according to the manufacturer's specifications. The belly of the FDS of the left arm was identified using palpation and EMG. The NIRS probe was secured along the belly of the FDS and was wrapped with an elastic bandage to prevent shifting of the probe. The placement of the NIRS probe was marked with permanent ink for reproducible positioning throughout the study. The NIRS data were collected at 50 Hz and stored for *post-hoc* analysis.

The $\dot{V}O_2$ (ml $O_2 \cdot min^{-1}$) of the FDS was estimated for each minute of exercise using the technique described previously (15), which integrates deoxy-[Hb + Mb] and $\dot{Q}_{BA}$. It was

assumed that the deoxy-[Hb + Mb] signal reflects exclusively deoxy-[Hb] [we acknowledge that the signal contains deoxy-[Mb] as well (28)] and that the entire signal arises only from the muscle (i.e., not from any interposing adipose or skin tissue). With these assumptions the deoxy-[Hb] may be converted into an estimated $\dot{V}O_2$. The deoxy-[Hb] values are in units of μmole heme/*l* tissue, where the tissue is assumed to be muscle. These deoxy-[Hb] units can be converted into μmole heme/*l* blood using the conversion 1.36% capillary blood volume/muscle volume [derived from 400 cap/mm^2, 28.3 μm^2 CSA, and a coefficient of 1.2 correcting for tortuosity and branching of the capillaries (85)]. These units can then be converted into mole O_2/*l* blood assuming 1 mole O_2/mole heme and further to *l* O_2/*l* blood using the conversion 22.4 *l* O_2/mole O_2. $\dot{V}O_2$ values in *l* O_2/min may then be obtained by multiplying this value by the measured $\dot{Q}_{BA}$ values.

Data analysis

Mean blood velocity ($\dot{V}_{mean}$; cm·s^{-1}) was defined as the time-averaged mean velocity over each 3 s contraction cycle. $\dot{Q}_{BA}$ (ml·min^{-1}) was calculated using the product of $\dot{V}_{mean}$ and vessel cross-sectional area (CSA = πr^2). CSA (cm^2) was calculated each minute of exercise using brachial artery diameters measured at the beginning of each minute. The $\dot{Q}_{BA}$ data were analyzed using three consecutive contraction cycles (i.e., 9 s) for rest and at the end of each minute of exercise. The NIRS data were analyzed using 1 s mean values that were converted to 30 s mean bins for resting values and 9 s time-binned mean values at the end of each minute of exercise and at exhaustion. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured at least three times at rest and once every 2 min during exercise and were then used to calculate MAP. Vascular conductance (VC) (ml·min^{-1}·(100 mmHg)$^{-1}$) was calculated using the quotient of $\dot{Q}_{BA}$/MAP, multiplied by 100.

Kinetics analyses were conducted for the $\dot{Q}_{BA}$ and $\dot{V}O_2$ data during 85% P_{peak} using 6 s time-binned mean values over the initial 120 s of exercise and 9 s time-binned mean values at 180 and 240 s with a mono-exponential model:

$$y(t) = y(b) + A(1 - e^{-(t - TD)/\tau})$$

where $y(t)$ is the $\dot{Q}_{BA}$ or $\dot{V}O_2$ at any point in time, $y(b)$ is the appropriate baseline before the onset of exercise, A is the peak amplitude of the response, TD is the time delay proceeding the increase in, and τ is the time constant.

Statistical analysis

All curve fitting and statistical analyses were performed using a commercially available software package (SigmaPlot, Systat Software, San Jose, CA, USA). Differences in resting values, kinetic parameters, and T_{lim} were analyzed using Student's paired t-tests. Differences within condition (i.e., 40% BR and 85% BR) at rest were compared and if no differences were found, these values were averaged to represent the mean resting value for that condition. All exercising values were analyzed using two-way ANOVAs with repeated measures (condition x time) using Tukey's *post hoc* tests when main effects were detected. Differences were considered significant when $p \leq 0.05$. Data are presented as mean ± standard deviation unless otherwise noted.

Chapter 4 - Results

Plasma [NO_3^-] & [NO_2^-] and resting blood pressure

Plasma [NO_3^-] was elevated 26-fold over PL after acute BR supplementation (795 ± 76 vs 29 ± 5 μM, $p < 0.001$). All subjects demonstrated an elevated plasma [NO_2^-] after acute BR supplementation (469 ± 139 vs 69 ± 17 nM, $p < 0.001$, Fig. 1), resulting in a 5.8-fold increase over PL. Resting blood pressure values are presented in Table 1. SBP, DBP, and MAP were significantly reduced after acute BR supplementation by 8.4%, 7.7%, and 8.7%, respectively.

40 $\%P_{peak}$ exercise

The mean power for 40 $\%P_{peak}$ was 2.3 ± 0.3 W. All subjects were able to sustain 10 min of exercise at 40 $\%P_{peak}$ in both conditions. $\dot{Q}_{BA}$ increased rapidly from exercise onset in both conditions before approaching a steady-state of approximately 285 ml·min^{-1} by 240 s. $\dot{Q}_{BA}$ was not significantly different after BR supplementation at rest or at any time during exercise compared to PL (Fig. 2). There was no main effect for BR on MAP during exercise compared to PL ($p = 0.102$, Fig. 3), although MAP was lowered by 4.6 mmHg on average throughout exercise. There was no effect of BR on VC ($p = 0.323$, Fig. 3).

Deoxy-[Hb + Mb] increased following exercise onset in both conditions, with PL showing an overshoot that quickly transitioned to a lower steady-state by 120 s (Fig. 4). The overshoot with BR was significantly greater and extended through 120 s (47.4 ± 13.8 vs 42.1 ± 12.0 μM, $p = 0.014$) and 180 s (46.3 ± 14.1 vs 42.2 ± 13.5 μM, $p = 0.049$). However, end exercise deoxy-[Hb + Mb] was not different between BR and PL (43.3 ± 9.6 vs 42.4 ± 15.1 μM, $p = 0.649$). Total-[Hb + Mb] was not significantly different after BR supplementation. The estimated $\dot{V}O_2$ was not significantly different between BR and PL at any min during exercise or at the end of exercise (21.8 ± 12.2 vs 22.4 ± 14.2 ml O_2·min^{-1}, Fig. 6).

85 %P_{peak} exercise

The mean power for 85 %P_{peak} was 4.8 ± 0.5 W. BR had no significant effect on T_{lim} compared to PL (342 ± 83 vs 382 ± 138 s, p = 0.382, Fig. 5). $\dot{Q}_{BA}$ was not significantly different at rest or any time during exercise after BR supplementation. $\dot{Q}_{BA}$ increased at exercise onset and attained end exercise values that were not significantly different between BR and PL (369 ± 155 vs 391 ± 135 ml·min^{-1}, p = 0.341, Fig. 2).

Deoxy-[Hb + Mb] increased at exercise onset in both conditions, with BR significantly elevated over PL for all common time points preceding end exercise (60 – 240 s, $p < 0.05$). At end exercise, BR and PL were not statistically different (54.4 ± 19.7 vs 49.1 ± 14.5 μM, p = 0.07, Fig. 4). Total-[Hb + Mb] was not significantly different after BR supplementation, both conditions showing a progressive increase toward the end exercise values. There was no difference for estimated end-exercise $\dot{V}O_2$ after BR supplementation (31.0 ± 10.4 vs 30.7 ± 10.9 ml O_2·min^{-1}, Fig. 6).

Kinetics analyses

The results of the kinetics analyses are presented in Table 2. Kinetics analysis of $\dot{V}O_2$ was conducted with 5 subjects, as two of the subjects exhibited a response to the onset of exercise (in opposite supplementations) which was atypical and determined to be an outlier (≥ 4 SDs from the mean). BR had no significant effect on Amp, τ, or TD for $\dot{Q}_{BA}$ or $\dot{V}O_2$ following exercise onset. Although no significant differences were detected for the Amp (p = 0.06) or τ (p = 0.196) of the $\dot{V}O_2$ response, large effect sizes of 1.14 and 0.7, respectively, were found.

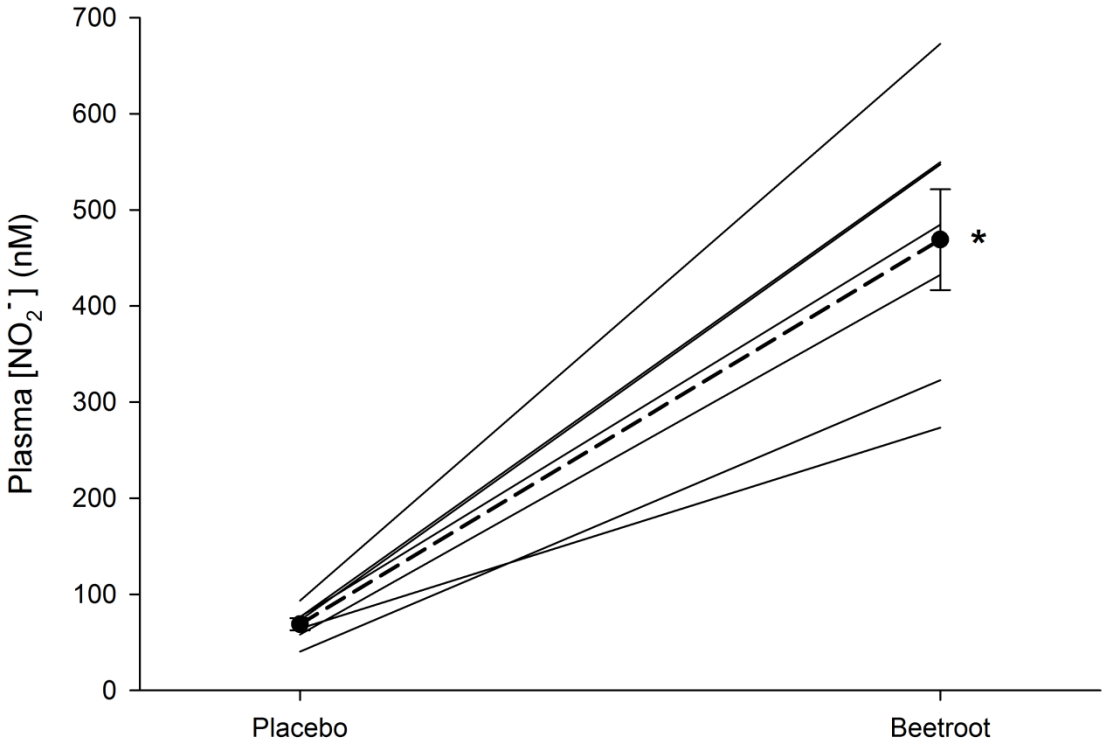

Figure 1. Resting nitrite concentrations

Plasma nitrite concentration ($[NO_2^-]$) for each individual subject (solid lines) and group mean (dashed line). Error bars represent SE. *****, significantly different from placebo ($p < 0.001$).

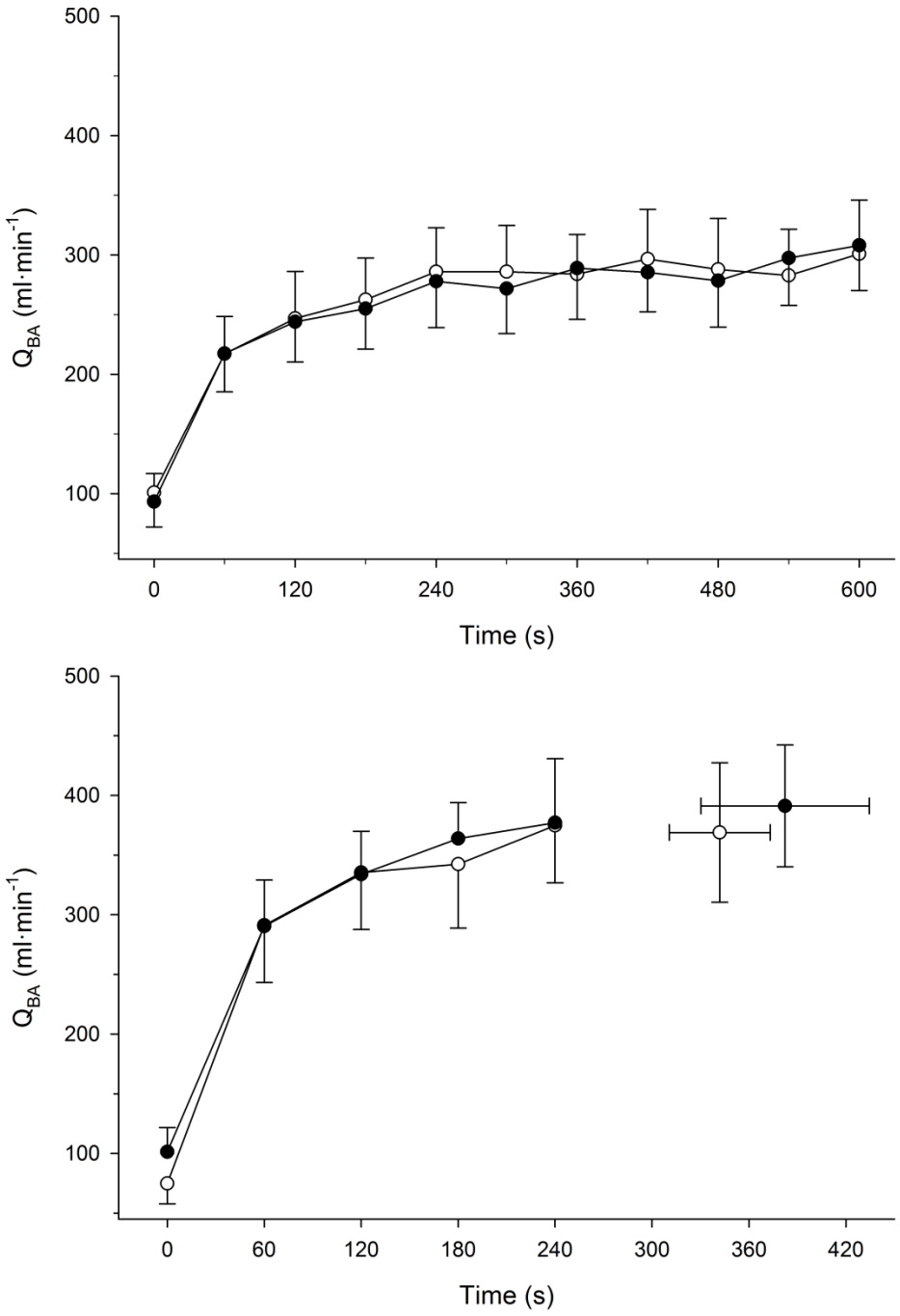

Figure 2. Brachial artery blood flow during exercise

Top: Mean brachial artery blood flow ($\dot{Q}_{BA}$) at the end of each minute of 40 $\%P_{peak}$ exercise. *Below*: Mean $\dot{Q}_{BA}$ at the end of each minute of 85 $\%P_{peak}$ exercise and the limit of exercise tolerance (T_{lim}). In both graphs, filled circles represent placebo and open circles represent beetroot supplementation. Error bars represent SE.

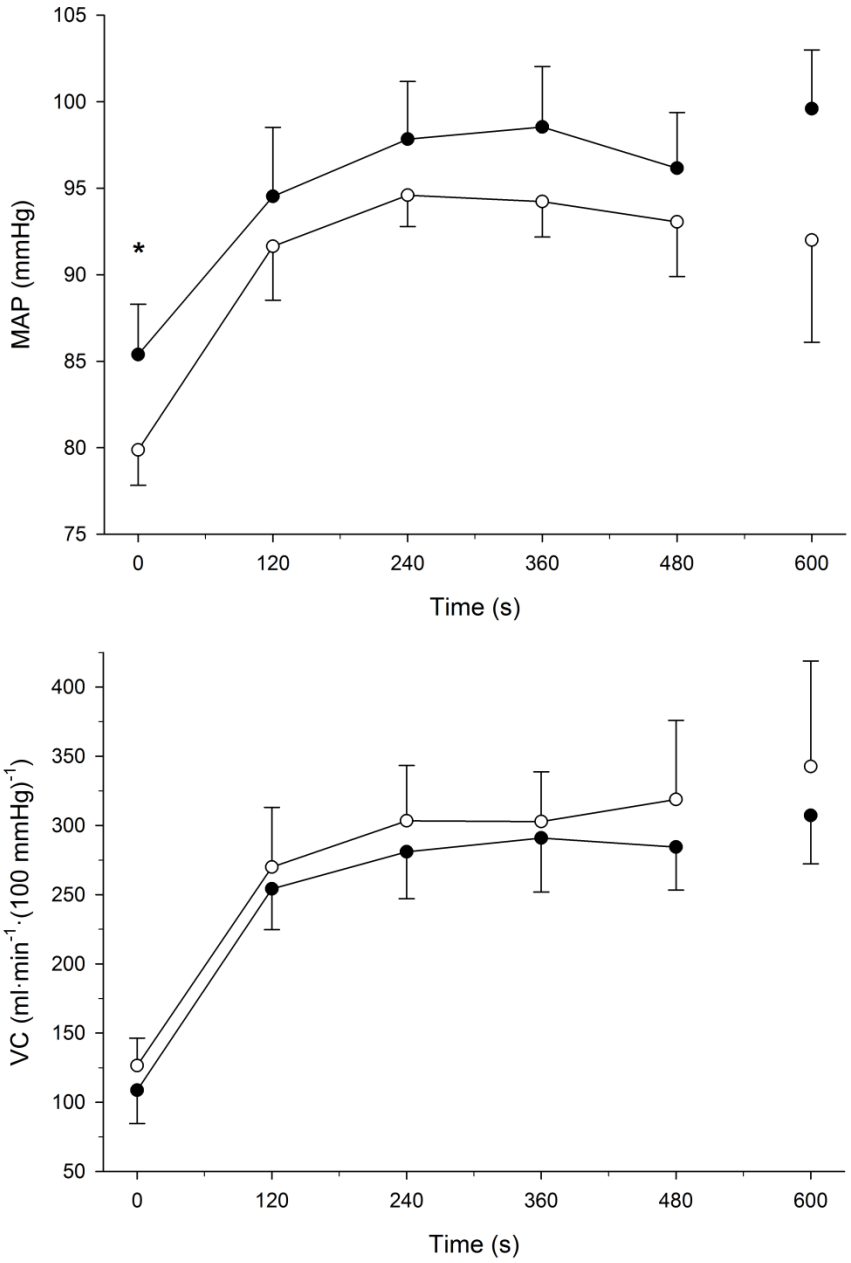

Figure 3. Mean blood pressure and vascular conductance responses to 40 %P_{peak} exercise
Top: Mean arterial pressure (MAP) taken every 120 s during exercise. *Below*: Vascular conductance (VC) calculated as the product of brachial artery blood flow and MAP every 120 s during exercise. In both graphs, filled circles represent placebo and open circles represent beetroot supplementation. Error bars represent SE. *****, significantly different from placebo ($p < 0.05$). The data point for 600 s represents the mean value in the absence of one different individual in each condition (i.e., a mismatched n = 6).

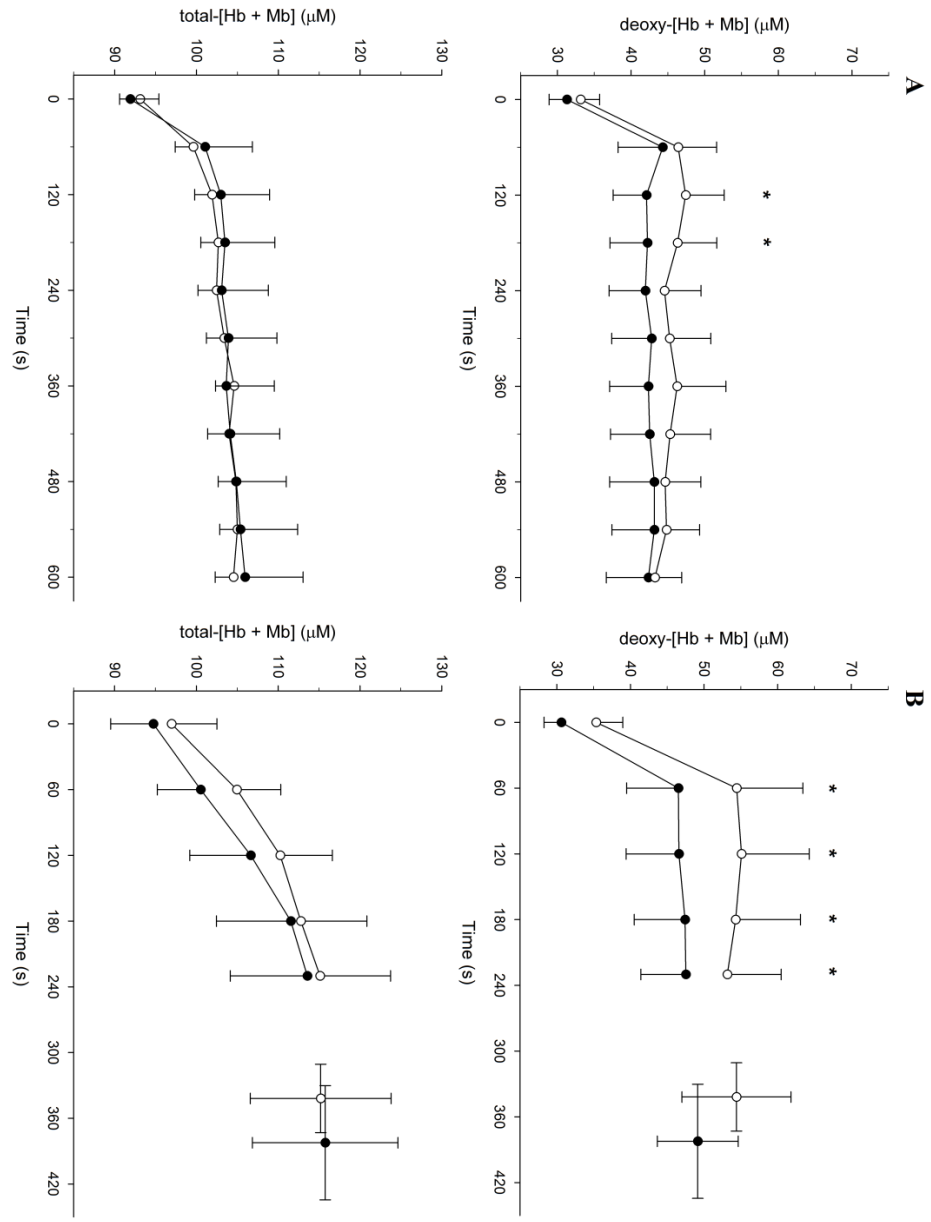

Figure 4. NIRS-derived muscle and microvascular oxygenation responses during exercise A: 40 %P_{peak} exercise *Top*: Mean deoxygenated-[hemoglobin + myoglobin] (deoxy-[Hb + Mb]) at the end of each minute of exercise. *Below*: Mean total-[hemoglobin + myoglobin] (total-[Hb + Mb]) at the end of each minute of exercise. **B: 85 %P_{peak} exercise** *Top*: Mean deoxy-[Hb + Mb] at the end of each minute of exercise and at the limit of exercise tolerance (T_{lim}). *Below*: Mean total-[Hb + Mb] at the end of each minute of exercise and at T_{lim}. In all graphs, filled circles represent placebo and open circles represent beetroot supplementation. Error bars represent SE. *****, significantly different from placebo ($p < 0.05$).

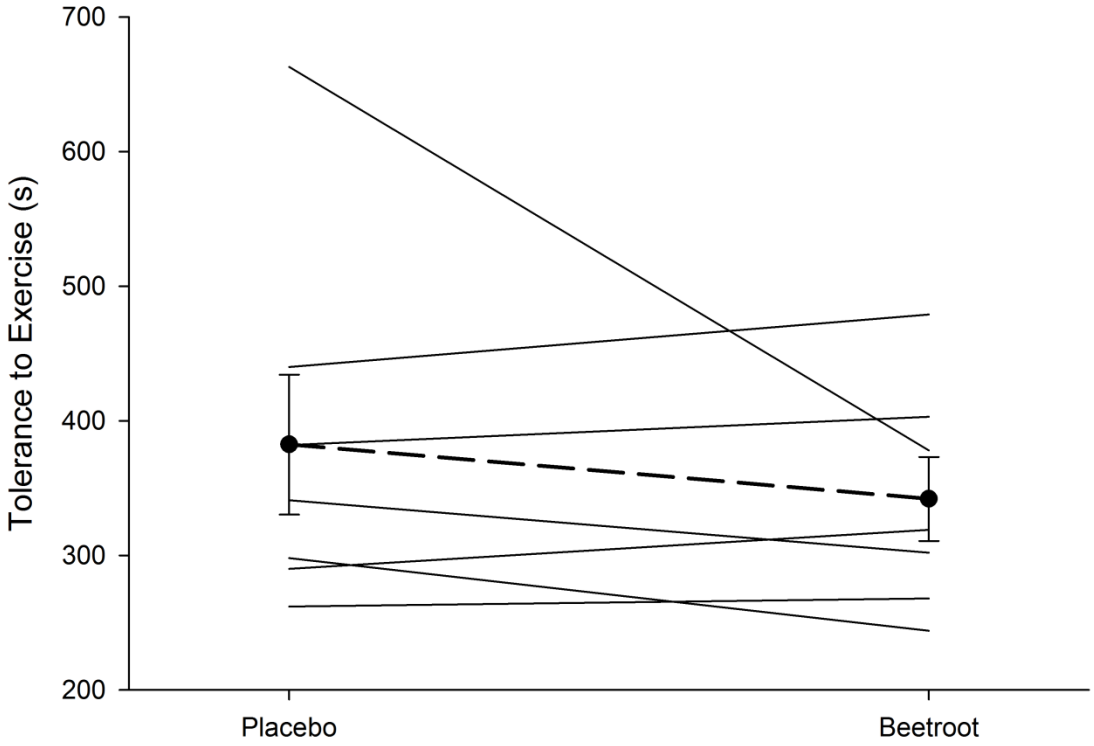

Figure 5. Effect of supplementation on tolerance to exercise

Individual (solid lines) and mean (dashed line) tolerance to exercise (T_{lim}) responses under both supplementations during 85 $\%P_{peak}$ exercise. Error bars represent SE.

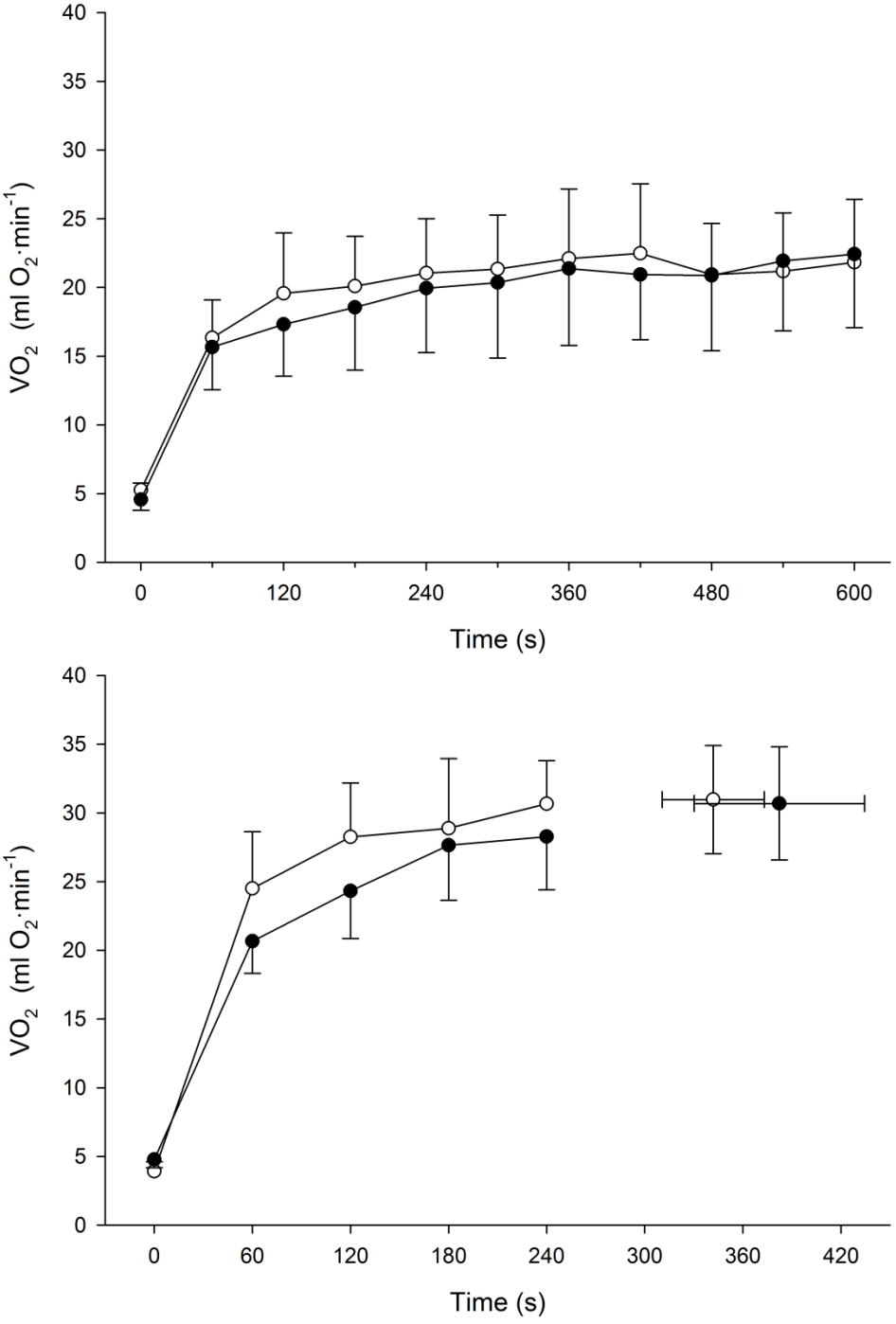

Figure 6. Estimated $\dot{V}O_2$ during exercise

Top: Mean estimated $\dot{V}O_2$ at the end of each minute of 40 $\%P_{peak}$ exercise. *Below*: Mean estimated $\dot{V}O_2$ at the end of each minute of 85 $\%P_{peak}$ exercise and at the limit of exercise tolerance (T_{lim}). Error bars represent SE.

Table 1. Resting blood pressure

	Placebo	Beetroot
SBP (mmHg)	133 ± 7	122 ± 8 ǂ
DBP (mmHg)	64 ± 8	59 ± 7 *
MAP (mmHg)	87 ± 7	79 ± 6 ǂ

SBP, DBP, and MAP denote systolic blood pressure, diastolic blood pressure, and mean arterial pressure, respectively. Values are expressed as mean ± SD

ǂ, significantly different from placebo ($p < 0.01$) *, significantly different from placebo ($p < 0.05$)

Table 2. Kinetics parameters

$\dot{Q}_{BA}$	Placebo	Beetroot
Amp ($ml \cdot min^{-1}$)	258 ± 53	278 ± 81
τ (s)	46 ± 43	43 ± 26
TD (s)	4 ± 5	3 ± 4
$\dot{V}O_2$		
Amp ($ml\ O_2 \cdot min^{-1}$)	21 ± 9	29 ± 14
τ (s)	38 ± 14	31 ± 8
TD (s)	1 ± 2	4 ± 4

Amp, τ, and TD denote the primary amplitude, time constant, and time delay, respectively. Values are expressed as mean ± SD

Chapter 5 - Discussion

The present study investigated the effects of acute BR supplementation on conduit artery $\dot{Q}$ concurrently with muscle and microvascular oxygenation characteristics during moderate and severe intensity handgrip exercise. The acute dosage utilized (~13 mmol NO_3^-), elevated plasma [NO_2^-] more than 5-fold higher than that seen with placebo and resulted in significant reductions in blood pressure at rest. In agreement with our first hypothesis, and previous findings (19, 62), BR had no significant effect on $\dot{Q}_{BA}$ at rest or any time point during moderate or severe intensity handgrip exercise compared to PL. The primary novel finding of the present study, in contrast to our second hypothesis, was that the $\dot{V}O_2$ was not significantly different after BR during moderate or severe intensity handgrip exercise. Additionally, BR had no significant effect on T_{lim} when exercise was performed in the severe intensity domain.

Protocol advantages

Handgrip exercise provides several advantages over commonly used whole body exercise modes (i.e., cycling and running) considering the measurements that can be made. The similar fiber type composition of the FDS and *vastus lateralis* (VL) (40, 55, 94) allows translation of metabolic findings between the two. With whole body exercise, measurement of $\dot{Q}$ (via ultrasound) through the conduit (femoral) artery is obstructed due to the dynamic movement of the legs and requires the use of invasive techniques such as thermodilution. Conversely, $\dot{Q}$ can be noninvasively and accurately measured from rest to maximal effort during handgrip exercise. Similarly, the use of NIRS to measure the oxygenation of the VL and other leg muscles is complicated by the presence of a sizable layer of adipose tissue (38) which thus limits penetration of the light into only the superficial portions of the muscles. To further complicate the interpretation of these signals in the thigh musculature, a distinct spatial heterogeneity of

deoxy-[Hb + Mb] has been observed during cycling exercise (67). Handgrip exercise allows for the use of NIRS on the active muscle with a reduced relative contamination of the adiposity to the overall signal and for the interrogation of the entire active muscle, both of which enable a more comprehensive description of the underlying physiology.

Effect on plasma [NO_2^-] and blood pressure

An acute dose of BR resulted in a 581% increase in plasma [NO_2^-] 2.5 hours after consumption, supporting the accumulating evidence that both acute and chronic BR supplementation can significantly increase the plasma concentrations of NO_3^- and NO_2^- in both healthy and patient populations (61, 100, 101, 105, 106) and animal preparations (36, 37). Often, although not ubiquitously, the increase in plasma [NO_2^-] is accompanied by a significant reduction in SBP, DBP, and/or MAP (for review see (51)). The present study showed an 8% decrease in all three variables at rest and a non-significant decrease in MAP during exercise, reminiscent of the significant reduction measured in exercising rats (37). Although the effects during exercise may have been underpowered in the present study, the effect of BR supplementation on blood pressure regulation suggests that BR may be valuable to acutely and chronically (at least 15 days (100)) control moderate hypertension.

Effect on control of blood flow

The discovery of NO and its critical importance as a signaling molecule in the cardiovascular system, coupled with the demonstration that dietary NO_3^- is reduced to NO_2^- and then NO (75), inspired the hypothesis that BR would increase $\dot{Q}$, particularly in hypoxic and acidic tissues such as contracting muscle. Before this was directly tested, Cosby and colleagues (26) performed a seminal study that investigated the effect of a direct injection of inorganic NO_2^- into the forearm vasculature of man, and found the injection increased $\dot{Q}_{BA}$ during both rest and

exercise. Later studies investigated the effect of BR on exercise performance and muscle oxygenation utilizing NIRS and found deoxy-[Hb + Mb] to be reduced, which was attributed to increased blood volume associated with vasodilation (7, 61). Subsequently, Ferguson and colleagues (36, 37) discovered that BR supplementation increased hindlimb $\dot{Q}$, and presumably microvascular red blood cell content (63), in a fiber type specific manner in rats.

To date, the previous studies (19, 62) and the present investigation that directly measured $\dot{Q}$ in humans, have be unable to replicate the findings of Ferguson *et al*. (36, 37) or Cosby *et al*. (26) after BR supplementation. It must be noted that the dose of BR given to the rats was much greater than that typically administered to humans and was dispersed chronically over 5 days. Further, the largest effect was seen in muscles composed of a high percentage of type IIb and IIx fibers. The dose administered was designed to account for the 7-fold higher resting $\dot{V}O_2$ that rats exhibit compared to humans (80). To match this dosing, the subjects in the present study would need to consume approximately 13 servings (~85 mmol NO_3^-) of the supplement utilized. This may be excessive because the plasma [NO_3^-] in the present investigation exceeded that of the high dose rats by 500% (36). The plasma [NO_2^-] was 22% lower than that in the high dose rats, suggesting possible differences in the conversion process between humans and rats, differences in the site of intravascular storage of NO_2^- (30), or an effect emerging as a consequence of the chronic supplementation. Although human plasma [NO_2^-] appears steady over 5 days of supplementation (100), there is evidence for intracellular changes to protein expression and mitochondria with long-term NO_2^- exposure (16, 23). How these alterations might function to increase $\dot{Q}$ during exercise is currently unclear.

Although data are sparse, the fiber type composition of the human forearm flexor muscles is closely matched to the composition of the VL with a distribution of 50-60% type II fibers (40,

55, 94). This distribution is quite disparate from the hindlimb of the rat, which is composed of >90% mixed type II fibers (3). Nevertheless, a recent study found that BR increased peak cardiac output and $\dot{V}O_2$ in CHF patients during a supine maximal incremental exercise test (106). However, that study was not designed to resolve the spatial distribution of the ~10% increase in cardiac output. If BR does favorably affect VC and $\dot{Q}$ to type II fibers, as suggested by Ferguson and colleagues (37), the increased proportion of type II fibers with ageing, CHF, and other diseases (43, 93) supports the notion that BR supplementation may be more effective in these populations. The absence of an effect during severe intensity exercise in the present study, when type II fibers are thought to be recruited more heavily, suggests a more complex relationship between plasma NO_2^- and vascular control than presently appreciated. However, any differences in control of the circulation between upper and lower limbs may exacerbate the complexity of this relationship.

Effect on tissue oxygenation and estimated $\dot{V}O_2$

Larsen *et al*. (72) were the first to show that a dietary NO_3^- supplement could reduce the $\dot{V}O_2$ associated with a given work rate. Subsequent studies utilizing BR supplementation have yielded mixed results across a variety of exercise modalities, with some showing modest reductions in $\dot{V}O_2$ (4, 7, 70, 100, 105), and others no change (13, 21, 59, 60) after supplementation. Initial evidence appeared to suggest that BR had the greatest effect during moderate intensity exercise and/or in untrained subjects (4, 7, 105), but again this finding has not been universally observed (69, 70, 102). NIRS-derived variables have been measured concurrently with $\dot{V}O_2$ in two studies to date (7, 13). Deoxy-[Hb + Mb], which represents the microvascular matching of $\dot{V}O_2$ to $\dot{Q}$, paralleled the change in $\dot{V}O_2$ when it occurred (7, 13).

Given the above, it was surprising that, in the present investigation, deoxy-[Hb + Mb] was elevated significantly after BR supplementation at min 2 and 3 during moderate exercise and throughout severe intensity exercise. Moreover, the total-[Hb + Mb] during both exercise bouts was not significantly impacted by BR supplementation. Changes in total-[Hb + Mb] from rest to exercise are thought to reflect the change in microvascular hematocrit (28), in that any change from rest to exercise would represent a net change in Hb concentration associated with red cells rather than a change in concentration of Mb, which presumably is fixed. To the best of our knowledge, the current study is the first to observe an increased deoxy-[Hb + Mb] after BR supplementation. When the increased deoxy-[Hb + Mb] is considered in relation to the unchanged total-[Hb + Mb] (and $\dot{Q}_{BA}$), it would suggest an increased fractional O_2 extraction for the same work being performed. However, there was no difference between the estimated $\dot{V}O_2$ after BR supplementation in the present study during exercise. It has been shown that duty-cycles ≥ 50% mechanically constrain $\dot{Q}_{BA}$ and $\dot{V}O_2$ during handgrip exercise (15, 99). This duty-cycle imposed mechanical limitation may mask any effect of BR supplementation.

Effect on kinetics parameters

No differences in the absolute amplitude of $\dot{Q}_{BA}$ or $\dot{V}O_2$ were found in the present study, but kinetics analyses revealed large effect sizes for the $\dot{V}O_2$ amplitude and time constant after BR supplementation. The primary amplitude of $\dot{V}O_2$ over the first 240 s of exercise at 85% P_{peak} approached a significant increase, before reaching similar end exercise values. These findings are in agreement with the speeding of pulmonary $\dot{V}O_2$ kinetics shown during whole body exercise (6, 13, 59) and the equivalent $P_{mv}O_2$ response discovered in rats (36). The ability to dissociate the effects of BR by implementing slow pedal cadence (and presumably high duty-cycle) reported

by Bailey and colleagues (6) may provide insight to the inconsistent findings of the efficacy of BR, in this regard.

Limitations

An acute bolus of BR was utilized herein, which showed similar effects on blood pressure regulation as seen with chronic supplementations. Chronic supplementation of BR augments $\dot{Q}$ in rats (36, 37); however, in agreement with previous studies in young adults (19, 62), an acute dose in humans has not yielded the same outcome. As such, future research might usefully employ chronic supplementation to determine if multiple days are required to observe this $\dot{Q}$ augmentation, possibly via protein modification (16). Similarly, exercise activating a larger muscle mass that still allows accurate measurement of $\dot{Q}$ (i.e., knee extension) would help to determine if the effect of BR is related in some fashion to the size of muscle mass recruited. It is feasible that handgrip exercise does not recruit a large enough group of muscles with a subsequently large enough bed of vasculature to impact upstream conduit $\dot{Q}$ measurements. Finally, it remains to be determined if the upper and lower body control $\dot{Q}$ and $\dot{V}O_2$ differently during exercise, and if differences that may exist are associated with dissimilar sensitivity to BR. We acknowledge that the method used to estimate $\dot{V}O_2$ herein utilizes several assumptions (15). We contend that these assumptions, held constant throughout, should not obscure an impact of BR on $\dot{V}O_2$.

Conclusions

The present study reaffirmed previous findings that an acute dose of BR is sufficient to lower SBP, DBP, and MAP in young adults, suggestive of its value as a method to control moderate hypertension and improve vascular health. However, the beneficial effects associated with BR supplementation during large muscle mass exercise were not seen when the

exercise was performed in small muscle mass handgrip exercise utilizing a 50% duty-cycle. These findings emphasize the limitations to the efficacy and utility of BR supplementation as it may have reduced effect during small muscle mass exercises. Similarly, the duty-cycle of a given exercise may largely attenuate any potential benefit imparted by BR supplementation as shown in the present study and the recent work by Bailey *et al*., (6). These factors, combined with the complex relationship between plasma NO_2^- and vascular control, may provide insight into the varying exercise performance outcomes of BR supplementation.

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