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**EFFECT OF ACUTE DIETARY NITRATE CONSUMPTION ON SUBMAXIMAL  
OXYGEN CONSUMPTION AND OXIDATIVE STRESS IN HYPOXIA**

**BY**

**COLIN R. CARRIKER**

**DISSERTATION**

Submitted in Partial Fulfillment of the  
Requirements for the Degree of

**Doctor of Philosophy**

**Physical Education, Sports and Exercise Sciences**

The University of New Mexico  
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**Effect of acute dietary nitrate consumption on submaximal oxygen consumption  
and oxidative stress in hypoxia**

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**ABSTRACT**

Reduced partial pressure of oxygen impairs exercise performance at altitude. Acute nitrate supplementation, at sea level may reduce oxygen cost during submaximal exercise in hypoxia. Therefore, we investigated the metabolic response during exercise at altitude following acute nitrate consumption. Ten well-trained ( $61.01 \pm 7.37$  ml/kg/min) males (age  $28 \pm 7$  yr) completed 3 experimental trials (T1, T2, T3). T1 included baseline demographics, a maximal aerobic capacity test ( $\dot{V}O_{2max}$ ) and five submaximal intensity cycling determination bouts at an elevation of 1600m. A 4-day dietary washout, minimizing consumption of nitrate rich foods, preceded T2 and T3. In a randomized, double-blind, placebo-controlled, crossover fashion, subjects consumed a placebo (PL) or nitrate rich (NR) beverage 2.5 hours prior to T2 and T3. Exercise at 3500m (T2 and T3) consisted of a 5-min warm-up (25%  $\dot{V}O_{2max}$ ) and four 5-min cycling bouts (40, 50, 60, 70% of  $\dot{V}O_{2max}$ ) each separated a 4-min rest period. Cycling RPM and watts for each submaximal bout were determined during T1. Pre-exercise plasma nitrite was elevated following NR consumption compared to PL ( $p < 0.05$ ). Oxygen consumption, respiratory exchange ratio, oxygen saturation, heart rate and rating of perceived exertion were not different at any submaximal intensity between NR and PL treatments. Blood lactate, however, was reduced following NR consumption compared to PL at 40 and 60% of  $\dot{V}O_{2max}$  ( $p < 0.05$ ). Following exercise, compared to rest, catalase and 8-isoprostane increased at 3500m in both the placebo and nitrate rich groups ( $p < 0.05$ ). There was no difference between groups (placebo vs nitrate rich) for either of these markers of oxidative stress. Our findings suggest that acute nitrate supplementation prior to exercise at 3500m does not reduce oxygen cost or oxidative stress, but may reduce lactate production at lower intensity workloads.

Keywords: beetroot juice, hypoxia, nitric oxide

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## **Chapter 1 Introduction to Study**

### **Introduction**

Initially, nitric oxide (NO) was deemed a potent vasodilator capable of increasing blood flow via relaxation of vascular endothelium (1). However, NO has also been implicated in a number of other functions such as improved or increased exercise induced glucose uptake (2), neurotransmission (3), immune response (4), regulation of mitochondrial respiration (5–7), and AMPK-mediated enhancement of glycolysis (8,9). The production of nitric oxide has been classified under two separate pathways: 1) the oxygen dependent L-arginine-nitric oxide pathway (10,11) and 2) the oxygen independent nitrate-nitrite-nitric oxide pathway (12–14). The oxygen dependent pathway occurs as nitric oxide and L-citrulline are synthesized from the oxidation of L-arginine. The oxygen independent pathway provides an important complement to the L-arginine-nitric oxide pathway especially under circumstances of hypoxia and/or impaired NOS availability (12,13,15,16). Moreover, during exercise, the nitrate-nitrite-nitric oxide pathway has been implicated in reduced oxygen cost and increased mitochondrial efficiency over a number of submaximal workloads (17–21). Exploitation of this pathway may provide potential ergogenic effects during endurance based activities.

### **NO production from Nitrate and Nitrite**

Nitrite ( $\text{NO}_2^-$ ) is both a product of endogenous NO oxidation and nitrate ( $\text{NO}_3^-$ ) reduction. Inorganic  $\text{NO}_3^-$  from dietary intake forms  $\text{NO}_2^-$  after interacting with facultative anaerobic bacteria in the mouth (22,23). Once swallowed,  $\text{NO}_2^-$  is converted to NO within the acidic environment of the stomach (24). This is contrary to original conclusions which postulated  $\text{NO}_2^-$  and  $\text{NO}_3^-$  were endogenously inert end products of



NO. It is now clear that  $\text{NO}_3^-$  and  $\text{NO}_2^-$  provide an auxiliary oxygen independent pathway for NO formation. This may be especially important under conditions of hypoxia (i.e. exercise) where the oxygen dependant L-arginine route may be limited by the decreased bioavailability of nitric oxide synthase (NOS), nicotinamide adenine dinucleotide phosphate (NADHP) flavin adenine dinucleotide (FAD) and other related co-factors (11).

Nitrate is absorbed directly from the gastrointestinal tract with plasma  $\text{NO}_3^-$  levels reaching their peak between 60 minutes (25) and 2.5 hours (26). After either sodium nitrate or inorganic  $\text{NO}_3^-$  ingestion, plasma concentration of  $\text{NO}_2^-$  increases (16, 26–28). Therefore, the initial reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  occurs in the mouth. As a result, if antibacterial mouthwash is administered prior to  $\text{NO}_3^-$  ingestion conversion of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  is attenuated. Antibacterial mouthwash abolishes commensal oral bacteria causing a decrement in nitrate reductase activity (30).

This oxygen independent pathway allows for NO production even during conditions of hypoxia, acidosis or other heavy exercise circumstances. Therefore, an increase in plasma  $\text{NO}_3^-$  and  $\text{NO}_2^-$  may increase NO production even when NOS and endothelial NOS (eNOS) expression are limited by hypoxia or related oxidative stress (31, 32). Therefore, dietary supplementation which elevates  $\text{NO}_2^-$  and  $\text{NO}_3^-$  plasma concentrations, increases NO syntheses over a wide range of exercise intensities as NO may be less impacted by down regulation of the L-arginine pathway during extreme exercise intensities or hypoxic conditions. Further, the alternative nitrate-nitrite-nitric oxide pathway may facilitate physical activity at altitude which is predicated by reduced partial pressure and a concomitant reliance on oxygen independent NO production (33).

### **High Altitude and Plasma Nitrite Concentration**

The plasma  $\text{NO}_2^-$  concentration of individuals residing at high altitude may be greater than values of those residing at or near sea level. Compared to residents at 206 m, Tibetans (residing at 4200m) show greater bioactive NO products of plasma and red blood cell  $\text{NO}_3^-$  as well as increased plasma  $\text{NO}_2^-$  (34). In addition, alongside greater forearm blood flow and lower vascular resistance, NO production was increased in highlanders compared to sea level residents (34). In the previously mentioned study, dietary intake was monitored and intake of dietary nitrate was “not at a level expected to significantly increase circulating nitrate or nitrite” (34). As a result of high altitude acclimatization, residents may experience an increase in plasma biomarkers of NO production ( $\text{NO}_3^-$  and  $\text{NO}_2^-$ ) as well as elevated cGMP. This may indicate that individuals acclimatized to high altitude may have increased NO activity (35). Reduction of circulating  $\text{NO}_2^-$  occurs as hypoxia increases (35). This decline in  $\text{NO}_2^-$  concentration may explain the importance of the oxygen independent conversion of plasma  $\text{NO}_2^-$  to NO.

Via allosteric  $\text{NO}_2^-$  reduction by hemoglobin,  $\text{NO}_2^-$  can also be converted to NO as evidenced by the formation of iron-nitrosylated hemoglobin. Forearm blood flow during exercise increased *in vivo*, following infusion of  $\text{NO}_2^-$  regardless of whether a nitric oxide synthase (NOS) inhibitor was present (36). Such findings highlight the complementary nature of the nitrate-nitrite-nitric oxide pathway when production of NO via NOS (i.e. conversion of L-arginine to NO and L-citrulline) is impaired. Interestingly, the maximal rate of NO conversion from  $\text{NO}_2^-$  (via deoxyhemoglobin) occurs when at 50% hemoglobin saturation (12). Shiva and colleagues (37) also reported that compared

to deoxyhemoglobin, deoxymyoglobin reduces  $\text{NO}_2^-$  to NO approximately 36 times faster.

### **Exercise Benefits (efficiency and performance)**

Nitrate supplementation via nitrate salts (sodium nitrate) or food sources such as fruits and vegetables (sources of inorganic nitrate) has been shown to increase both plasma  $\text{NO}_3^-$  and  $\text{NO}_2^-$  concentration in as little as 2 (27) or 3 (20, 21) days of loading. While loading protocols vary in duration from 2-6 days, acute supplementation within 2-3 hours of exercise has also demonstrated marked increases in both plasma  $\text{NO}_3^-$  and  $\text{NO}_2^-$  (28, 29, 33, 34). Such protocols generally initiated loading with approximately 4-6 mmol/day of  $\text{NO}_3^-$  although amounts as large as 16.8 mmol have been used (40).

The dietary nitrate induced increase in plasma nitrite allows for greater NO bioavailability via the nitrate-nitrite-nitric oxide pathway. While resting cyclic guanosine monophosphate (cGMP) is not altered with 3 days of sodium nitrate supplementation (0.1 mmol/kg/day), increased mitochondrial efficiency is present due to reduced proton leakage across the inner mitochondrial membrane; possibly due to reduced ADP/ATP translocase (ANT) protein expression (21). Mitochondrial efficiency is increased as the amount of oxygen consumed per ATP produced (P/O ratio) has been shown to increase after nitrate supplementation. Therefore, less proton leak occurs in the presence of higher maximal ATP synthesis rate (21).

In contrast to the thought that mitochondrial P/O ratio impacts oxygen cost after nitrate supplementation, reduced total ATP cost during muscle contraction has also been implicated as the cause of reduced oxygen cost (lower  $\dot{V}\text{O}_2$  for a given submaximal workload) during exercise (41). This theory posits that muscle force production requires

less total ATP as opposed to the converse theory suggesting the mitochondrial P/O ratio increases. To further support the reduced ATP cost theory, results have shown reduced PCr degradation in addition to reduced ADP and Pi accumulation. The authors (41) suggested if oxygen cost was reduced as a result of a change in mitochondrial efficiency, there would not have been differences between placebo and nitrate supplementation (beet juice) for the concentration of PCr and ADP accumulation during exercise.

While principle biochemical mechanisms require additional investigation, nitrate supplementation has been shown to reduce  $\dot{V}O_2$  over a number of submaximal exercise intensities (17, 19–21, 27–29, 37). Research has found nitrate supplementation improves performance during cycling-, rowing- and running-based activities (17, 34, 38–40), although, others have found no performance-based ergogenic effects post-nitrate supplementation (45–47).

### **Practical Application**

It is possible that oxygen cost is reduced under a number of submaximal workloads for both recreationally trained and elite athletes alike at sea level (17, 19–21, 27–29, 37). An increase in plasma nitrite,  $NO_2^-$ , occurs in response to nitrate supplementation via both nitrate salts ( $NaNO_3^-$ ) or whole foods high in nitrate content; such foods include: celery, cress, chervil, lettuce, red beetroot, spinach, and rocket (rucola) (48). The increased plasma nitrite content is thought to play a role in NO production; increased NO production has been associated with increased mitochondrial efficiency and/or reduced ATP cost during submaximal activity. As such, the ergogenic potential of nitrate supplementation has been established (17, 34, 38–40). Results from a

number of studies, however, provide conflicting evidence with regards to the performance benefits linked to nitrate supplementation.

Despite a documented reduction in oxygen cost, performance may not be affected in some individuals. The ergogenic qualities linked to dietary nitrate supplementation require further investigation for both aerobic and anaerobic activities. Further, a comparison between normoxic and hypoxic environments may better explain the contribution of the oxygen independent nitrate-nitrite-nitric oxide pathway during submaximal exercise.

The possibility exists that after a dietary nitrate loading period, individuals may actually fall into one of two distinct performance categories: responders and non-responders (36, 43). Responders were classified as those participants who exhibited an increase (>30%) in plasma  $\text{NO}_2^-$  following  $\text{NO}_3^-$  supplementation (47). Individuals who responded to supplementation, exhibited a 2% reduction in completion time of a 50 mile time trial ( $p < 0.05$ ). In addition, the chronic effect of nitrate supplementation requires further investigation. While a majority of studies employ a 2-6 day loading period (approximately 4-6 mmol/day  $\text{NO}_3^-$ ), supplementation for 15 days did not demonstrate any indications of reduced sensitivity to nitrate supplementation (28). Findings illustrated that oxygen cost was reduced (approximately 4%) during moderate-intensity exercise (approximately 90% lactate threshold) after the initial ingestion 2.5 hours prior to exercise and this reduction remained similar throughout the 15 day protocol (measures taken at 5 and 15 days) (28).

### **Importance of Nitrate Availability During Exercise**

While consumption of dietary nitrate increases plasma  $\text{NO}_2^-$  concentrations, there is a paucity of literature investigating the change in plasma levels in response to exercise duration (or changes in plasma  $\text{NO}_2^-$  concentration over time while exercising). Previous work by Larsen et al. (20) demonstrated no change in plasma  $\text{NO}_3^-$  with a decline in plasma  $\text{NO}_2^-$  levels in both the placebo and nitrate loaded groups after approximately 35 minutes of exercise with intensity increasing in stages from 45%  $\dot{V}\text{O}_{2\text{max}}$  to maximal work. Post-exercise no difference was found for plasma  $\text{NO}_2^-$  concentration between control and  $\text{NO}_3^-$  loaded groups. Perhaps, the decline of  $\text{NO}_2^-$  over time could be a potential cause of the non-significant changes found in oxygen cost at higher workloads ( $\geq 85\% \dot{V}\text{O}_{2\text{max}}$ ).

The current study seeks to examine the  $\text{NO}_2^-$  concentration at each submaximal intensity to further dissect whether a cumulative decline in  $\text{NO}_2^-$  could be a cause of the non-significant findings found by Larsen (20). In addition, plasma  $\text{NO}_2^-$  concentration will be examined alongside reduced oxygen cost to determine whether any correlation exists between the total  $\text{NO}_3^-$  concentration and the total reduction in  $\dot{V}\text{O}_2$ . Larsen and colleagues established that there was no difference in oxygen cost at higher exercise intensity workloads ( $>85\% \dot{V}\text{O}_{2\text{max}}$ ). The lack of significance was partially attributed to the following reasons: 1) “well above lactate threshold in several subjects” 2)  $\dot{V}\text{O}_2$  did not reach a steady-state level 3) After the maximum effort test, the plasma  $\text{NO}_2^-$  values were not different between the  $\text{NO}_3^-$  loaded and control group. These conclusions are limited in that the post-exercise measure of  $\text{NO}_2^-$  was taken only after the maximal/exhaustive bout. Therefore,  $\text{NO}_2^-$  changes after each workload were unknown. It is possible that the non-significant findings at 85%  $\dot{V}\text{O}_{2\text{max}}$  were a result of inadequate  $\text{NO}_2^-$  concentration, and

not necessarily the higher intensity of the workload (above lactate threshold). Larsen et al. (27) illustrated a decline in  $\text{NO}_2^-$  after a maximal exercise bout, while plasma  $\text{NO}_2^-$  then rose during the 30-minute post-exercise recovery. Thus, further research is necessary to determine the changes in plasma  $\text{NO}_2^-$  that occur over time. Change in plasma  $\text{NO}_2^-$  may be further impacted by exercise intensity; this too merits further investigation.

### **Conclusions**

Supplementation with dietary nitrate does not appear to harm endurance related performance. However, the impact of dietary  $\text{NO}_3^-$  as an ergogenic aid is less supported given the conflicting literature (17,38,42–47), although, others have found no performance-based ergogenic effects post-nitrate supplementation. The oxygen cost at various submaximal workloads are consistently reduced after  $\text{NO}_3^-$  supplementation, although this has not been correlated to time trial performance or changes in power output. While the ergogenic effect of dietary nitrate supplementation remains inconclusive, the literature does not establish any reason to believe there would be a decline in performance using supplementation periods of less than 15 days.

The present study seeks to examine the physical response which dietary nitrate supplementation incurs during exercise at submaximal intensities while acutely (<3 hours) exposed to high altitude (approximately 3500m). These findings can be utilized by a number of populations including coaches, athletes, researchers, nutritionists and dietitians to enhance exercise quality, optimize training, improve health and increase exercise performance.

## **Purpose**

The purpose of this study is to investigate the impact that consuming inorganic dietary nitrate has on both the caloric cost and physical stress experienced by people who exercise and work at high altitude (HA, 3500m, in this study). The current study seeks to directly identify what benefit dietary nitrate conveys during submaximal exercise (increasing in difficulty) under HA conditions as compared to normobaric (approximately 1600m) conditions. We hypothesize that participants who consume this commercially available dietary supplement (Beet It, James White Drinks Ltd, Ipswich, UK) two to three hours prior to their test session will undergo less insult to the body. Using the Hypo/Hyperbaric (“altitude”) chamber belonging to UNM’s Department of Health, Exercise and Sports Sciences, “insult” will be determined by measuring rating of perceived exertion, heart rate, blood pressure, number of calories expended (via oxygen consumption), markers of oxidative stress (cellular damage or other disturbances of the molecular signaling pathways) and production of blood lactate (before, during and after exercise). Dietary inorganic nitrate is found in a variety of foods including root vegetables, green leafy vegetables, and some cured or processed meats (48). The present study intends to control for dietary nitrate sources while raising blood plasma nitrate and nitrite ( $\text{NO}_3^-$  and  $\text{NO}_2^-$ , respectively) via acute supplementation two to three hours before exercise (26, 28, 34, 35).

In the last 5 years, dietary nitrate research has dramatically increased. Dietary nitrate has been implicated in reduced oxygen cost (less calories required to sustain exercise at a particular intensity) and increased mitochondrial efficiency (more energy production for every oxygen molecule consumed) over a number of submaximal workloads (17, 19–21). This is important because, at a particular intensity, reducing the



amount of oxygen consumed by the body may improve performance, especially when participants are exposed to HA where oxygen transport from the blood to the working muscle is reduced. Research has found that nitrate supplementation improves performance during cycling, rowing and running at sea-level (17,38,42–44), although nitrate supplementation's impact while exercising at HA is less well known. This study seeks to fill the knowledge gap surrounding nitrate supplementation and HA exposure.

Previous research has investigated the effect of nitrate consumption on metabolic cost during exercise; however, limited research is available examining exercise and nitrate consumption during hypoxic exposure. This study seeks to fill the knowledge gap surrounding hypoxia and nitrate supplementation during submaximal exercise.

### *Specific Aim 1*

To determine the effect of  $\text{NO}_3^-$  supplementation on metabolic oxygen cost at submaximal exercise intensities (25, 40, 50, 60, 70% of  $\dot{V}\text{O}_{2\text{max}}$ ). It is hypothesized that participants who consume a commercially available  $\text{NO}_3^-$  rich dietary supplement (Beet It, James White Drinks Ltd, Ipswich, UK) two to three hours prior to their test session will:

- 1) Reduce oxygen cost compared to a placebo control
- 2) Reduce oxidative stress compared to a placebo control

Using a randomized, crossover, double-blind, placebo-controlled design (placebo vs.  $\text{NO}_3^-$  supplementation), we will test these hypotheses.

*Rationale:* Nitrate is absorbed directly from the gastrointestinal tract with plasma  $\text{NO}_3^-$  levels reaching their peak between 60 minutes (25) and 2.5 hours (26). After either sodium nitrate or inorganic  $\text{NO}_3^-$  ingestion, plasma concentration of  $\text{NO}_2^-$  increases (16,

26–28). Therefore, the initial reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  occurs in the mouth. As a result, if antibacterial mouthwash is administered prior to  $\text{NO}_3^-$  ingestion conversion of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  is attenuated. Antibacterial mouthwash abolishes commensal oral bacteria causing a decrement in nitrate reductase activity (30).

This oxygen independent pathway allows for NO production even during conditions of hypoxia, acidosis or other heavy exercise circumstances. Therefore, an increase in plasma  $\text{NO}_3^-$  and  $\text{NO}_2^-$  may increase NO production even when NOS and endothelial NOS (eNOS) expression are limited by hypoxia or related oxidative stress (31, 32). Therefore, dietary supplementation which elevates  $\text{NO}_2^-$  and  $\text{NO}_3^-$  plasma concentrations, increases NO syntheses over a wide range of exercise intensities as NO may be less impacted by down regulation of the L-arginine pathway during extreme exercise intensities or hypoxic conditions. Further, the alternative nitrate-nitrite-nitric oxide pathway may facilitate physical activity at altitude which is predicated by reduced partial pressure and a concomitant reliance on oxygen independent NO production (33). Therefore, this nitrate-nitrite-nitric oxide pathway is potentially highlighted during hypoxia (14).

### ***Specific Aim 2***

To examine whether characteristics of oxygen cost vary between participants who responded to  $\text{NO}_3^-$  supplementation compared to participants categorized as “non-responders” to  $\text{NO}_3^-$  supplementation. Non-responders will be identified if they fall into one or more of the following categories:

- 1) Participants exhibit a minimal alteration in plasma  $\text{NO}_2^-$  concentration following  $\text{NO}_3^-$  supplementation (increase anticipated) (47).
- 2) Minimal alteration in plasma  $\text{NO}_2^-$  throughout exercise (decrease anticipated) (26)
- 3) Non Sig. change in  $\text{O}_2$  consumption between NR and PL trials (40)

It is hypothesized that participants who fall into a category of “non-responders” will not exhibit a significant reduction in oxygen cost at any submaximal intensities.

*Rationale:* Although the bottom line is different, my thought is somewhat based off of the work of Robert Chapman who “retrospectively divided [participants] into responders (n=17) and non-responders (n=15) to altitude training on the basis of the change in sea-level 5,000-m run time determined before and after 28 days of living at moderate altitude and training at either low or moderate altitude” (49).

In line with this retrospective analysis, Wylie (40) classified participants as responders and non-responders in a dose-response study. Analysis revealed three non-responders when 4 mmol dietary  $\text{NO}_3^-$  was provided while there were two in the 8 mmol  $\text{NO}_3^-$  treatment and only one in the 16.8 mmol  $\text{NO}_3^-$  treatment. Some individuals may, therefore, require greater acute doses of dietary  $\text{NO}_3^-$  to stimulate any positive effects on exercise capacity.

Therefore, participants will be retrospectively classified into responders and non-responders. This will allow for comparisons to be made between all participants (nitrate rich vs. placebo) and separately between responders or non-responders to nitrate supplementation vs. placebo. It is hypothesized that “responders” to the nitrate rich

beverage, when compared to the “non-responders”, may present with significant changes in oxygen consumption during submaximal exercise.

### *Specific Aim 3*

To determine the relationship between nitrate levels found in the blood and resulting oxygen cost. To accomplish this aim, blood nitrate levels will be determined following each submaximal exercise bout. Values from the  $\text{NO}_3^-$  loaded trials will be compared to plasma  $\text{NO}_3^-$  values found following the submaximal bouts in the placebo trials.

It is hypothesized that there is a direct correlation between plasma  $\text{NO}_2^-$  concentration and oxygen cost. Therefore, if plasma  $\text{NO}_2^-$  falls throughout exercise, there may be an associated change in the oxygen cost difference between the NR and PL (lower plasma  $\text{NO}_2^-$  concentration may result in less change, or reduction, in  $\dot{V}\text{O}_2$ ). It will also be necessary to determine the relationship between  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and oxygen cost above and below lactate threshold. To accomplish this aim,  $\text{NO}_2^-$  levels will also be determined alongside blood lactate values during lower and higher intensity exercise. Plasma  $\text{NO}_2^-$  is expected to significantly increase after  $\text{NO}_3^-$  supplementation and during recovery from exercise. Previous work (26) has determined that participants whose plasma  $\text{NO}_2^-$  concentration declined following high-intensity intermittent exercise exhibited improvement in performance following dietary  $\text{NO}_3^-$  supplementation

Plasma  $\text{NO}_2^-$  concentration will be assessed following each submaximal bout.

*Rationale:* Previous research by Larsen (20) performed a similar protocol to determine oxygen cost at different exercise intensities and failed to determine a

significant impact only at higher levels of intensity that were performed at the end of the protocol (85%  $\dot{V}O_{2\max}$  and 100%  $\dot{V}O_{2\max}$ ). This lack of change may have been due to a decrease in the available plasma nitrate when these higher intensities were performed.

### **Scope of the study**

The purpose of this study is to investigate the submaximal metabolic response initiated after acute inorganic nitrate consumption in recreationally trained cyclists during five minute exercise bouts of increasing intensities while exposed to simulated altitude (approximately 3500m) via hypobaric hypoxia. A total of 12 male trained cyclists ( $\dot{V}O_{2\max} >70\%$  ACSM age predicted normative value) will complete this study. All subjects will be healthy and free of disease and risk stratified as low risk according to American College of Sports Medicine criteria.

Participants will be recruited via flyers and word of mouth. For potential inclusion in the study, participants must be men between 18 and 45 years of age. These ages correspond to the “low risk” category as identified by the American College of Sports Medicine (50). Based on the responses to the health history questionnaire, only participants who are free of cardiovascular, pulmonary, and metabolic diseases will be invited to continue with the study.

Further, participants entering into the study will be categorized as trained cyclists. Categorization will be based on 1) prescreening questionnaire responses indicating a minimum of 150 minutes/week cycling or cardiovascular-based activity for a minimum of 8 weeks as indicated on a self-reported physical activity history questionnaire and 2)  $\dot{V}O_{2\max}$  test results placing the participant above the 70<sup>th</sup> percentile for his age and sex

(50). Participants will also attend an informal orientation discussing the study protocol, at which time interested participants will sign an informed consent document.

After completing IRB-approved consent forms, each participant will schedule three experimental trials (T1, T2 and T3) to be conducted in the UNM Exercise Physiology Laboratories. T1 will include baseline demographics, a maximal aerobic capacity test on an electronically braked cycle ergometer, and a series of submaximal exercise bouts to establish the intensity (watts) on the bike which corresponds to 25, 40, 50, 60, 70% of  $\dot{V}O_{2max}$ . T2 and T3 will consist of exercise at 3500m using a hypobaric chamber. Participants will exercise for 5 minutes at each of the predetermined exercise intensities corresponding to 25, 40, 50, 60, 70% of  $\dot{V}O_{2max}$  with a 4-minute period of active rest (pedaling against no resistance) between each submaximal exercise bout.

This study will follow a randomized, double-blind, placebo-controlled, crossover fashion as participants will be assigned to either a placebo (PL) or inorganic nitrate rich (NR) group. Throughout all supplementation and/or washout periods, to minimize any confounding effects of their dietary intake, subjects will be asked to minimize their intake of foods containing high nitrate content. A document outlining nitrate-containing foods will be provided to each subject to encourage successful compliance.

Participants will be asked to complete a food and activity log 2 days prior to completing their initial altitude trial (second visit). The participant will be asked to replicate this food and activity log during the 2 days prior to their second altitude trial (third visit).

### **Significance of the Study**

This is one of the first studies to examine the relationship between dietary nitrate supplementation and reduced oxygen cost during submaximal exercise (25, 40, 50, 60, 70%  $\dot{V}O_{2max}$ ) during hypobaric hypoxia (3500m). Previous research found nitrate supplementation reduced oxygen cost under hypoxic conditions of 11% ambient inspired oxygen (approximately 5,000 m altitude) while exercising at approximately 45%  $\dot{V}O_{2peak}$  (51). Other research reports that  $NO_3^-$  supplementation (consuming 3 equal doses at 24 h, 12 h and 2.5 h prior to exercise; totaling 9.3 mmol of  $NO_3^-$ ) under hypoxic conditions of 14.5% ambient inspired oxygen (approximately 3000 m) restores knee extension (48 watts at 40 pulses/min) time to exhaustion to values similar to those attained under normoxic conditions (52). Still, further investigation is necessary regarding different exercise intensities under hypoxic conditions. Findings from the present study examining nitrate supplementation could be utilized by a number of groups including coaches, athletes, researchers, nutritionists and dietitians seeking enhanced exercise quality, optimized training, and improved health during acute exposure (<3 hours) to high altitude (3500m). Research findings from the present study will also report on topics of clinical interest including blood pressure and rate-pressure product at rest, during, and after exercise under hypoxia.

This study will make use of biochemical assays including blood lactate, oxidative stress, plasma volume, hemoglobin content, hematocrit and  $NO_3^-$  and  $NO_2^-$ . This will adequately determine the efficacy of dietary nitrate consumption at five different submaximal exercise intensities while performed under HA conditions.

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## Chapter 2 Literature Review

This chapter presents a review article, which has been accepted for publication: Carriker, C., Gibson, A., & Mermier, C. (2013). The role of the nitrate-nitrite-nitric oxide pathway during hypoxia. *Journal of Sport and Human Performance*, 1(4), 63–78.

### The role of the nitrate-nitrite-nitric oxide pathway during hypoxia

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#### Introduction

Human movement and cognition rely on cerebral processing via an intricate neuronal network. The physiology of cerebral energy metabolism has been recently debated regarding an astrocyte-neuron lactate shuttle or conventional glucose oxidation as viable substrates (1,2). Reduced cerebral oxygenation may occur in ischemic stroke patients or during environmental exposure to high altitude. Cerebral ischemia, under severe cases, may result in not only impaired cognitive and motor function but neuronal damage or degeneration including either neuronal apoptosis or necrosis (3). However, if blood flow to regions of the brain is increased, greater oxygen flux occurs, which may ameliorate the consequences of cerebral ischemia outlined above (4,5). Recently, dietary nitrate has been shown to reduce oxygen cost during submaximal sea level exercise (6). Further, the conversion of nitrate to nitrite to nitric oxide has been implicated as an oxygen independent pathway (7), and as such, may be a potent ergogenic aid during hypoxic conditions as occurs during high altitude exposure.

Initially, nitric oxide (NO) was deemed a potent vasodilator capable of increasing blood flow via vascular smooth muscle relaxation and cyclic GMP accumulation (8). However, NO has also been implicated in a number of other functions such as improved or increased exercise induced glucose uptake in skeletal muscle (9), neurotransmission (10), immune response (11), regulation of mitochondrial respiration (12–14), and glycolysis as mediated by AMPK (15,16). Clinically, NO may ameliorate some of the negative consequences of ischemia/reperfusion injury

(reduced infarct size and endothelial dysfunction), especially in the heart (17) and brain (5) .

Several studies have noted improved vascular and myocardial function/outcomes when NO was activated prior to ischemia or reperfusion injury (18–20). Therefore, increased NO formation/availability during incidence of hypoxia or otherwise impaired blood flow may reduce neuronal damage, improve cognition, and further improve motor function in individuals experiencing ischemic conditions.

### **Brain blood flow during exercise**

In addition to pathology surrounding stroke or other vascular compromise, as exercise intensity nears maximum, the onset of total body exhaustion is initiated. Such a phenomenon is often commonly recognized as an ‘I need to stop’ feeling. During such maximal exercise, a slight decline in cardiac output may precede fatigue (21) with a concomitant reduction in brain blood flow (22) while oxygen extraction may be enhanced in cerebral tissue (23). Under such maximal exercise conditions, cerebral oxygen demand remains high and in some instances, oxygen supply may be outpaced by cerebral demand. When this occurs, to avoid damage or other catastrophic failure, motor unit recruitment is reduced (22). A meta-analysis examining cardiovascular training status found that untrained participants had reduced oxygen delivery to the frontal cortex which was determined to be insufficient for demand during high intensity exercise. As a result, untrained participants incurred insufficient cerebral oxygenation when compared to trained counterparts (22).

Over a wide range of exercise intensities from rest through high intensity, global cerebral oxygenation follows a quadratic trend (22). Therefore, cerebral oxygenation increases during low to moderate intensity exercise and begins to level off when approaching high intensity exercise. As exercise intensities reach maximum, cerebral oxygenation falls (22). During actual

and imagined exercise, an increase in perceived exertion may initiate an increase in regional cerebral blood flow (within the thalamic region, insular cortex and anterior cingulate cortex or the medial prefrontal region). Participants who experienced an increase in perceived exertion during both actual and imagined exercise also incurred an elevated heart rate and blood pressure (24).

Additionally, when effort/activity is imagined, insular cortex activation increases when a cardiovascular response occurs simultaneously (25). Homeostatic feedback is afferently relayed to the dorsal posterior insula, while the appropriate sensation is manufactured within the anterior insula based on said afferent feedback (26). Given the quadratic trend for cerebral oxygenation mentioned above, during maximal exercise (when the sensation to cease exercise occurs) feedback pertaining to cerebral blood flow/oxygenation may initiate protective signaling mechanisms to down-regulate or stop the exercise/activity prior to catastrophic failure, organ ischemia or injury.

During maximal intensity exercise, the content of deoxygenated hemoglobin in the brain rises while cerebral oxygenation in the prefrontal cortex declines (27). The prefrontal cortex relays information to the motor cortex, and in the presence of decreased prefrontal oxygenation results in decreased muscle function (28). Under such a relay mechanism, cerebral oxygen desaturation precedes voluntary exhaustion (29). Ultimately, if brain blood flow were increased, perception of effort may be reduced and, during maximal exercise, performance may be improved as motor unit recruitment could remain high for extended durations prior to initiating volitional cessation.

## Pathways implicated in nitric oxide production

The production of NO has been classified under two distinct pathways (Figure 1). The first is an oxygen dependent pathway: L-arginine-nitric oxide (30,31). This pathway allows synthesis of nitric oxide and L-citrulline from the oxidation of L-arginine by nitric oxide synthase (NOS) enzymes. Three different isoforms that generate NO have been previously found, including: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) enzymes (10).

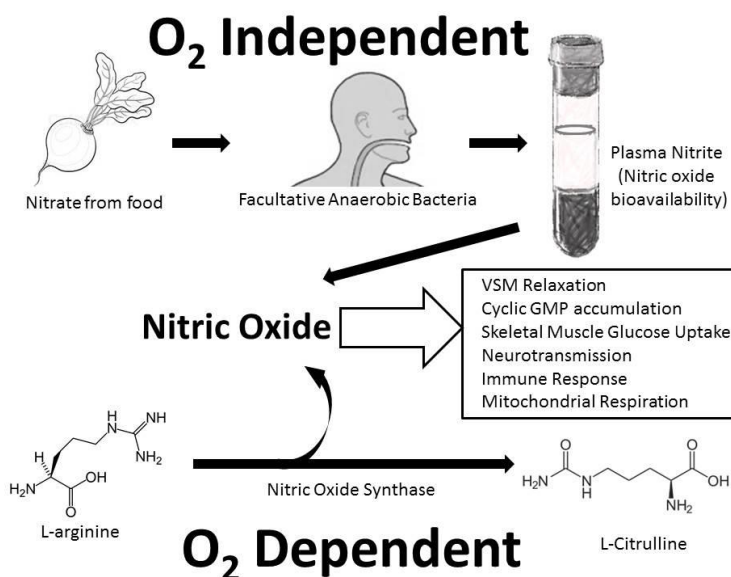


Figure 1. Pathways producing nitric oxide via an oxygen independent (top branch) or oxygen dependent (bottom branch) mechanism. Dietary nitrate is reduced to nitrite via facultative anaerobic bacteria while nitrite is further reduced to nitric oxide in acidic and hypoxic tissues. L-arginine also produces NO and L-citrulline via nitric oxide synthase. VSM: Vascular Smooth Muscle

The second pathway for NO generation is an oxygen independent pathway: nitrate-nitrite-nitric oxide (6,32,33). The oxygen independent pathway provides an important complement to the L-arginine-nitric oxide pathway especially under circumstances of hypoxia and/or impaired NOS availability (33–37). Moreover, during exercise, the nitrate-nitrite-nitric oxide pathway has been implicated in reduced oxygen cost and increased mitochondrial efficiency over a number of sub-maximal workloads (38–42). Exploitation of this pathway may provide potential ergogenic effects for exercise activities conducted during conditions of hypoxia such as altitude or pathology (coronary artery disease or congestive heart failure).

Because oxygen is required for NO synthesis via the L-arginine-nitric oxide pathway, during incidence of ischemia or hypoxia, drugs which increase NOS activity may be less efficacious (43–45) and the nitrate-nitrite-nitric oxide pathway may become enhanced during reduced oxygen availability (33,35). As a result, increased NO production via nitrate/nitrite availability may provide a key complement during incidence of reduced oxygen tensions. Therefore, during acute hypoxia (as encountered during unacclimatized high altitude exposure), it is hypothesized that inorganic nitrate supplementation may reduce incidence of ischemia by activating NO production via the nitrate-nitrite-nitric oxide pathway. In addition, because increased plasma nitrite concomitantly reduces oxygen cost during submaximal activity, it is possible that nitrate supplementation will improve performance despite the reduced oxygen partial pressure.

Consuming dietary nitrate may result in increased NO formation as nitrate consumption has previously been shown to increase plasma nitrite (46–48) (an *in vivo* marker of NO production (49,50)). Increased NO formation during incidence of hypoxia may negate some of the negative consequences of the reduced oxygen diffusion gradient between blood and tissues.

### **NO production from Nitrate and Nitrite**

Both the L-arginine-NO pathway (44) and the diet (51,52) contribute to elevated plasma nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ) levels in the body. An increase in plasma  $\text{NO}_3^-$  occurs in response to consumption of either whole foods and/or nitrate salts ( $\text{NaNO}_3^-$ ) which contain high levels of  $\text{NO}_3^-$ . Natural foods high in  $\text{NO}_3^-$  content include the following: celery, cress, chervil, lettuce, red beetroot, spinach, and rocket (rucola) (51). Meats that have been cured or processed may contain  $\text{NO}_2^-$  as an additive to inhibit bacterial growth. In addition,  $\text{NO}_2^-$  is a product of endogenous NO oxidation and  $\text{NO}_3^-$  reduction.



Inorganic  $\text{NO}_3^-$  from dietary intake forms  $\text{NO}_2^-$  after interacting with facultative anaerobic bacteria in the mouth (53,54); this process is also referred to as bacterial nitrate reductase activity (32). Subsequently, once  $\text{NO}_2^-$  swallowed, it is converted to NO within the acidic stomach (55). This is contrary to original conclusions which postulated  $\text{NO}_2^-$  and  $\text{NO}_3^-$  were endogenously inert end products of NO (56). It is now clear that  $\text{NO}_3^-$  and  $\text{NO}_2^-$  provide an auxiliary oxygen independent pathway for NO formation. This may be especially important under conditions of hypoxia (i.e. intense or near maximal exercise) where the oxygen dependant L-arginine route may be limited by the decreased bioavailability of NOS, nicotinamide adenine dinucleotide phosphate (NADHP), flavin adenine dinucleotide (FAD) and other related co-factors (31). In addition, during incidence of hypoxia and ischemia,  $\text{NO}_2^-$  has a demonstrated capacity to reduce tissue injury (35,57–59) and inhibit the generation of reactive oxygen species (ROS)(60).

Nitrate is absorbed directly from the gastrointestinal tract with plasma  $\text{NO}_3^-$  levels reaching their peak 60 minutes after ingestion (61). After either  $\text{NaNO}_3^-$  or inorganic  $\text{NO}_3^-$  ingestion, plasma  $\text{NO}_2^-$  increases (38,62–64). The initial reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  occurs in the mouth. Therefore, an antibacterial mouthwash administered prior to  $\text{NO}_3^-$  ingestion, attenuates the downstream conversion of  $\text{NO}_3^-$  to  $\text{NO}_2^-$ . Antibacterial mouthwash results in the removal of the commensal oral bacteria and subsequent decrement in nitrate reductase activity (65).

An increase in plasma  $\text{NO}_3^-$  and  $\text{NO}_2^-$  may increase NO production even when NOS and eNOS expression are limited by hypoxia or related oxidative stress (66,67). Dietary intake which elevates  $\text{NO}_2^-$  and  $\text{NO}_3^-$  plasma concentration, also increases NO synthesis over a wide range of exercise intensities. During high intensity exercise or instances of hypoxia, NO production via the NOS catalyst may be compromised due to reduction in the oxygen substrate

(68). Further, the alternative  $\text{NO}_3^-$  to  $\text{NO}_2^-$  to NO pathway facilitates activity at altitude which is predicated with reduced partial pressure and a concomitant reliance on oxygen independent NO production (69).

### **High Altitude and Plasma Nitrite concentration**

Individuals residing at high altitude may have greater plasma nitrite values than lowlanders. Tibetans residing at 4200m show greater bioactive NO products of plasma and red blood cell  $\text{NO}_3^-$  as well as increased plasma  $\text{NO}_2^-$  compared to residents at 206m (70). This suggests NO production was increased in highlanders alongside a greater forearm blood flow and lower vascular resistance compared to residents near sea-level. (70). Dietary intake was monitored in the previously mentioned study and dietary nitrate was “not at a level expected to significantly increase circulating nitrate or nitrite” (70). Further, acclimatization to high altitude results in increased plasma biomarkers of NO production ( $\text{NO}_3^-$  and  $\text{NO}_2^-$ ) as well as elevated cGMP indicating increased NO activity (71). In fact, enhanced circulatory extraction of  $\text{NO}_2^-$  occurs as hypoxia increases (increasing altitude) (71) which may explain the importance of the oxygen independent conversion of plasma  $\text{NO}_2^-$  to NO.

$\text{NO}_2^-$  can also be converted to NO via allosteric  $\text{NO}_2^-$  reduction by hemoglobin as evidenced by the formation of iron-nitrosylated hemoglobin. *In vivo*, infusion of nitrite both with and without a NOS inhibitor present resulted in increased forearm blood flow during exercise (72). This points toward the complementary nitrate-nitrite-nitric oxide pathway when production of NO via NOS is impaired. Further, there was an inverse relationship between iron-nitrosylated hemoglobin formation and the oxyhemoglobin saturation ( $r = -0.7$  and  $P < 0.0001$ ) (72). The maximal rate of NO conversion from  $\text{NO}_2^-$  (via deoxyhemoglobin) occurs when

hemoglobin is 50% saturated (32). In support of this finding, Shiva and colleagues (73) noted deoxymyoglobin reduces  $\text{NO}_2^-$  to NO approximately 36 times faster than deoxyhemoglobin.

### **High Altitude and Blood Flow**

Sea level residents acclimatizing to high altitude also incur a reduction in blood flow within microcirculatory blood vessels  $<50\mu\text{m}$  in diameter (74). Similar reductions in the blood flow of microcirculatory small ( $<25\ \mu\text{m}$ ) and medium (26–50  $\mu\text{m}$ ) blood vessels was also found in lowlanders exposed to altitude ( $>3500\text{m}$ ) with an explanation that the cause was perhaps due to the decreased hematocrit (reduced plasma volume) and the subsequent increase in blood viscosity (75). The authors add that the reduced blood flow may aid in oxygen delivery as the time for diffusion is increased at the capillary bed and is therefore a favorable adaptation in lowlanders acclimatizing to high altitude (75). Participants who supplement with inorganic nitrate may therefore increase plasma  $\text{NO}_2^-$  and subsequently increase the availability of NO in light of the reduced NO production from L-arginine (32).

As early as 15 minutes of acute altitude exposure (76), systemic vasodilation occurs, in part, mediated by NO production to ensure adequate oxygenation of tissues. In lowlanders acclimatized to high altitude ( $>3500\text{m}$ ), both the production and availability of NO is enhanced (71). Conversely, in the pulmonary circuit, acute altitude exposure results in hypoxic pulmonary vasoconstriction. In compensation for the hypoxic encounter, alveolar ventilation increases to offset hypoxemia thereby resulting in respiratory alkalosis (77). The increased pulmonary vasoconstriction can increase pulmonary capillary pressure which increases capillary leakage leading to high altitude pulmonary edema (HAPE) (78). Interestingly, pulmonary artery systolic blood pressure was greatest in individuals who developed HAPE after altitude exposure at 4959m (measurements made over 2 day sojourn). Importantly, those individuals not exhibiting

HAPE criteria had greater concentrations of nitrate-nitrite, measured via bronchoalveolar lavage fluid within one day of exposure to 4959m (79). Additionally, the concentration of expired NO was found to be lowest in individuals susceptible to HAPE during exposure to altitude ( at 12, 24, 36, and 48 hours at 4,559 m); indicative of a dysfunction in pulmonary NO synthesis (80). Therefore, impaired pulmonary epithelial NO synthesis may result in decreased bioavailability of NO, and increased pulmonary vasoconstriction may predicate the susceptibility of developing HAPE.

Given NO's effect on vascular tone and mitochondrial efficiency, greater NO availability during altitude exposure has increasing importance as ROS production increases. During altitude acclimatization, NO production/availability is associated with circulating elevations in cGMP concentrations (in the absence of changes in natriuretic peptide levels) (71). As reported by Levett (71), elevations in cGMP at 5,300m were positively correlated with microvascular blood flow in small (<25 mm diameter) and medium-sized (26–50 mm) vessels ( $p=0.06$  and  $p=0.025$  respectively;  $r$  value not reported), yet the cGMP concentrations were insufficient to normalize microcirculatory blood velocity at 5,300m (71). During altitude exposure, pharmacologic intervention such as with tadalafil or sildenafil (both phosphodiesterase-5 inhibitors) inhibit cGMP degradation, thereby increasing cGMP and subsequently preventing the onset of HAPE (81,82).

## **Critique**

It is hypothesized that during exposure to high altitude, maximal exercise intensity, or cardiovascular pathology such as associated with stroke, inorganic nitrate supplementation may reduce incidence of ischemia and improve performance by activating NO production via the nitrate-nitrite-nitric oxide pathway thereby reducing oxygen cost. The NO production via the

nitrate-nitrite-NO pathway acts complementary to the L-arginine pathway and may, therefore, increase NO bioavailability. In addition, nitrate supplementation has previously been shown to increase mitochondrial efficiency in humans at sea level; however, the benefits at altitude are less well known.

Increased plasma  $\text{NO}_2^-$  concentration occurs both during altitude acclimatization (>3500m) (71) and during short term (3 day) dietary nitrate supplementation (62). During exercise in conditions of reduced oxygen partial pressures, increased ROS may result.  $\text{NO}_3^-$  supplementation increases  $\text{NO}_2^-$ , and  $\text{NO}_2^-$  has been reported to have cytoprotective capabilities (i.e. improved mitochondrial oxidative phosphorylation) and to reduce mitochondrial ROS generation (60).  $\text{NO}_2^-$  has been previously established as a reservoir for NO (72) during hypoxia as  $\text{NO}_2^-$  is converted to NO (45).

While a number of physiological adaptations (increased oxygen carrying capacity, mitochondrial density, ventilatory response etc.) occur in response to altitude acclimatization, these particular changes occurring in response to  $\text{NO}_3^-$  supplementation have not been researched. Altitude acclimatization induces a number of hematological and non-hematological changes to improve performance/tolerance during hypoxia. However, the same physical changes have not been reported during short term  $\text{NO}_3^-$  supplementation (<15 days). Therefore, performance benefits similar to those resulting from altitude acclimatization may not be as likely despite the increased  $\text{NO}_2^-$  and NO bioavailability.

The limited research on dietary  $\text{NO}_3^-$  and its influence on cGMP is equivocal.  $\text{NO}_3^-$  supplementation has been shown to increase cGMP (83,84), but there is also evidence that suggests dietary  $\text{NO}_3^-$  has no influence on plasma levels of cGMP (42,62). Research supporting

$\text{NO}_3^-$  supplementation and resultant increases in cGMP at altitude is currently unavailable and is therefore, an area open for future research. Examination of soluble guanylyl cyclase activation during hypoxia could provide an explanation for the benefit of increased bioactivation of NO via  $\text{NO}_3^-$  supplementation. Mice lacking NO-sensitive guanylyl cyclase exhibited increased hypertension at rest due to the inability to appropriately vasodilate (85). Therefore, regardless if NO production is normal, when guanylyl cyclase loses sensitivity to NO or is unable to bind/interact with NO, vascular tone impairments may result in reduced blood flow/oxygen flux to working muscles. Such a condition may be a primary component of reduced performance during exercise at altitude.

It is possible that an overabundance of NO via nitrate supplementation (elevated plasma nitrite) could induce receptor desensitization or hypotension, a negative consequence. While  $\text{NO}_3^-$  supplementation has been shown to reduce blood pressure (83), these effects appear to be therapeutic in both healthy patients (i.e. reducing blood pressure but not reported to induce problematic hypotension)(63,86) and patients with peripheral artery disease (64). In fact, the consumption of natural sources of dietary nitrate, including certain fruits and vegetables (51), has been generally associated with decreased blood pressure, reduced oxygen cost during submaximal exercise, and increased exercise tolerance (87).

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### **Chapter 3 Research Manuscript**

This chapter presents a research manuscript, entitled “Effect of acute dietary nitrate consumption on submaximal oxygen consumption at altitude”. This manuscript will be submitted to the *International Journal of Sport Nutrition and Exercise Metabolism*.

**Effect of acute dietary nitrate consumption on submaximal oxygen consumption at altitude.**

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## Introduction

Dietary inorganic nitrate is found in a variety of foods including root vegetables, green leafy vegetables, and some cured/processed meats (Hord, Tang, & Bryan, 2009). Nitrate supplementation via nitrate salts (sodium nitrate) and food sources have both been shown to increase plasma nitrate ( $\text{NO}_3^-$ ) and plasma nitrite ( $\text{NO}_2^-$ ) concentrations in as little as two (Larsen, Weitzberg, Lundberg, & Ekblom, 2010) or three (Larsen, Weitzberg, Lundberg, & Ekblom, 2007) days of supplementation or consumption. While nitrate loading protocols vary in duration, i.e. two to six days, acute supplementation within two to three hours of exercise has been shown to increase both plasma  $\text{NO}_3^-$  and  $\text{NO}_2^-$  (Bescós et al., 2011; Lansley, Winyard, Bailey, et al., 2011; Vanhatalo et al., 2010).

Plasma  $\text{NO}_2^-$  is recognized as an *in vivo* biomarker of nitric oxide (NO) production (Kleinbongard et al., 2003; Lauer et al., 2001). The nitrate-nitrite-nitric oxide pathway may provide potential ergogenic effects during endurance activities during hypoxia (Carriker, Gibson, & Mermier, 2013). Little research has explored the ergogenic potential of dietary nitrate at altitude; however, nitrate supplementation appears to reduce  $\dot{V}\text{O}_2$  during submaximal exercise near sea level (Bailey et al., 2009; Lansley, Winyard, Bailey, et al., 2011; Lansley, Winyard, Fulford, et al., 2011; Larsen et al., 2007, 2010, 2011). Nonetheless, these sea level investigations may only represent a recreationally trained population ( $\dot{V}\text{O}_{2\text{max}}$  40-60 ml/kg/min) (Cermak, Gibala, & van Loon, 2012; Lansley, Winyard, Bailey, et al., 2011; Lansley, Winyard, Fulford, et al., 2011; Larsen et al., 2007, 2011). Studies using subjects with mean  $\dot{V}\text{O}_{2\text{max}}$  values >60 ml/kg/min demonstrated no change in submaximal oxygen cost and no improvement in performance following nitrate supplementation vs placebo (Bescós et al., 2012; Cermak, Res, et al., 2012; Peacock et al., 2012; Wilkerson et al., 2012). Interestingly, unpublished data from our

lab (1600m) showed that untrained subjects (mean  $\dot{V}O_{2\max}$  43.79 ml/kg/min) consistently consumed less oxygen during exercise at workloads  $<60\%$   $\dot{V}O_{2\max}$  following a 4-day nitrate supplementation loading protocol.

During altitude acclimatization at 3500m, plasma  $\text{NO}_3^-$  and  $\text{NO}_2^-$  have been shown to be elevated in as little as 2-5 days (Janocha et al., 2011; Levett et al., 2011). Moreover, the plasma  $\text{NO}_2^-$  concentration of individuals residing at high altitude may be greater than values of those residing at or near sea level after controlling for dietary  $\text{NO}_3^-$  (Erzurum et al., 2007). This may lead to enhanced metabolic signaling such as an increase in cyclic guanosine monophosphate, potentially increasing NO activity (Levett et al., 2011). When exposed to various elevations, ranging from sea level to 5300m, for 2 to 5 days, participants incurred the greatest increases in plasma  $\text{NO}_3^-$  and  $\text{NO}_2^-$  content at  $\sim 3500\text{m}$  (Janocha et al., 2011; Levett et al., 2011). Participants exposed to elevations from 4250m to 5300m had less  $\text{NO}_2^-$  concentration than when exposed 3500m, likely as a result of the reduction of circulating  $\text{NO}_2^-$  to NO in response to the reduced partial pressure of oxygen (Janocha et al., 2011; Levett et al., 2011). This decline in  $\text{NO}_2^-$  concentration may explain the importance of the oxygen independent conversion of plasma  $\text{NO}_2^-$  to NO as the body encounters a hypoxic environment.

The purpose of this study was to examine the effect of acute dietary nitrate supplementation on submaximal oxygen cost at 3500m for well-trained cyclists exercising at 40, 50, 60 and 70% of  $\dot{V}O_{2\max}$ . We predict that supplementation with dietary nitrate would accelerate the acclimatization process by raising plasma nitrite prior to altitude exposure at 3500m. We further predict that the increased levels of plasma nitrite would reduce oxygen consumption and improve oxygen saturation ( $\text{SaO}_2$ ) while exercising under high altitude conditions.

## Methods

### *Subjects*

Ten healthy trained male cyclists ( $28 \pm 7$  years;  $\dot{V}O_{2\max}$   $61.01 \pm 7.37$  ml/kg/min) residing at ~1600m for the previous 6 months volunteered for the study. All subjects provided written consent for this institutional review board approved study. Subjects were excluded if they indicated any known cardiovascular, pulmonary or metabolic disease on a health history questionnaire or incurred extended travel resulting in a change of altitude greater than 400m.

### *Exercise Tests*

All tests were carried out on an electronically braked cycle ergometer (Velotron, RacerMate, Seattle, WA, USA) with a racing saddle and pedal system similar to those used during training. The computer-controlled workload allowed subjects to self-select a comfortable cadence between 70-90 rpm. Preferred cadence and cycle ergometer configuration (seat height, handle bar height and distance to seat, and pedal preference), were recorded during trial 1 (T1) and replicated during trial 2 (T2) and trial 3 (T3) to minimize alteration in the muscle recruitment patterns.

Following T1, subjects were randomized to either the NR or PL intervention following a 4-day nitrate washout (Figure 1a). Subjects were instructed to consume the NR or PL beverage 2.5 hours prior to beginning exercise during T2 and T3. Throughout the 24 hours preceding T2, subjects were asked to record their dietary intake. Subjects were then instructed to replicate this diet during the 24 hours prior to T3.

To minimize diurnal variation, subject testing times and days were kept consistent between trials. Subjects were asked to avoid strenuous exercise, alcohol, chewing gum, and

mouthwash 24 hours prior to each trial as well as caffeine 12 hours before each trial (items which may alter  $\text{NO}_2^-$  availability) (Govoni, Jansson, Weitzberg, & Lundberg, 2008).

### *Experimental Protocol*

Trial 1: Maximal oxygen consumption test and pre-test determination of submaximal intensities

T1 exercise was completed in a normobaric (1600m) and thermoneutral environment (~18-21 °C) (Figure 1b). All subjects completed a standardized cycling test to exhaustion which began with 3 minutes of unloaded cycling followed by a 30 watt/min ramp protocol. Breath-by-breath metabolic gases were continuously collected (True One, ParvoMedics, Sandy, Utah, USA) and averaged over 11-breath sequences. The  $\dot{V}\text{O}_{2\text{max}}$  was recorded as the highest value collected over the 30 seconds prior to the subject's volitional exhaustion. Following the maximal exertion test, subjects completed a brief active cool down (~3-5 minutes) and then rested for a minimum of 15 minutes until the post-exercise heart rate (HR) recovered to within 10 beats of the resting (baseline) value. During this recovery period, subjects were permitted to sit or ambulate at their leisure. Subjects then completed five submaximal intensity cycling bouts. This served to further familiarize the subject with the electronically braked bike and also establish the resistance and cadence for each T2 and T3 bout (25, 40, 50, 60, and 70% of  $\dot{V}\text{O}_{2\text{max}}$ ).

Trial 2 and 3: Submaximal exercise at altitude

The T2 and T3 exercise sessions were conducted at 3500m via hypobaric hypoxia (Figure 1c). Each submaximal bout was performed for 5 minutes with a 4-minute period of rest separating the subsequent intensity. The resistance and cadence for T2 and T3 (corresponding to 25, 40, 50, 60, and 70% of  $\dot{V}\text{O}_{2\text{max}}$  workloads) were determined during T1.



$\dot{V}O_2$  was assessed over the final 30 seconds of each submaximal exercise bout (True One, ParvoMedics, Sandy, Utah, USA). HR (short range radiotelemetry via Polar Electro T31, New York, USA), pulse oximetry ( $SaO_2$ ; Go2 Achieve, Nonin Medical Inc., Plymouth, Minnesota, USA) and rating of perceived exertion (RPE; Borg, 1970), were recorded during the final 10 seconds of each exercise bout. Blood lactate was determined (Lactate Plus, Nova Biomedical, MA, USA) from venous blood collected immediately after each submaximal intensity.

### ***Dietary Intervention***

Subjects consumed either a  $NO_3^-$  rich (NR) ( $\sim 12.8$  mmol/day) or  $NO_3^-$  depleted (PL) commercially available beverage (Beet It, James White Drinks Ltd, Ipswich, UK) using a double-blind, placebo-controlled, cross-over design. During the dietary washout, subjects were asked to avoid foods high in nitrate content or supplements containing nitrate/nitric oxide/L-arginine (Hord et al., 2009). Subjects followed an otherwise normal diet; self-recorded prior to T2 and then repeated prior to T3.

### ***Venous Blood Sampling***

Prior to the maximal exertion test (T1),  $\sim 10$  mL of venous blood was drawn via venipuncture into a tube containing ethylenediaminetetraacetic acid (EDTA) for later analysis of baseline plasma nitrite ( $NO_2^-$ ) concentration. Upon arrival at the lab for T2 and T3, an intravenous catheter (Venflon IV cannula; Becton-Dickinson, Franklin Lakes, New Jersey, USA) was placed in a prominent forearm vein. The catheter was removed following the post-70%  $\dot{V}O_{2max}$  blood draw. Blood samples ( $\sim 10$  ml) were drawn before exercise and following each intensity (25, 40, 50, and 60% of  $\dot{V}O_{2max}$ ) for analysis of  $NO_2^-$  during both T2 and T3.

### ***Biochemical Measurements***

All tubes were inverted approximately ten times and then centrifuged at 4,000 rpm at 4°C for 6 minutes. Plasma samples were then stored at -80°C for subsequent analysis. Plasma nitrite was determined in duplicate using a commercially available microplate-based colorimetric assay kit (Cayman Chemical, protocol # 780001).

### **Statistical Analysis**

Results are presented as mean  $\pm$  standard deviations. Data were analyzed using a repeated-measures analysis of variances (ANOVA) with two factors: time and condition. An alpha level of 0.05 was set for determination of statistical significance.

### **Results**

#### *Plasma Nitrite following consumption of PL or NR beverage*

Approximately 2.5 hours after beverage consumption, plasma nitrite ( $\text{NO}_2^-$ ) was elevated following supplementation with NR ( $1.39 \pm 1.21$  uM) compared to PL ( $0.70 \pm 0.34$  uM;  $p < 0.05$ , Figure 2).

#### *Oxygen consumption and respiratory exchange ratio*

Following NR supplementation, oxygen consumption, recorded as the final 30-sec average of each stage, was not different at any intensity while exercising at 3500m ( $p = 0.13$ ) when compared with PL (Figure 3a). Similarly, respiratory exchange ratio was not different between treatments at any exercise intensity ( $p = 0.17$ ) (Figure 3b).

### *Oxygen Saturation and Blood Lactate*

During the 70%  $\dot{V}O_{2\max}$  intensity, all participants (in both treatments) were above the lactate threshold. Exercising at 3500m initiated a decline in SaO<sub>2</sub> for both conditions. SaO<sub>2</sub> was not different at any intensity when comparing NR and PL (p=0.15) (Figure 4a). However, the SaO<sub>2</sub> of the NR group began to level off at 50%  $\dot{V}O_{2\max}$  and remained greater than PL during the 60 and 70%  $\dot{V}O_{2\max}$  intensities. During exercise, lactate was lower following NR administration compared to PL at the 40 and 60%  $\dot{V}O_{2\max}$  workloads (p<0.05) (Figure 4b).

### *Heart rate and rating of perceived exertion*

HR was not different between treatments at any exercise intensity (p=0.67) (Figure 5a). Similarly, rating of perceived exertion was not different in response to NR supplementation compared with PL at any intensity (p=0.84) (Figure 5a).

## **Discussion**

Acclimatization at 3500m results in a number of physiologic changes including increased serum and plasma nitrate and nitrite levels (Levett et al., 2011). Increased plasma nitrate following supplementation has been shown to increase performance in recreationally trained individuals at sea level; however, the findings at altitude are less conclusive. We hypothesized exploitation of the nitrate-nitrite-nitric oxide pathway during hypoxia would result in reduced oxygen cost, which may translate to improved performance. Our study examined oxygen cost at various intensities (% $\dot{V}O_{2\max}$ ) in an effort to determine when dietary nitrate consumption may be most advantageous during exercise at altitude (3500m).

Following a similar protocol to that of Larsen (Larsen et al., 2007), the current study implemented 5-minute bouts of exercise at increasing intensities, each followed by four minutes of rest. Larsen and colleagues, however, utilized successive 5-minute bouts without rest between each intensity. The current study administered NR or PL 2.5 hours prior to exercise while subjects ( $\dot{V}O_{2\max}$   $55 \pm 3.7$  ml/kg/min) in Larsen's study consumed sodium nitrate (0.1 mmol/kg) over two separate 3-day periods (NR and PL). While hypoxia was not incorporated into Larsen's study design, they found that nitrate supplementation reduced normobaric oxygen cost during the lowest 4 workloads (45, 60, 70 and 80% of  $\dot{V}O_{2\max}$ ). While our results differed in that we found no change in submaximal  $\dot{V}O_2$  comparing NR to PL, our results were similar in that we found no difference in exercise HR. Previously, an acute dose of nitrate ~2.5 hours prior to exercise at sea level has been shown to reduce oxygen cost during moderate intensity exercise (Lansley, Winyard, Bailey, et al., 2011; Larsen et al., 2010; Vanhatalo et al., 2010; Wylie et al., 2013). Acute dietary nitrate loading 3 hours prior to exercise has shown increases in plasma nitrite similar to loading protocols between 3 and 6 days (Bescós et al., 2011; Webb et al., 2008).

Other research has also used similar exercise methodology to that used in the present study. Under normobaric conditions, Bescós (Bescós et al., 2011) administered an acute dose of NR 3 hours prior, similar to our administration of NR 2.5 hours prior to exercise. Our subjects ( $\dot{V}O_{2\max}$   $61.01 \pm 7.37$  ml/kg/min) completed successive 5-minute exercise bouts (40, 50, 60 and 70% of  $\dot{V}O_{2\max}$ ) separated by 4 minutes of passive recovery; while Bescós' subjects ( $\dot{V}O_{2\max}$   $65.01 \pm 6.2$  ml/kg/min) engaged in 6 minutes of exercise (2.0, 2.5, 3.0, and 3.5 watts/kg of body mass), interspersed with 3 minutes of passive recovery. Our results were similar in that neither study found a difference in submaximal oxygen cost during low- to moderate-intensity cycling exercise. Our data also are consistent with their hypothesis that the physiological response to

cardiorespiratory training, including: increased mitochondrial volume, enzyme activity and aerobic capacity (Hopker et al., 2009; Tonkonogi & Sahlin, 2002), may reduce the efficacy of acute nitrate supplementation when compared to lesser or moderately trained subjects (Hoon, Johnson, Chapman, & Burke, 2013).

Dietary nitrate supplementation prior to exercise in normobaric hypoxia exercise has been explored with mixed results. A single dose of nitrate consumed 3 hours prior to normobaric hypoxic exercise (15% O<sub>2</sub>; equivalent to 2500m) was found to lower  $\dot{V}O_2$  during minute 12 and 15 of a 15-minute exercise bout conducted at 60% of  $Watt_{max}$  (determined at altitude), with no difference in SaO<sub>2</sub> (Muggeridge et al., 2014). Time-trial performance (16.1km) during hypoxic exposure was significantly improved following nitrate supplementation compared to placebo ( $p < 0.05$ ).  $\dot{V}O_{2peak}$  of subjects was measured at simulated altitude ( $51.9 \pm 5.8$  ml/kg/min) (Muggeridge et al., 2014). Beyond ~500m,  $\dot{V}O_{2max}$  declines 7-9% for every 1000m increase in elevation up to 6300m (Robergs, Quintana, Parker, & Frankel, 1998). The current study measured  $\dot{V}O_{2max}$  under normobaric conditions (1600m). If the  $\dot{V}O_{2max}$  of the present study was equated to 2500m, the estimated mean  $\dot{V}O_{2max}$  (~56.74 ml/kg/min) would be greater than those reported for the Muggeridge study. Perhaps the reduction in O<sub>2</sub> cost found by Muggeridge can be partially attributed to the lower training status ( $\dot{V}O_{2max}$ ) of their participants. It is also possible that the shorter submaximal bout duration of the present study (5-minute bouts) was not long enough to initiate mechanistic benefit from the conversion of NO<sub>3</sub><sup>-</sup> to NO<sub>2</sub><sup>-</sup> to NO. Separately, Vanhatalo used normobaric hypoxia of 14.5% O<sub>2</sub> (~3000m), and found that, following NR consumption, high-intensity ( $48 \pm 4$  watt) knee extensor time to exhaustion was restored to values similar to those found during normoxia (Vanhatalo et al., 2011). The duration of hypoxic exercise for the placebo ( $393 \pm 169$  s) was compared to the nitrate group ( $477 \pm 200$  s;  $p < 0.05$ ).

Neither  $\dot{V}O_{2\max}$  nor  $\dot{V}O_{2\text{peak}}$  values of the subjects were reported for this study, which makes it difficult to directly compare our results to theirs. Two distinct differences were also present; our study employed hypobaric hypoxia and was conducted at a greater simulated elevation. Still other research using 11% inspired  $O_2$ , corresponding to ~5000m, determined nitrate supplementation reduced oxygen consumption during a 20-minute exercise bout at a workload corresponding to 45%  $\dot{V}O_{2\max}$  (Masschelein et al., 2012). Masschelein found subjects ( $61.7 \pm 2.1$  ml/kg/min) had higher  $SaO_2$  and lower lactate following nitrate supplementation (0.07 mmol nitrate/kg body wt/day for 6 days prior to exercise) compared to the placebo while exercising at 45%  $\dot{V}O_{2\max}$ . Their findings for lactate are similar to ours at intensities below 60%  $\dot{V}O_{2\max}$  (3500m), and while our  $SaO_2$  data was not statistically different between conditions, we did determine  $SaO_2$  was greater in the highest two workloads (60 and 70%  $\dot{V}O_{2\max}$ ) following NR consumption. The simulated elevation of Masschelein's study was markedly higher and, therefore, we must use caution when comparing our results to theirs. We suspect exercise greater than their chosen 45% intensity would be quite difficult to maintain over 20 minutes at 5000m.

While there are distinct differences between normobaric and hypobaric hypoxia (Millet, Faiss, & Pialoux, 2013; Richard & Koehle, 2012), non-significant changes in  $\dot{V}O_2$  during submaximal exercise in this study and several others could be attributed to the training status of the participants. Sea level studies reporting a reduction in submaximal exercise  $\dot{V}O_2$  following nitrate supplementation consistently reported subjects with mean  $\dot{V}O_{2\max}$  values below 60 ml/kg/min. It is therefore plausible that well-trained participants are already receiving the maximal benefit from physiologic adaptations to endurance training. It is also possible that a difference in  $\dot{V}O_2$  may have been found if the duration of each intensity in the present study was

extended; as Muggeridge (Muggeridge et al., 2014) only found a difference during the final minutes of their 15-minute protocol.

It was hypothesized that NR supplementation would be an effective strategy for reducing  $O_2$  cost during submaximal exercise at altitude, due to the increased potential for conversion of  $NO_2^-$  to NO (Carriker et al., 2013; Lundberg, Weitzberg, & Gladwin, 2008; Zweier, Wang, Samouilov, & Kuppusamy, 1995). However, during submaximal exercise at 3500m, NR supplementation does not appear to provide any benefit when compared to a placebo for well-trained subjects. Future studies should assess the change in oxygen consumption while exploring different endurance capacities ( $\dot{V}O_{2max}$ ) of subjects, longer duration exercise, and different elevations. While the cohort used by Masschelein (Masschelein et al., 2012) was similarly trained compared to ours, their altitude exposure was much higher (~5000m via normobaric hypoxia). It is therefore possible that, in well-trained participants, the benefit of NR is not evident until individuals are exposed to much lower barometric pressures. In such a case, a subject would encounter greater physiologic stress pertaining to gas exchange due to the reduced partial pressure of oxygen. Therefore, our findings suggest that individuals who are planning to compete at higher altitudes (or those who seek other physiological advantages) use traditional altitude acclimatization strategies rather than acute nitrate supplementation (Levine & Stray-Gundersen, 1997; Wilber, Stray-Gundersen, & Levine, 2007; Wilber, 2011). While nitrate supplementation may provide benefit during sea level exercise, our research does not support its efficacy at altitude (3500m). Prior to deriving definitive conclusions regarding dietary nitrate's ergogenic potential above sea level, more research is needed.

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The study was designed by CC, CM, CW and AG; data were collected and analyzed by CC, CM, TV, KJ, NB, RV, NC, and AG; data interpretation and manuscript preparation were undertaken by CC, CM, RV, CW, and AG. All authors approved the final version of the paper”.



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## Figure Legends

FIGURE 1—A) Schematic timeline: Trial 1 (1600m) and Trial 2+3 (3500m). B) Trial 1 protocol beginning with  $\dot{V}O_{2max}$  test followed by familiarization trials (25, 40, 50, 60, and 70% of  $\dot{V}O_{2max}$ ) C) Trial 2 and 3 submaximal exercise (25, 40, 50, 60, and 70% of  $\dot{V}O_{2max}$ ) during hypobaric hypoxia (3500m).

FIGURE 2—A) Plasma nitrite concentration (uM) following consumption of placebo (white bars) or nitrate rich (black bars) beverage 2.5 hours prior to exercise. B) Counterbalanced placebo consumed prior to either Trial 2 or Trial 3. C) Counterbalanced nitrate rich beverage consumed prior to either trial 2 or trial 3. Data are presented as the mean  $\pm$  SD (N=10). \*p<0.05 compared with placebo.

FIGURE 3—Oxygen consumption (L/min) during submaximal exercise at 40, 50, 60 and 70%  $\dot{V}O_{2max}$  after placebo (white bars) and nitrate rich (black bars) supplementation 2.5 hours prior to exercise. B) Respiratory exchange ratio during submaximal exercise after placebo (white bars) and nitrate rich (black bars) supplementation. Data are presented as the mean  $\pm$  SD (N=10).

FIGURE 4—A) Oxygen saturation (%) during submaximal exercise at 40, 50, 60, and 70%  $\dot{V}O_{2max}$  after placebo (white bars) and nitrate rich (black bars) supplementation 2.5 hours prior to exercise. B) Lactate (mmol/L) during submaximal exercise after placebo (white bars) and nitrate rich (black bars) supplementation. Data are presented as the mean  $\pm$  SD (N=10). \*p<0.05 compared with placebo.

FIGURE 5—A) Heart rate (bpm) during submaximal exercise at 40, 50, 60, and 70%  $\dot{V}O_{2max}$  after placebo (white bars) and nitrate rich (black bars) supplementation 2.5 hours prior to exercise. B) Rating of perceived exertion (Borg 6-20) during submaximal exercise after placebo (white bars) and nitrate rich (black bars) supplementation. Data are presented as the mean  $\pm$  SD (N=10).

Figure 1:

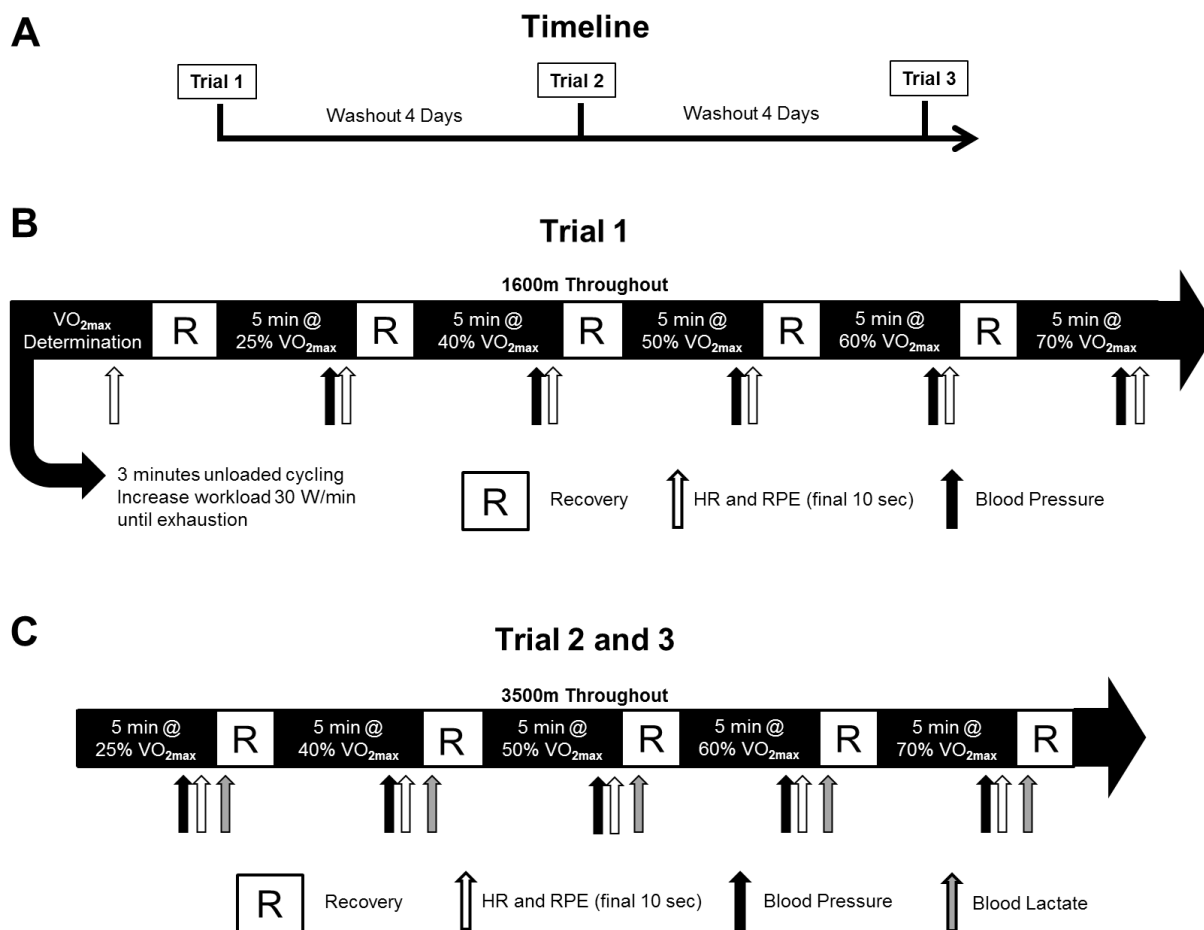


Figure 2:

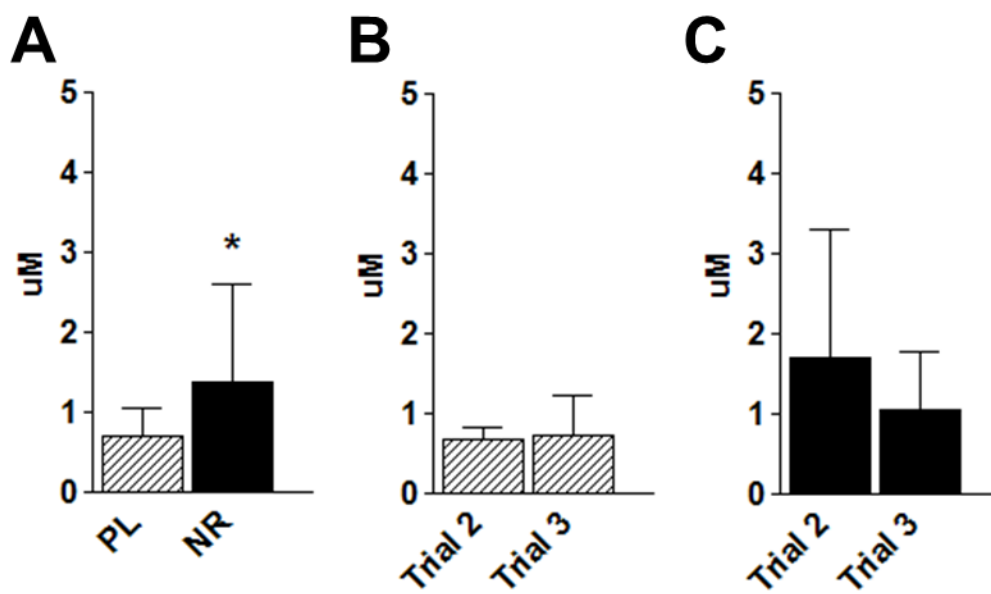


Figure 3:

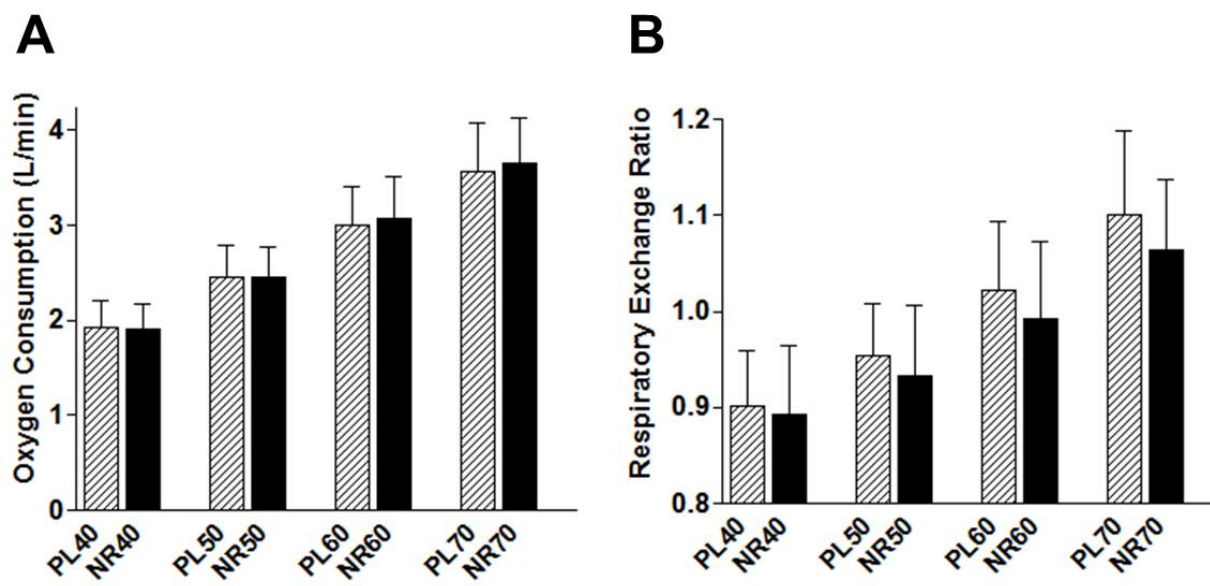




Figure 4:

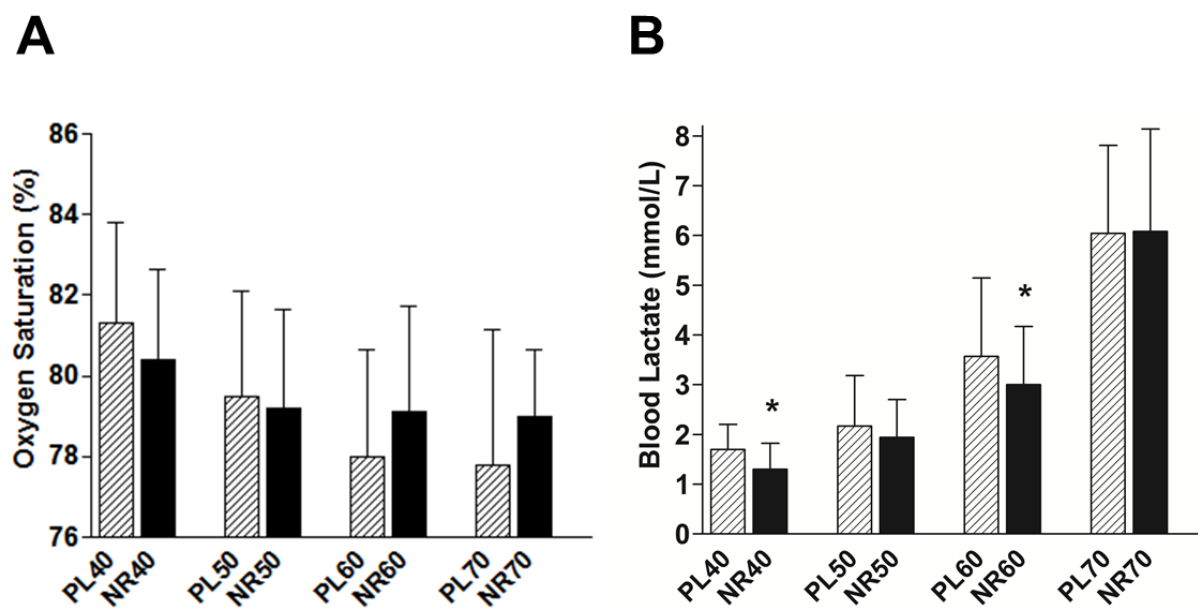
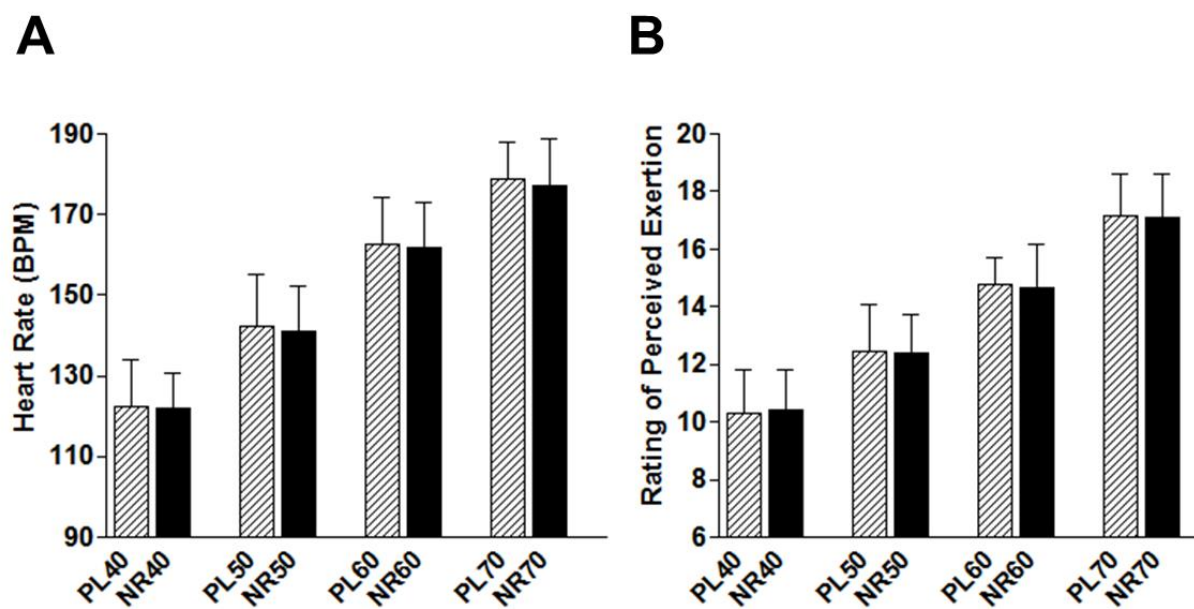


Figure 5:



## Chapter 4 Summary, Conclusions, Recommendations

### Summary

The review manuscript entitled “The role of the nitrate-nitrite-nitric oxide pathway during hypoxia,” provides insight into the potential role which dietary nitrate may play in individuals exposed to hypoxia. Previous research at sea level alludes to the benefits of dietary nitrate supplementation including reduced submaximal oxygen consumption, improved exercise time trial performance, increased time to exhaustion and reduced resting blood pressure. The oxygen independent reduction of nitrate to nitrite to nitric oxide, in the presence of deoxyhemoglobin and deoxymyoglobin, points to the ergogenic potential of dietary nitrate while exercising at altitude. The review paper highlights the effect of dietary nitrate on exposure to high altitude, maximal exercise, as well as cardiovascular pathology. The underlying mechanistic or molecular signaling responsible for the physiological benefits of dietary nitrate has yet to be determined. Larsen’s group (Larsen et al., 2011) previously pointed toward an improvement in mitochondrial efficiency resulting in improved oxidative phosphorylation efficiency (i.e. the amount of oxygen consumed per ATP produced; P/O ratio). The limited research surrounding dietary nitrate and altitude exposure or hypoxia leaves room for many future areas of investigation.

The research manuscript entitled “Effect of acute dietary nitrate consumption on submaximal oxygen consumption at altitude” seeks to fill the knowledge gap surrounding the ergogenic potential of dietary nitrate at altitudes above sea level. This research provides evidence that dietary nitrate may not possess the same benefits at altitude as previously identified during exercise at sea level. While exercise and dosing protocols used during our study were similar to those previously performed, we failed to identify benefits of nitrate consumption in

variables such as oxygen consumption, oxygen saturation, heart rate, rating of perceived exertion and exercise blood pressure, which were not different between the placebo and nitrate rich trials. Therefore, dietary nitrate consumption may not be a viable means of performance enhancement during exercise at altitude in well-trained subjects.

This study also explored the changes in oxidative stress associated with exercise following dietary nitrate consumption. Our data showed a significant increase in both catalase and 8-isoprostane following exercise at 3500m in both the placebo and nitrate rich groups (Figure 1 and 2). However, there was no difference between groups (placebo vs nitrate rich) for either of these markers of oxidative stress. Given the acute altitude exposure, the impact which dietary nitrate has on oxidative stress over prolonged exposure is unknown. Future research should explore the changes in oxidative stress in individuals residing at high altitude compared to lowlanders acutely exposed to high altitude. In addition, dietary nitrate may also affect different populations to various extents. Therefore, future studies should recruit samples of differing training status, gender, and health (high blood pressure, peripheral arterial disease, etc.).

## **Conclusions**

The principle finding of this research was that an acute dose of dietary nitrate, consumed 2.5 hours prior to exercise at 3500m, does not reduce oxygen consumption at exercise intensities ranging from 40 to 70%  $\dot{V}O_{2max}$  for well-trained subjects. While other research conducted at sea level has toted dietary nitrate as ergogenic in nature, the same conclusions are far from conclusive for dietary nitrate consumed prior to exercise at higher altitudes.

## **Recommendations**

As previously mentioned, future research should examine different samples including trained vs untrained, male vs female, and healthy vs unhealthy (high blood pressure or peripheral artery disease etc.). The duration of exercise should be examined as shorter duration exercise bouts may not provoke pronounced differences in measured variables. Exercise durations of 15-20 minutes appear to be suitable for determining an effect of acute dietary nitrate consumption. In addition, the altitude examined for this research was 3500m, while other research has explored the exercise response following dietary nitrate at 2500m, 3000m and 5000m. Further exploration of these and other elevations is necessary to determine the efficacy of nitrate supplementation, particularly for sojourners to higher altitudes. In addition, much of the previous research has used acute (2.5 hour prior) to short term (up to 15 days) nitrate supplementation. The benefit of dietary nitrate beyond 15 days is unknown. Therefore, prolonged or chronic supplementation should be examined to determine whether dietary nitrate maintains any benefits seen during the early stages of supplementation. The literature surrounding dietary nitrate has gained profound interest in the last 5 years and as a result, this new topic has many additional avenues to explore.

## Figure Legends

FIGURE 1—Catalase activity (nmol/min/ml) immediately following all submaximal exercise bouts after placebo (white bars) and nitrate rich (black bars) supplementation 2.5 hours prior to exercise. Data are presented as the mean  $\pm$  SD (n=9).

FIGURE 2—Isoprostane activity (pg/ml) 60 minutes after completing all submaximal exercise bouts after placebo (white bars) and nitrate rich (black bars) supplementation 2.5 hours prior to exercise. Data are presented as the mean  $\pm$  SD (n=9).

Figure 1:

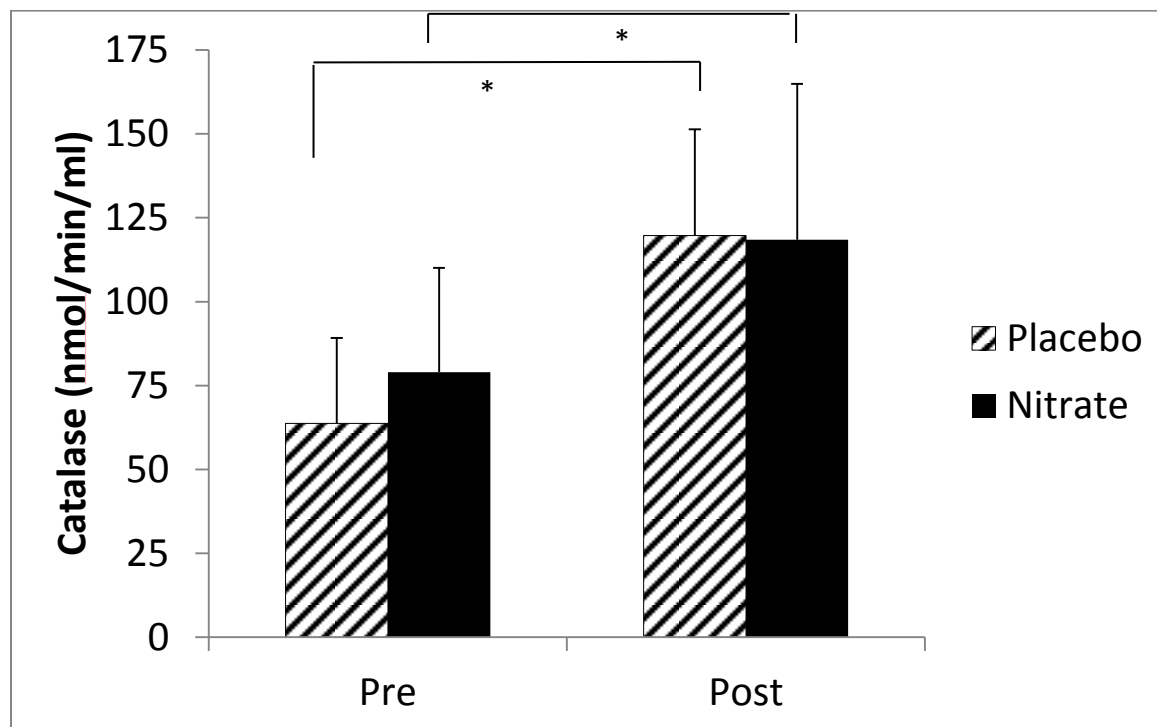
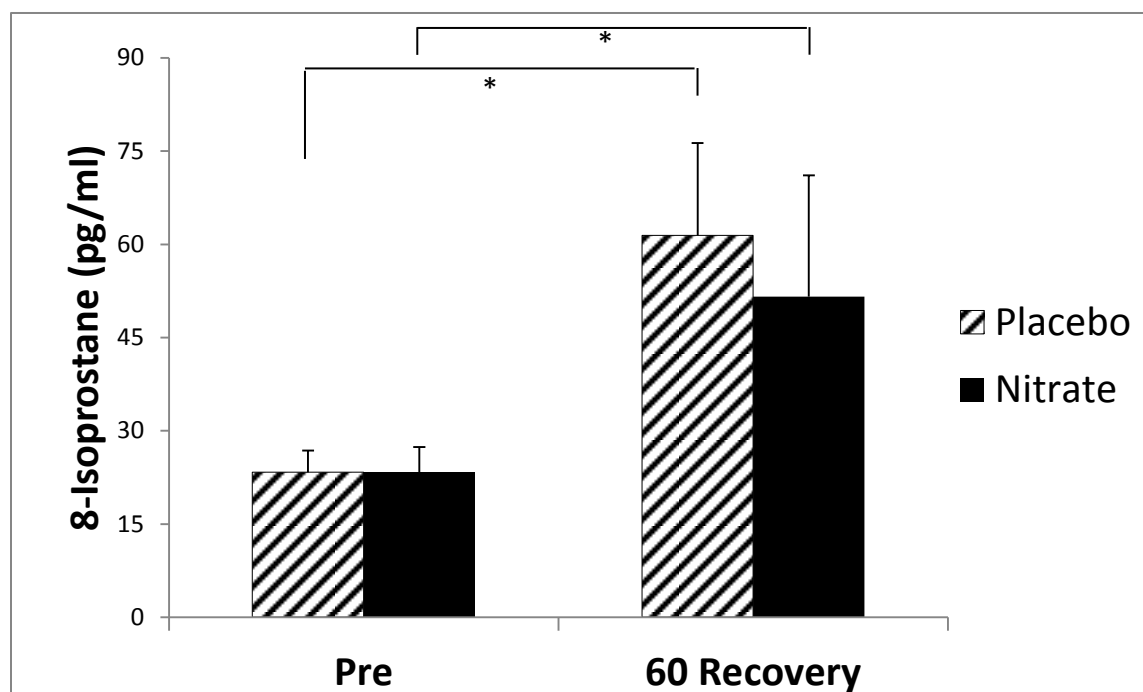


Figure 2:





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## Appendix A. HIPAA

### UNIVERSITY OF NEW MEXICO HEALTH SCIENCES CENTER HIPAA<sup>1</sup> AUTHORIZATION TO USE AND DISCLOSE PROTECTED HEALTH INFORMATION FOR RESEARCH PURPOSES

**Title of Study:** Effect of acute dietary nitrate consumption on submaximal oxygen consumption and oxidative stress in hypoxia

**Principal Investigator:** Ann Gibson, Ph.D.  
**UNMHSC Department:** Heath, Exercise, and Sports Sciences  
**Mailing Address:** MSC 04-2610, Johnson Center, University of New Mexico,  
 Albuquerque, NM 87131  
**Co-Investigator:** Colin Carriker, M.S.

**Sponsor:** N/A

1. **What is the purpose of this form?** You have been asked to take part in a research study. The consent form for this study describes your participation, and that information still applies. This extra form is required by the federal Health Insurance Portability and Accountability Act (HIPAA).
2. **What if I don't want my personal health information (PHI) to be used in this research study?** You do not have to give this permission. Your decision not to sign this form will not change your ability to get health care outside of this research study. However, if you do not sign, then you will not be allowed to participate in the study.
3. **What PHI am I allowing to be used for this research?** The information that may be used includes:
4. a.)  $VO_{2max}$ - maximal aerobic capacity  
 b.) Height, weight, and resting blood pressure  
 c.) Oxygen consumption during five (5) different intensities of exercise on a treadmill.
5. **Where will researchers go to find my PHI?** We will ask you to fill out a questionnaire about your health.
6. **Who will be allowed to use my information for this research and why?** The researchers named above and their staff will be allowed to see and use your health information for this research study. It may be used to check on your progress during the study, or analyze it along with information from other study participants. Sometimes research information is shared with collaborators or other institutions. Your records may also be reviewed by representatives of the research sponsor or funding agency, the Food and Drug Administration (FDA) to check for quality, safety or effectiveness, or the Human Research Review Committee (HRRC) for the purposes of oversight and subject safety and compliance with human research regulations.

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<sup>1</sup> HIPAA is the Health Insurance Portability and Accountability Act of 1996, a federal law related to privacy of health information.

7. **Will my information be used in any other way?** Your information used under this permission may be subject to re-disclosure outside of the research study and be no longer protected under certain circumstances such as required reporting of abuse or neglect, required reporting for law enforcement purposes, and for health oversight activities and public health purposes.
8. **What if I change my mind after I give this permission?** You can change your mind and withdraw this permission at any time by sending a written notice to the Principal Investigator at the mailing address listed at the top of this form to inform the researcher of your decision. If you withdraw this permission, the researcher may only use and share your information that has already been collected for this study. No additional health information about you will be collected by or given to the researcher for the purposes of this study.
9. **What are the privacy protections for my PHI used in this research study?** HIPAA regulations apply to personal health information in the records of health care providers and other groups that share such information. There are some differences in how these regulations apply to research, as opposed to regular health care. One difference is that you may not be able to look at your own records that relate to this research study. These records may include your medical record, which you may not be able to look at until the study is over. The HIPAA privacy protections may no longer apply once your PHI has been shared with others who may be involved in this research.
10. **How long does this permission allow my PHI to be used?** If you decide to be in this research study, your permission to access and use your health information in this study may not expire, unless you revoke or cancel it. Otherwise, we will use your information as long as it is needed for the duration of the study.

I am the research participant. By signing this form, I am giving permission for my personal health information to be used in research as described above. I will be given a copy of this authorization form after I have signed it.

\_\_\_\_\_  
Name of Research Subject

\_\_\_\_\_  
Signature of Subject

\_\_\_\_\_  
Date

\_\_\_\_\_  
Name of Person Obtaining Authorization

\_\_\_\_\_  
Signature

\_\_\_\_\_  
Date

## Appendix B. Informed consent

The University of New Mexico Health Sciences Center  
Consent to Participate in Research

Effect of acute dietary nitrate consumption on submaximal oxygen consumption and oxidative stress in hypoxia

### *Introduction*

You are being asked to participate in a research study conducted by Ann Gibson, Ph.D., the Principal Investigator, Colin Carriker, M.S., and their associates from the Department of Health, Exercise, and Sports Sciences. This research is studying the effect of a commercially available inorganic nitrate beverage on oxygen consumption during sub-maximal exercise.

Previous research has studied the effect of nitrate supplementation during several exercise intensities at or near sea level elevations. More research is needed to determine if there are benefits of nitrate supplementation for submaximal exercise performed at high altitude (~11,500 feet). At high altitudes, it is more difficult to get oxygen to the muscles; that can make the same exercise effort seem harder than it was at a lower elevation. Based on results from research performed at lower elevations, we believe the nitrate supplement will improve the body's ability to deliver oxygen during sub-maximal exercise at high altitude. If we are correct, altitude performance will improve and there will be fewer markers of oxidative stress (by-products made as the body makes the energy needed to fuel sub-maximal exercise). Therefore, the purpose of this study is to examine how the inorganic nitrate supplement affects oxygen consumption and by-product formation during sub-maximal cycling exercise at a simulated altitude of ~11,500 ft. This research may add new knowledge about high altitude exercise performance following supplementation with this nitrate-rich beverage.

You are being asked to participate in this study because you are a trained male cyclist who is free from any heart, lung, or metabolic diseases. Approximately 30 people will take part in this study at the University of New Mexico.

This form will explain the research study, and will also explain the possible risks as well as the possible benefits to you. If you have any questions, please ask one of the study investigators.

### *What will happen if I decide to participate?*

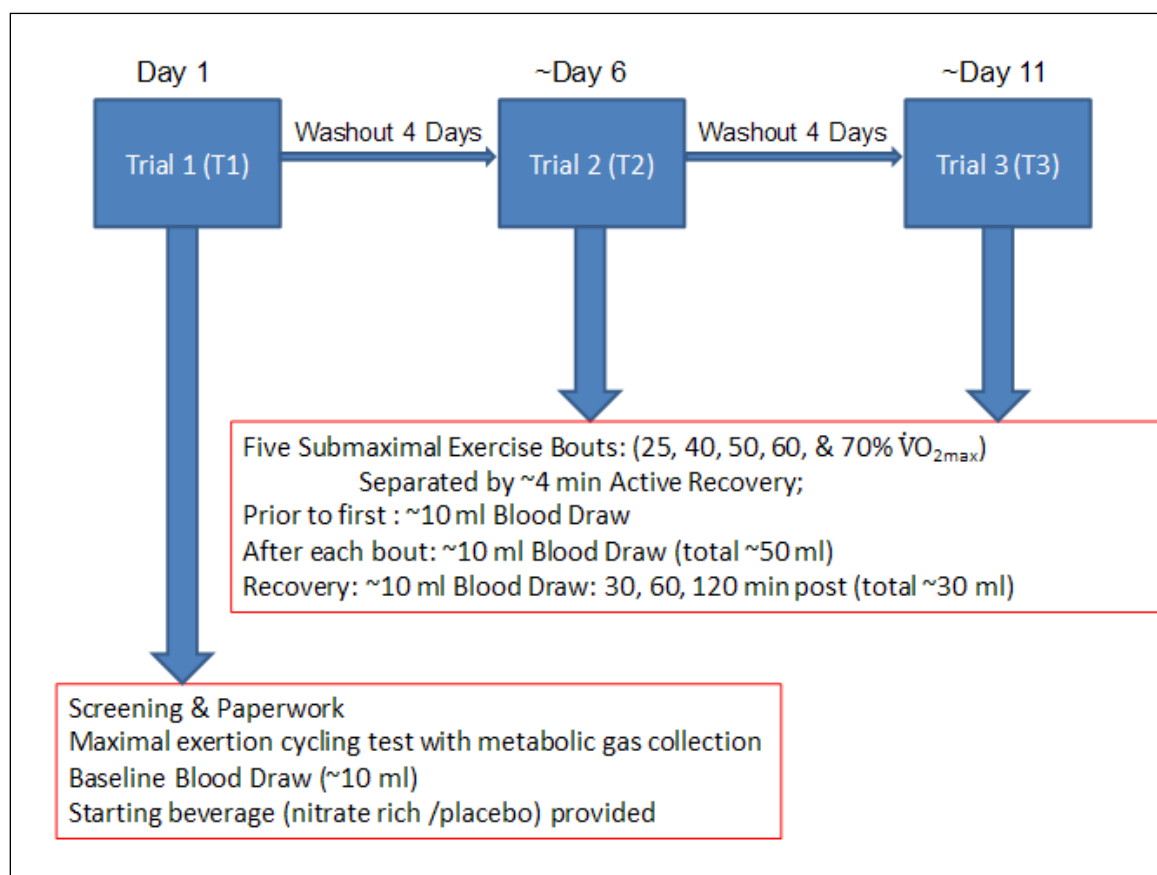
If you agree to participate, the following things will happen:

#### **Overview**

1. You will be asked to come to the Exercise Physiology Lab in Johnson Center on the University of New Mexico main campus three times in approximately 2 weeks.
2. As shown in Figure 1, the first visit (T1), is when all the paperwork will be completed and baseline information gathered. You will be given the beverage you are to drink before the next visit. You will be given either the nitrate-rich beverage (Beet It, James White, Drinks Co.) or a placebo (containing little or no nitrate) beverage. We will schedule your next visit, give you instructions about how and when to consume your

assigned beverage, and instruct you on keeping a food log. You will also be given a list of foods to avoid. Details about the T1 procedures are outlined below (First Visit section).

3. Figure 1 also shows that the second visit (T2) follows a 4-day period during which you will consume a low nitrate diet (avoiding the foods on the list we give you). Two hours before you come to the Exercise Physiology Lab for your scheduled appointment, you will drink your designated beverage according to the instructions given to you. We will walk together to the altitude chamber (near Carlisle Gym on the UNM main campus) and start the preparations for the day's testing. At the end of T2, we will schedule the third visit, give you the other beverage, and remind you of the importance to continue avoiding certain foods and following your food log as closely as possible.
4. The third visit (T3) also follows a 4-day period during which you will consume a low nitrate diet (avoiding the foods on the list we give you). Two hours before you come to the Exercise Physiology Lab for your scheduled appointment, you will drink the designated beverage according to the instructions given to you. We will walk together to the altitude chamber and start the preparations for the day's testing.



**Figure 1: Proposed Timeline (Start to Finish)**

### First Visit

1. During your first visit, you will sign this consent form, HIPAA form and fill out a health history questionnaire.
2. We will review your medical history form and determine if you qualify to participate, we will also take your resting blood pressure.
3. You will be directed to the bathroom so you can void your bladder and bowels.
4. Following this, you will put on the heart rate monitor we issued to you and then change into your exercise attire.
5. After we take your height and weight, we will take a blood sample from one of your forearm veins.
6. You will then perform a maximal exertion cycling test; this test will last approximately 8-12 minutes. The test will begin with a warm-up. After that, the workload will be adjusted so that the pedaling becomes progressively harder. This increase in workload will continue until you can go no more. Then the workload will be reduced so that you can cool down for a while.
7. Throughout the maximal exertion cycling test, you will have a breathing mouthpiece in your mouth and a nose clip over your nose so that we can measure your maximal aerobic capacity (also known as maximal oxygen consumption or  $VO_{2max}$ ). There may be some discomfort associated with the mouthpiece or the nose clip.
8. When the cycling test and cool down are finished, you will rest in a seated position for approximately 15 minutes. You will then perform five sub-maximal cycling bouts at different workloads. Each of these bouts will be separated from the next by four minutes of easy cycling (Recovery). Using these five sub-maximal cycling bouts, we will determine the workloads for your T2 and T3 sessions.

## **Second and Third Visit**

1. Your second (T2) and third (T3) appointments will start much like T1 did.
2. We will then walk together to the altitude chamber and measure your resting blood pressure after you have been sitting inside the chamber for ~10 minutes.
3. Afterwards, we will close the altitude chamber door and begin our “ascent” to ~11,500 feet. This will be done by changing the pressure within the chamber (this “ascent” will take approximately 7-10 minutes). Similar to airplane travel, your ears may ‘pop’ as the pressure changes. This should not be painful, but may cause some temporary discomfort until we stabilize the pressure at the simulated altitude of 11,500 feet.
4. While we are “traveling” to our targeted altitude, we will place an indwelling catheter into one of your forearm veins. This may cause some temporary pain or discomfort after the initial needle stick. This technique will allow for a small, flexible tube (catheter) to stay in place and be used for drawing blood. That way we will not need to stick you every time we need a blood sample. We anticipate that the catheter placement will only take one needle stick. Should we need to reposition the catheter or make other necessary adjustments including individual venipuncture (single needle stick), this may require an additional needle stick(s) throughout the duration of the visit.
5. You will be fitted with a breathing mouthpiece and nose clip just like in T1.
6. You will then cycle for 5-minute bouts. These 5-minute cycling bouts (25, 40, 50, 60 and 70% of your  $VO_{2max}$ ) are the same as those determined in T1. The 5-minute bouts will be separated by 4-minute intervals of easy pedaling (Recovery). During the 4-minute

- periods, a blood sample will be taken through the catheter. The catheter will then be flushed with a standard saline solution to decrease the likelihood a clot will form in it.
7. After the final exercise bout and blood draw, the catheter, mouthpiece, and nose clip will be removed. We will “descend” to Albuquerque’s elevation and walk back to Johnson Center.
  8. You will have your blood drawn at 30 minutes, 1 hour and 2 hours following the removal of the catheter. Each of these blood draws will require an additional needle stick
  9. During this 2-hour period, you will be free to walk around inside the Exercise Physiology Lab, but we ask that you avoid additional physical activity. You may wish to bring a book/computer to occupy your time. We will provide a desk and couch for your convenience.
  10. After the final 2 hour blood draw, your testing session is finished.
  11. There may be discomfort during exercise of increasing workloads. Should you need to stop at any time during the testing process, just let us know and slow down your pedaling.

### *How long will I be in this study?*

Participation in this study will take a total of approximately 8-10 hours over a period of 2 weeks.

Summary of anticipated time:

Visit 1: ~1.5 hours

Visit 2: ~3 hours

Visit 3: ~3 hours

Given the nature of this study, the visits are scheduled at particular intervals; if you anticipate a scheduling conflict, please let us know immediately so we can best accommodate the conflict.

### *What are the risks or side effects of being in this study?*

#### **Exercise Risks**

- Possible side effects of maximal exertion exercise include nausea, lightheadedness, muscle cramps, or dizziness after completion of the exercise.
- In people with heart disease, exercise testing to the point of fatigue has a very low risk of sudden death (1 in 10,000) and complications of the heart (4 in 10,000). Because you are a trained cyclist accustomed to exercise, the risk is expected to be much less.

There are additional possible risks of physical stress, emotional distress, inconvenience and loss of privacy and confidentiality (see below) associated with participating in a research study.

Although you may already be accustomed to the cycling intensities used in this study, you may not be accustomed to the change in altitude. While exercising at ~11,500 feet of elevation, your maximal intensity exercise capacity (what you could normally perform at the Albuquerque, NM elevation), may be reduced. Therefore, it may feel more difficult while exercising at a given workload. Should you need to stop exercising at any time, you may.

While exercising, we will be measuring the amount of oxygen your body is using; you will, therefore, exercise while breathing through a special mouthpiece that is connected to a flexible

hose integrated into the computer. There may be some discomfort while exercising with the mouthpiece in place as your nostrils will be “pinched” shut so that you will be breathing only through your mouth. It is unlikely that this setup will cause any pain; although, it may be uncomfortable.

Regarding possible side effects due to the beverages, Beet It, James White, Drinks Co. reports “The most obvious is that Beet It shots may turn urine pink. There is some anecdotal evidence that consuming large quantities of beet juice can cause upset stomachs.”

### **Venous Blood Draw**

- There may be some pain associated with the needle stick. Some people will experience only a prick or stinging sensation while others feel a moderate level of momentary pain. Some people (less than 1 in 10,000) may faint or feel light headed during venous catheterization. Bruising at the site of the needle stick is possible as is localized infection. We anticipate that catheterization will require only one needle stick; after that the tubing remains in place during the exercise testing (while you are completing the cycling trials on the bike). This allows for easy access during each additional blood draw. While every effort will be made to keep the blood flowing through the catheter so we can get the samples we need, there is a possibility a clot will form in it. That would require that we place another catheter or use single needle sticks to get the blood samples). To reduce the likelihood of infection, an experienced technician will wear gloves and use an alcohol swab to sterilize the area where the catheter will be placed or where needle sticks will be made. To reduce the likelihood of bruising, we will apply pressure at the site once the needle or catheter has been removed.
- After the final exercise bout and blood draw, the catheter will be removed. After the exercise period this is considered the Recovery Period (Figure 1). You will have your blood drawn at 30 minutes, 1 hour and 2 hours following the removal of the catheter. Each of these blood draws will require an additional needle stick

It should also be noted, there may be unforeseen risks to participating in this study. For more information about risks and side effects, ask the investigator.

### *How do I know if I am eligible to participate in this study?*

You must be a male between the ages of 18 to 45 yrs. Participants must also:

- 1) Indicate that you performed at least 150 minutes of aerobic activities (predominantly cycling-based activities) per week for each of the past 8 weeks.
- 2) Your maximal oxygen consumption from T1 must be higher than that of 69% of the men in your age group (reference values provided by the American College of Sports Medicine).

### *What are the benefits to being in this study?*

This research may provide insight into the possible benefits of this nitrate-rich supplement for individuals exercising at sub-maximal intensities at high altitude Elevations.



While there is no guarantee that you will have a direct benefit as a result of your participation in the study, all participants will be given the results of their VO<sub>2</sub>max test upon completion of T3. Upon request, you will be informed of your performance outcomes for your individual treatment/trials. As a result, this may be of potential benefit to you.

*What other choices do I have if I do not want to be in this study?*

Your participation is voluntary, and if you decide to not be in the study then you will not be contacted again.

*How will my information be kept confidential?*

We will take measures to protect the security of all your personal information, but we cannot guarantee confidentiality of all study data.

Information contained in your study records is used by study staff. The University of New Mexico Health Sciences Center Human Research Review Committee (HRRC) oversees human subject research and may be permitted to access your records. There may be times when we are required by law to share your information. However, your name will not be used in any published reports about this study.

Your information will be stored in a locked file cabinet. In addition, you will be given a study number that will be used for data collection and analysis. Your information will not be associated with your study number after the completion of data analysis.

*What are the costs of taking part in this study?*

The primary cost for participating in this study is your time. If you park on the University campus, you will be responsible for all parking fees. All nitrate and placebo beverages will be provided at no cost.

*Will I, as a research participant, receive monetary compensation for my time?*

No, you will not be compensated for your participation in this study.

*What will happen if I am injured or become sick because I took part in this study?*

You are responsible for all medical and transport expenses associated with any adverse event, illness, or accident occurring as a result of your participation in this study.

No commitment is made by the University of New Mexico Health Sciences Center (UNMHSC) to provide free medical care or money for injuries to participants in this study.

In the event that you have an injury or illness resulting from your participation in this study, UNMHSC will provide emergency treatment; however, reimbursement for all related costs of care will be sought from your insurance company, managed care plan, or other benefits program. If you do not have insurance, you may be responsible for these costs. You will also be responsible for any associated co-payments or deductibles required by your insurance.

It is important for you to tell the Principal Investigator immediately if you have been injured or become sick because of taking part in this study. If you have any questions about these issues, or

believe that you have been treated carelessly in the study, please contact the Human Research Review Committee (HRRC) at the University of New Mexico Health Sciences Center, Albuquerque, New Mexico 87131, (505) 272-1129 for more information.

*How will I know if you learn something new that may change my mind about participating?*

You will be informed of any significant new findings that become available during the course of the study. Such findings may be related to changes in the risks or benefits associated with this study or new alternatives to participation that might change your mind about participating.

*Can I stop being in the study once I begin?*

Your participation in this study is completely voluntary. You have the right to choose not to participate or to withdraw your participation at any point in this study without affecting your standing in the community or other services to which you are entitled.

*Whom can I call with questions or complaints about this study?*

If you have any questions, concerns or complaints at any time about the research study, Ann Gibson, Ph.D. or her research project associates will be glad to answer them if you call (505)-277-2658.

If you need to contact someone after business hours or on weekends, please email Carriker@unm.edu for communication with Colin Carriker.

If you would like to speak with someone other than the research team, you may call the UNMHSC HRRC at (505) 272-1129.

*Whom can I call with questions about my rights as a research participant?*

If you have questions regarding your rights as a research participant, you may call the UNMHSC HRRC at (505) 272-1129. The HRRC is a group of people from UNM and the community who provide independent oversight of safety and ethical issues related to research involving human participants. For more information, you may also access the HRRC website at <http://hsc.unm.edu/som/research/hrrc/>.

## Consent to Participate

By signing this consent form, you are not giving up any legal rights. Your signature means that you understand the study plan, have been able to ask questions about the information given to you in this form, and you agree to join the study.

We will give you a copy of this consent form to keep for your personal records.

\_\_\_\_\_  
Participant Printed Name

\_\_\_\_\_  
Signature

\_\_\_\_\_  
Date

\_\_\_\_\_  
Principal Investigator

\_\_\_\_\_  
Signature

\_\_\_\_\_  
Date

**All of the following components are optional, please indicate whether you agree to consent by checking the yes or no box. In addition, please print and sign your name, only if you selected the YES checkbox.**

### Consent to retain de-identified blood samples for academic purposes

**Yes**       **No**

By signing below, you are not giving up any legal rights. Your signature indicates you are willing to allow your blood samples to be de-identified and kept beyond the study period. These samples would be used for academic purposes such as future student practice of biochemical assay techniques. If consent is not provided below, your blood samples will be de-identified and destroyed according to OSHA regulations at the end of the study period.

_____	_____	_____
Participant Printed Name	Signature	Date
_____	_____	_____
<b>Principal Investigator</b>	<b>Signature</b>	<b>Date</b>

---

### Consent to contact participant for alternative/continuation studies

**Yes**       **No**

By signing below, you are not giving up any legal rights. Your signature indicates you would like to be contacted for any follow-up studies similar to the present study. If consent is not provided below, you will not be contacted for anything outside the purposes of the present study.

_____	_____	_____
Participant Printed Name	Signature	Date
Preferred email address: _____		

_____	_____	_____
<b>Principal Investigator</b>	<b>Signature</b>	<b>Date</b>

---

#### FOR RESEARCH TEAM ONLY

I have explained the research to the participant and answered all of his questions. I believe that he understands the information described in this consent form and freely consents to participate.

\_\_\_\_\_  
Name of Investigator/ Research Team Member (type or print)

_____	_____
Signature of Investigator/ Research Team Member)	Date



## Appendix D. Flyer

# University of New Mexico Research Study In Albuquerque, NM

HRPO study #13-583      Version 12/05/2013

**Effect of inorganic nitrate (Beet It; James White Drinks Ltd., Ipswich, United Kingdom, U.K.)  
supplementation on oxygen consumption during submaximal exercise in trained cyclists.**



Are you a trained male cyclist?

Do you have a very high aerobic capacity for your age and gender  
We'd love to have you participate in our exercise research study!

- We are looking for males age 18 to 45 years
  - Participants cannot have cardiac, peripheral vascular, cerebrovascular disease, chronic obstructive pulmonary disease, asthma, interstitial lung disease, or cystic fibrosis, diabetes mellitus (type I or II), thyroid disorders, renal or liver disease.
- Participants must meet the following pre-requisites:

- 1) Achieve a  $VO_{2max}$  value tested on a cycle ergometer above the 70<sup>th</sup> percentile for your age and sex
- 2) Primarily reside at ~1600 m for the previous 6 months and agree to avoid extended-travel which will incur a change in altitude greater than 500 m.
- 3) Self-report engaging in cardio based activities >150 minutes/week for a minimum of 8 consecutive weeks preceding enrollment in the study.

- You will be asked to exercise on 3 different days over a period of approximately 2 weeks
- You will consume a beverage provided at no cost (2 hours prior to Trial 2 and Trial 3)
- Trial 1: Complete a maximal aerobic effort stationary cycle test ( $VO_{2max}$ ).
- Trial 2: Complete five (5) different intensity 5-minute cycle exercise bouts separated by a 4-min break.
- Trial 3: Complete five (5) different intensity 5-minute cycle exercise bouts separated by a 4-min break.
- At each trial we will draw your blood. There is no cost to participate in this study.

**Contact: Colin Carriker at [Carriker@unm.edu](mailto:Carriker@unm.edu) for more information about this voluntary research study: **Please email with subject line: UNM Nitrate Research** OR call 505.277.2658**



Are you taking any medications, vitamins or dietary supplements now? Y N

If yes, what are they? \_\_\_\_\_

Do you have allergies to any medications? If yes, what are they? \_\_\_\_\_

Are you allergic to latex? Y N

Have you been seen by a health care provider in the past year? Y N

If yes, elaborate \_\_\_\_\_

Have you had a prior stationary cycle test? Y N. If yes, when? \_\_\_\_\_

What were the results? \_\_\_\_\_

Have you ever experienced any adverse effects during or after exercise (fainting, vomiting, shock, palpitations, hyperventilation)? Y N If yes, elaborate. \_\_\_\_\_



**LIFESTYLE FACTORS**

Do you now or have you ever used tobacco? Y N If yes: type \_\_\_\_\_

How long? \_\_\_\_\_ Quantity \_\_\_/day Years since quitting \_\_\_\_\_

How often do you drink the following?

Caffeinated coffee, tea, or soda \_\_\_\_\_oz/day Hard liquor \_\_\_\_\_oz/wk Wine \_\_\_\_\_oz/week Beer \_\_\_\_\_oz/wk

Indicate your current level of emotional stress. High \_\_\_\_\_ Moderate \_\_\_\_\_ Low \_\_\_\_\_



**PHYSICAL ACTIVITY/EXERCISE**

**Physical Activity**

How many times per week do you exercise 30 minutes/day or more? (CIRCLE ONE)

01 2 3 4 5 6 7

Please explain the type of exercise or activities you regularly participate in.

Cardiovascular \_\_\_\_\_

Strength Training \_\_\_\_\_

Flexibility/Stretching \_\_\_\_\_

Do you train in any activity (eg. stationary cycling, road cycling, spin class)? Y N

Rate your current cycling ability on a scale from 0 to 5 (CIRCLE ONE)

Poor -- 0 1 2 3 4 5 -- Excellent

**In a given week, how often do you engage in Vigorous Exercise (>20 Minute sessions)**

\_\_\_\_\_ times per week



## Appendix E. List of food sources rich in nitrate

### List of Food Sources Rich In Nitrates

\* Hord, Norman. "Food Sources of Nitrates and Nitrites: The Physiologic Context for Potential Health Benefits." *The American Journal of Clinical Nutrition* 90 (2009): 1-10. Print.

During the course of your participation, you are asked to avoid food sources classified as Middle, High, or Very High in Nitrates. These levels are described in Table 1 with suggestions of what foods to avoid. These foods high in nitrates are strictly found in vegetables, and according to Hord et al... (referenced in Table 1) nitrates are low to very low in fruits and meats, as demonstrated in table 2. Therefore, should avoid foods listed in Table 1 while you are in this study.

**Table 1**

Classification of vegetables according to nitrate content<sup>1</sup>

Nitrate content (mg/100 g fresh weight)	Vegetable varieties
Very low, <20	Artichoke, asparagus, broad bean, eggplant, garlic, onion, green bean, mushroom, pea, pepper, potato, summer squash, sweet potato, tomato, watermelon
Low, 20 to <50	Broccoli, carrot, cauliflower, cucumber, pumpkin, chicory
Middle, 50 to <100	Cabbage, dill, turnip, savoy cabbage
High, 100 to <250	Celeriac, Chinese cabbage, endive, fennel, kohlrabi, leek, parsley
Very high, >250	Celery, cress, chervil, lettuce, red beetroot, spinach, rocket (rucola)

**Table 2**

Mean nitrate and nitrite contents of a convenience sample of fruit, vegetables, meats, and processed meats<sup>1</sup>

	Nitrates	Nitrites
	mg/100 g	mg/100 g
<b>Fruit</b>		
Apple sauce	0.3	0.008
Banana	4.5	0.009
Fruit mix	0.9	0.08
Orange	0.8	0.02
<b>Vegetables</b>		
Broccoli	39.5	0.07
Carrots	0.1	0.006
Cole slaw	55.9	0.07
French fries	2.0	0.17
Ketchup	0.10	0.13
Mustard greens	116.0	0.003
Salad mix	82.1	0.13
Spinach	741	0.02
Tomato	39.2	0.03
Vegetable soup	20.9	0.001
Desiccated vegetable dietary supplement <sup>2</sup>	27,890	10.5
<b>Meats/processed meats</b>		
Bacon	5.5	0.38
Bacon, nitrite-free	3.0	0.68
Ham	0.90	0.89
Hot dog	9.0	0.05
Pork tenderloin	3.3	0