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Running head: Dietary nitrate and exercise efficiency

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ABSTRACT

Purpose: Humans can reduce inorganic nitrate (NO_{3}^{-}) to nitrite (NO_{2}^{-}), nitric oxide (NO) and other bioactive nitrogen oxides. The purpose of this study was to test the hypothesis that a single dose of inorganic nitrate before exercise might enhance the tolerance of endurance athletes to high intensity exercise. **Methods:** Eleven cyclists (age: 34.3 ± 4.8 yrs; VO_{2peak}: 65.1 ± 6.2 $mL \cdot kg^{-1} \cdot min^{-1}$) participated in this randomized, double-blind, crossover study. Subjects received dietary supplementation with nitrate ($NaNO_3$ 10 $mg \cdot kg^{-1}$ of body mass) or a placebo (NaCl) three hours before exercise. They then performed a cycle ergometer test which consisted of four 6-min submaximal workloads, corresponding to 2.0, 2.5, 3.0 and 3.5 $W \cdot kg^{-1}$ of body mass, interspersed with 3 min of passive recovery. After a 5-min recovery period, the subjects performed one incremental exercise test until exhaustion. **Results:** Plasma nitrate and nitrite were significantly higher ($P < 0.05$) three hours after supplementation (nitrate: 250 ± 80 μm; nitrite: 2,313 ± 157 nM) than after the placebo (nitrate: 29 ± 8 μm; nitrite: 1,998 ± 206 nM) at resting conditions. Nitrate supplementation significantly reduced VO_{2peak} (nitrate:4.64 ± 0.35; placebo: 4.82 ± 0.33 $L \cdot min^{-1}$; $P = 0.010$) and the ratio between VO_2/power at maximal intensity (nitrate: 11.2 ± 1.1; placebo: 11.8 ± 1.1 $mL \cdot min^{-1}$/W; $P = 0.031$). This reduction of VO_2 occurred without changes in the time to exhaustion (nitrate:416 ± 32; placebo: 409 ± 27 seconds) or in the maximal power (nitrate:416 ± 29; placebo: 410 ± 28 W). **Conclusion:** A single oral dose of inorganic nitrate acutely reduces VO_{2peak} without compromising the maximal exercise performance.

Keywords: nitric oxide, nitrate, nitrite, exercise performance, exercise economy, oxygen uptake

Introduction

Paragraph Number 1 Nitrate (NO_3^-) and nitrite (NO_2^-) have been known predominantly as undesired molecules in the food chain with potentially harmful effects, or as inert oxidative end products of endogenous nitric oxide (NO) metabolism (21). However, research carried out over the last decade has shown that nitrate and nitrite are physiologically recycled in blood and tissues to form NO and other bioactive nitrogen oxides (20). When inorganic nitrate is ingested it is rapidly absorbed in the upper gastrointestinal tract and its bioavailability is almost 100%. The vast majority of absorbed inorganic nitrate is ultimately excreted in the urine, but up to 25% of plasma nitrate is actively taken up by the salivary glands and excreted in the saliva (32). In the mouth, facultative anaerobic bacteria on the surface of the tongue reduce nitrateto nitrite (10). Salivary nitrite then can be further converted to NO in the stomach (24), but it is also clear that a substantial part of swallowed nitrite is absorbed intact to increase circulating plasma nitrite(20). This nitrite can be converted to NO and other bioactive nitrogen oxides in blood and tissues under appropriate physiological conditions (22). This pathway complements the classical L-arginine NO synthase pathway and is especially enhanced during tissue acidosis and hypoxia, when NO formation by NO synthases may be compromised (20). A recent study showed that when this circuit was interrupted by not swallowing saliva for 3 h after ingestion of nitrate-rich beverages, the rise in plasma nitrite, but not nitrate, was blocked (36). Hence, this pathway is required to increase circulating nitrite concentration after nitrate load. A picture is now emerging of the important functions of the nitrate–nitrite–NO pathway in regulation of blood pressure and blood flow (16), gastric integrity (22) and tissue protection against ischemic injury (28). The nutritional aspect of these findings is intriguing since diet constitutes the main source of nitratein humans, with vegetables accounting for 60–80% of our daily intake.

*Paragraph Number 2*Tissue acidosis and low oxygen tension are present during physical exercise. In this metabolic state, the reduction of nitrite is probably greatly enhanced. Recent studies have reported that dietary nitrate supplementation decreases whole body oxygen consumption (VO_2) at low and moderate intensities of exercise in healthy subjects (1,2,15). Additionally, two recent studies showed that VO_2 values decreased significantly at higher intensities of exercise after several days of dietary nitrate supplementation (14,18). Although several hypotheses have attempted to explain how nitrate administration reduces the O_2 cost of exercise, the exact mechanism is currently unclear. The first research group to report the effects of nitrate supplementation on cardiorespiratory adaptation to exercise suggested that much of the O_2 reduction is due to the improvement in mitochondrial respiration with an increase in the P/O ratio (17). A recent study by Bailey et al. (1) suggested that this response could be derived from a reduction in phosphocreatine (PCr) degradation, which diminishes the ATP cost of muscle force production.

Paragraph 3 Currently, it is known that the exercise response is different in highly trained athletes and the untrained population. Chronic exercise training induces improvements in vascular structures, muscle tissues and the metabolism of NO (23,31). To date, studies have failed to report an improvement in the cardiorespiratory response in an athletic population using the classical precursor of NO (L-arginine) (4,19). However, in elderly populations with endothelial dysfunction, L-arginine supplements effectively enhance exercise capacity (9). Additionally, a recent study by Koppo et al. (13) reported that L-arginine supplementation speeds VO_2 kinetics in healthy males. Other studies using supplementation with L-citrulline (an alternative precursor of NO) showed a significant increase in plasma L-arginine concentration, but no effects on performance, in well-trained cyclists (29,30). Moreover, previous studies of

nitrate supplementation (1,2,14,15,18) assessed the effect of prolonged supplementation (between 3 and 6 days) in an attempt to increase the systemic levels of nitrate and nitrite. However, there is evidence of acute effects of nitrate on the cardiovascular system, since it lowers blood pressure 3 h after ingestion in healthy subjects (36). One very recent study assessed the effect of acute ingestion of nitrateon physically active people, but these subjects were not highly-trained (34).

*Paragraph Number 4*Accordingly, in this study we aimed to assess the effect of a single dose of nitrate given before cycling exercise on the cardiorespiratory and metabolic response in endurance athletes at different intensities. Moreover, we investigated the influence of nitrate supplementation on plasma levels of nitrateandnitrite over time. We hypothesized that dietary nitrate may not be effective in improving the cardiorespiratory adaptation to exercise at low to moderate intensities who are highly adapted to cycling. However, at higher intensities, at which acidosis and low oxygen tension occur, the nitrate–nitrite–NO pathway could be activated and increase tolerance to high intensity cycling, which is measured as the time to task failure.

Methods

Subjects

*Paragraph Number 5*Eleven male cyclists and triathletes (age 34.3 ± 4.8 yrs; body weight 73.3 ± 5.6 kg^{-1}; body mass index 23.7 ± 1.5 $kg \cdot m^{-2}$;VO_{2peak}65.1 ± 6.2 $mL \cdot kg^{-1} \cdot min^{-1}$; sum of six skinfolds [triceps, subscapular, supraspinal, abdominal, medial calf and front thigh] 55.5 ± 13.8 mm) volunteered to participate in this study. Athletes were members of competitive cycling or triathlon squads and none of them reported any medical conditions at the time of the study. None of the subjects smoked tobacco. The procedures employed in this study were approved by the Ethics Committee of the Catalonian Sports Council. All subjects gave their written informed

consent after an explanation of the experimental procedures and before the commencement of the study.

Nitrate supplementation

*Paragraph Number 6*Subjects were randomly assigned in a double-blind, crossover design to receive a single dose of either sodium nitrate (10 $mg \cdot kg^{-1}$ of body mass; Acofarma, code 18211, Spain) or the placebo (sodium chloride) dissolved in 250 mL of water. The two drinks could not be distinguished by taste or appearance. The beverage was ingested 3 h before the test, since this period of time is consistent with the pharmacokinetics of nitrate and the peak of circulating nitrite indicated in previous studies (36). During this period, the subjects remained under resting conditions in the laboratory and did not ingest food and fluids, apart from water, to guarantee hydration status. A diet with low levels of moderate or high nitrate content foods (green vegetables, beetroot, strawberries, grapes and tea) was followed for three days prior to the tests. During this time, athletes received nutritional guidelines and were encouraged to follow a high carbohydrate diet to optimize glycogen deposition. In addition, they were told to avoid alcohol, caffeine products and dietary supplements 48 h prior to the exercise test. A 7-day washout separated the supplementation periods.

Ergometry test

*Paragraph Number 7*The subjects were required to report to the laboratory on three occasions. The first test was carried out to familiarize the subject with the bicycle ergometer, gas analyzer and the testing procedure. The next two tests were performed under identical conditions and used to assess the effect of the dietary nitrateand placebo. Tests were carried out during the cycling off-season in November and December, to ensure that training or competitions would not affect the results of study. All tests were performed at the same time of day (± 1 h) on an electronically

braked cycle ergometer (Lode Excalibur Sport, Netherlands) under controlled conditions (22 ± 1ºC, 40-60% relative humidity, P_b 760-770 mmHg). Before and after the study, the cycle ergometer was calibrated for power outputs of 25–1000 W at different cadences and was found to be within 1% of a true value. The participants cycled at a self-selected pedal rate of between 70-100 rpm. This pedal rate, along with saddle and handle bar height and configuration, was recorded and reproduced in subsequent tests. The protocol of the test was divided into two parts: submaximal and maximal exercise intensity. Initially, the subjects completed four submaximal workloads corresponding to 2.0, 2.5, 3.0 and 3.5 $W \cdot kg^{-1}$ of body mass with every load lasting for 6 min, interspersed with three minutes of passive recovery. Five minutes after completion of the submaximal workloads, subjects performed a continuous incremental exercise test to volitional exhaustion. Starting at 3.0 $W \cdot kg^{-1}$, the work rate increased by 0.5 $W \cdot kg^{-1}$ every minute until task failure as a measure of exercise tolerance. The maximal power output (W_{max}) was calculated using theformula:

$$W_{max} = W_E + (W_I / t \cdot t_E)$$

Where W_{max}= maximal power output (W); W_E = power output of the last stage completed (W); W_I = work rate increment (W); t = workload duration (s); t_E= duration of the final stage (s).

Gas analysis

*Paragraph Number 8*During all the tests, oxygen uptake (VO_2), minute ventilation (V_E), carbon dioxide production (VCO_2) and the respiratory exchange ratio (RER) were measured breath-by-breath by a computerized gas analyzer (Cosmed Quark PFT-Ergo, Italy). Before each test, ambient conditions were measured and the gas analyzers and respiratory flowmeter were calibrated with high-precision calibration gases (16.00 ± 0.01% O_2 and 5.00 ± 0.01% CO_2, Scott

Medical Products, USA) and a 3-L calibration syringe (Hans Rudolph, USA), respectively, following the manufacturer's instructions.

Data analysis procedures

*Paragraph Number 9*Breath-by-breath VO_2 data from submaximal bouts of exercise were initially examined to exclude errant breaths caused by coughing, swallowing, sighing, etc. Values lying more than 4 SDs from the local mean were removed. The first 20 s of data after the onset of exercise (i.e. the phase I, cardiodynamic component) were deleted, and a nonlinear least squares algorithm was used to fit the data thereafter (SigmaPlot 8.0, SPSS Inc., USA). A single-exponential model was used to analyze the oxygen uptake kinetics of four submaximal rates of exercise, as described in the following equation:

$$VO_2\,(t) = VO_{2baseline} + A_p\,[1 - e^{-(t-TDp/\tau p)}]$$

*Paragraph Number 10*Where VO_2 (t) represents the absolute VO_2 at a given time; $VO_{2baseline}$ represents the mean VO_2 in the baseline period; A_p, TD_P and τ_p represent the amplitude, time delay, and time constant, respectively, describing the phase II (i.e. primary component) increase in VO_2 above baseline. $VO_{2baseline}$ and end-exercise VO_2 were defined as the mean VO_2 measured over the final 30 s before starting each submaximal workload and over the final 30 s of each submaximal workload, respectively. In addition, the gross efficiency (GE) was calculated as the mean of the data collected in the last 180 s of every submaximal workload in the steady-state with RER < 1.0 using the formula:

$$GE\ (\%) = \text{Work Rate (W)} / \text{Energy Expended } (J \cdot s^{-1}) \cdot 100$$

The energy expenditure was in turn calculated with the Brouwer equation (7):

$$\text{Energy expenditure } (J \cdot s^{-1}): [(3.869 \cdot VO_2) + (1.195 \cdot VCO_2)] \times (4.186 / 60) \cdot 1000$$

*Paragraph Number 11*The VO_{2peak} during the incremental test was determined as the mean VO_2 measured over the final 60 s of exercise. To determine the ventilatory threshold (VT) and the respiratory compensation point (RCP), the data were averaged at 30 s intervals and analyzed by two independent reviewers, according to methods described by Wasserman et al (35). Heart rate (HR) was continuously recorded during the test with a portable heart rate monitor and HR_{max} was defined as the HR at the point of exhaustion (Polar RS800 SD, Finland).

Blood sampling

*Paragraph Number 12*A small catheter was inserted into an antecubital vein for venous blood sampling. Four blood samples were collected to analyze nitrateand nitrite: 1) during resting conditions; 2) 3 h after supplement or placebo ingestion; 3) in the first minute after the fourth submaximal load; 4) in the first minute after the maximal test. Venous blood was drawn with a 5-mL syringe EDTA and was immediately centrifuged at 1,000 g for 20 min to separate plasma from blood cells. Plasma samples were then centrifuged for 30 min at 14,000 g in 10K filters (Amicon Ultra, Millipore) to remove proteins.The supernatant was recovered and used to measure nitrite and nitrate levels by detecting the liberated NO in a gas-phase chemiluminescence reaction with ozone using a nitric oxide analyzer (NOA 280i, Sievers).

*Paragraph Number 13*Nitrate levels were determined following an adaptation of the method described by Braman(6). Briefly, the purge vessel was loaded with a saturated VCl_3 solution in 1M HCl and heated to 90ºC with a current of hot water. To prevent damage to the NOA from the hydrochloric acid vapor, a gas bubbler filled with 1M NaOH was installed between the purge vessel and the NOA. A nitrate standard (5 - 200 μM) was used to calculate the nitrate concentration. Ten microliters of the filtered sample or standard were injected into the purge

vessel and the area under the curve of NO peaks was recorded and processed using NOAnalysis™ Liquid software v. 3.2 (IONICS, Boulder, CO, USA).

*Paragraph Number 14*Nitrite levels were determined following an adaptation of the method described by Castegnaro(8). Briefly, the purge vessel was loaded with 50 mM KI in glacial acetic acid and 400 µL of antifoam. A nitrite standard (0.5 – 10 µM) was used to calculate the nitrite concentration. One hundred microliters of the filtered sample or standard were injected into the purge vessel and the area under the curve of NO peaks was recorded and processed using NOAnalysis™ Liquid software v. 3.2 (IONICS, Boulder, CO, USA).

*Paragraph Number 15*In addition, seven samples of capillary blood (10 µL) were collected from the ear lobe to analyze lactate ([Hla]) using a Lactate Photometer plus DP100 (Diaglobal GmbH, Germany): 1) during resting conditions; 2) in the first minute after each submaximal load, and 3) at three and five minute after the maximal test.

Statistics

*Paragraph Number 16*Results are expressed as means ± standard error of the mean. A paired *t*-test was used to evaluate the differences between the placebo and the nitrategroups, where appropriate. To investigate the influence of time and treatment, the data were treated with two-way analysis of variance (ANOVA) with repeated measures on both time and treatment. The data were assessed to determine the normal distribution, and post-hoc analyses were performed via Tukey's HSD. The significance level was set at $P < 0.05$, while a trend was noted when $P < 0.10$.

Results

Plasma nitrateand nitrite kinetics

*Paragraph Number 17*The concentrations of nitratewere similar (nitrate: 30 ± 12 µM; placebo: 28 ± 10 µM) prior to intake. Three hours after ingestion, the plasma levels of nitrate had

increased significantly in the nitrategroup (250 ± 80 μM; $P < 0.001$), but remained unchanged in the placebo group (29 ± 8 μM). The nitrate concentrations in plasma were not affected at any sample point after placebo treatment (Figure 1). After nitrate supplementation, the plasma levels were significantly lower after submaximal (234 ± 82 μM; $P = 0.027$) and maximal (237 ± 85 μM; $P = 0.045$) exercise compared with the peak value reached 3 h post-supplementation (250 ± 80 μM) (Figure 1).

*Paragraph Number 18*There were no differences between treatments in the levels of nitrite under fasting conditions (nitrate: 2,005 ± 158; placebo: 2,053 ± 278 nM). Conversion of nitrate to nitrite was evident from the increased plasma nitrite levels 3 h after nitrate supplementation (2,313 ± 157 nM; $P = 0.017$) compared to the placebo (1,998 ± 206 nM). During nitrate treatment, nitrite levels were significantly lower after maximal exercise (2,126 ± 251 nM; $P = 0.044$) than the peak value reached 3 h post-supplementation (2,313 ± 157 nM) (Figure 1). Nitrite also tended to be lower after the placebo treatment and maximal exercise (1,916 ± 168 nM) than under fasting conditions (2,053 ± 278 nM; $P = 0.056$) (Figure 1).

Submaximal work parameters

*Paragraph Number 19*The cardiorespiratory values during the four bouts of exercise after nitrate supplementation and the placebo are shown in Table 1. There were no significant differences between the nitrate and placebo in VO_2, VCO_2, VE, RER, HR and Gross Efficiency. In addition, we did not find changes in the time constant and primary amplitude of VO_2 at any submaximal load (Table 1). The mean work rate was 147 ± 11 W at 2 $W \cdot kg^{-1}$, 183 ± 14 W at 2.5 $W \cdot kg^{-1}$, 220 ± 17 W at 3 $W \cdot kg^{-1}$ and 257 ± 20 W at 3.5 $W \cdot kg^{-1}$. The chosen cadence was 87 ± 8 rpm on the two occasions (nitrate and placebo).

Maximal work parameters

*Paragraph Number 20*After nitratesupplementation, VO_{2peak} dropped from 4.82 ± 0.33 to 4.64 ± 0.35 $L \cdot min^{-1}$ (P = 0.010) (Table 2). In addition, VO_2 tended to be lower at the respiratory compensation point after nitrate supplementation (4.31 ± 0.28 $L \cdot min^{-1}$) than after the placebo (4.44 ± 0.23 $L \cdot min^{-1}$; P = 0.068) (Table 2). The ratio between oxygen consumption and power was significantly decreased at the VO_{2peak} level after nitrate ingestion (P = 0.031) (Figure 2). Other cardiorespiratory parameters such as heart rate, pulmonary ventilation and carbon dioxide production were unaffected by nitrate supplementation. There was no significant difference in time to exhaustion between treatments (nitrate: 416 ± 32 s; placebo: 409 ± 27 s; P = 0.169) at the maximal intensity of exercise. The workload at VO_{2peak} was 416 ± 29 W for nitrate and 410 ± 28 W for the placebo (P = 0.318).

Blood lactate concentration

*Paragraph Number 21*No differences were found in blood lactate accumulation between conditions at any point of submaximal or maximal exercise intensity (Figure 3).

Discussion

*Paragraph Number 22*In agreement with our first hypothesis, this research showed that cardio-respiratory adaptation at low to moderate intensities of exercise was not modified by a single administration of nitrate (10 $mg \cdot kg^{-1}$) in well-trained cyclists. Although our second hypothesis of nitrate-induced enhancement of tolerance to high intensity cycling was not confirmed, we found that the VO_{2peak} was significantly reduced without affecting the maximal attainable work, blood lactate or other cardiorespiratory parameters. This was coupled with consumption of plasma nitrite mainly in the nitrate group, which probably indicates a reduction of this anion to NO and other bioactive nitrogen species.

Effects of an acute dose of nitrate on blood levels of nitrate and nitrite

*Paragraph Number 23*The levels of plasma nitrate had increased by 86.9 ± 8.4% ($P < 0.05$) 3 h after supplementation compared to the placebo, which is consistent with previous studies (1,2, 15,18,36). Additionally, we found that plasma nitrate was significantly lower after submaximal (234 ± 82 μM; $P = 0.027$) and maximal (237 ± 85 μM; $P = 0.045$) exercise than its values at 3 h post-supplementation (250 ± 80 μM). This fact is difficult to attribute to the effect of exercise alone, as the level of nitratewas no different after the incremental than after submaximal exercise. Previous research showed that nitrateremained stable after exercise (15). One likely explanation for this finding is related to the pharmacokinetics of nitrateafter dietary ingestion. There is evidence that the plasma levels of nitrateincreased rapidly within 30 minutes after nitrate supplementation to peak at 1.5 h (18,36). The half-life of plasma nitrate in humans is approximately 5 h and there is a substantial decrease after 4 h of ingestion (36). In this study, the timing was at the borderline of the nitrate half-life, since athletes completed submaximal and maximal workloads at 3 h and 45 min (± 10 min) and 4 h and 5 min (± 14 min), respectively. Additional studies are needed to pinpoint the exact mechanisms behind this finding.

*Paragraph Number 24*Nitrite takes longer to appear in the circulation than nitrate, peaking between 2.5 to 3 h (36). This delay is due to the enterosalivary circulation of these compounds. The vast majority of absorbed nitrate is ultimately excreted in the urine, but up to 25% of plasma is also excreted in the saliva (22). In the oral cavity, commensal facultative anaerobic bacteria reduce nitrate to nitrite by the action of nitrate reductase enzymes. The nitrite is swallowed and, in the acidic environment of the stomach, it is reduced to NO or reenters the circulation as nitrite (21). As inorganic nitrite is the main precursor of NO and other bioactive nitrogen oxides, we decided on a 3 h period between supplement ingestion and the start of exercise to ensure that the

nitrite in plasma had peaked. Curiously, basal levels of nitrite in this study were higher than in previous studies of healthy populations (1,15,18,34). These differences may be due to methodological issues or the present subjects' high level of training. Accordingly, Rassaf et al. (25) showed that plasma nitrite is directly proportional to exercise capacity. Another recent study showed higher levels of nitrite in the Tibetan population, as a consequence of adaptation to altitude (11). Interestingly, inhabitants of high altitudes had higher maximal work rates than inhabitants of lower altitudes (11). To sum up, more studies are needed to establish the normal levels of plasma nitritein highly trained athletes.

Effects of an acute dose of nitrate on the physiological response to low to moderate exercise

*Paragraph Number 25*We found no statistical differences in cardiorespiratory adaptation to exercise at low to moderate intensities between the nitrateand placebo groups (Table 1). In contrast, previous studies showed significant improvements in exercise efficiency at low to moderate intensities after dietary nitratesupplementation (1,2,15). The present study design differed from these studies in two main aspects. The first is the duration of the treatment. While previous studies followed several days (between 3 and 6) of nitrate supplementation, we assessed the effect of only one dose before exercise. Interestingly, we showed that an acute dose of sodium nitrateequivalent to 10 $mg \cdot kg^{-1}$, produced a similar increase in plasma nitrate (218 ± 68 μM) as a 3-day supplement of sodium nitrate (212 ± 28 μM), in which 8.5 $mg \cdot kg^{-1} \cdot day^{-1}$ were ingested (18). Thus, the difference in VO_2 response to submaximal exercise between studies is probably not due to the availability of plasma nitrate. The second difference was the characteristics of the subjects analyzed in each study. All previous studies have been carried out in healthy volunteers with a VO_{2peak} between 45 and 58 $mL \cdot kg^{-1} \cdot min^{-1}$(1,2,14,15,18,34). In our study, all subjects were endurance athletes with high VO_{2peak} (65.1 ± 6.2 $mL \cdot kg^{-1} \cdot min^{-1}$). In this

regard, training may alter the physiological response to exercise. Mitochondrial volume and aerobic capacity in type II fibers increase greatly in endurance athletes (12). Decreases in submaximal oxygen uptake after endurance training may be due to changes in the working muscle's oxidative capacity and metabolic processes, represented by an increase in the activity of the mitochondrial enzymes (33). Evidence to support this argument is that NO production appears to be a temporary response to chronic exercise that progresses to structural vascular and muscle adaptations (23). Nevertheless, other anatomical, biochemical and biomechanical (pedaling technique) factors, among others, should not be excluded as they may contribute to the improvement of movement efficiency when normal and athletic populations are compared (12). Collectively, the data obtained at this moment suggest that the effects of acute nitrate supplementation at low to moderate intensities of exercisemight be more limited in endurance trained athletes than in moderately trained subjects.

Effects of an acute dose of nitrate on the physiological response to maximal exercise

*Paragraph Number 26*Nitrate supplementation showed atrend towards reducing O_2 cost of exercise when athletes exceeded the RCP point. Differences between nitrate and placebo conditions became significant at maximal intensity of exercise (VO_{2peak}). Additionally, we found that the mean ratio between VO_{2peak} and W_{peak}fell significantly after dietary nitrate ingestion. These findings confirm results reported by two recent studies when moderately trained subjects were supplemented for 3 and 6 days with inorganic nitrate and nitrate-rich beetroot juice, respectively (14,18). However, these surprising reductions in VO_{2peak} and in the ratio of VO_{2peak} and W_{peak}were not linked with impairment of performance. We found that tolerance to exercise measured as time to exhaustion was maintained after nitrate supplementation (nitrate: 416 ± 32 s; placebo: 409 ± 27 s). This physiological change occurred without any effect on other

cardiorespiratory parameters (HR, VE, VCO_2 and RER), as well as lactate concentrations, which suggests that the reduction inVO_{2peak}could not be originated from alterations in the energetic cost of cardiorespiratory support processes.

Paragraph Number 27 However, the mechanistic bases for the reduced VO_{2peak} following nitrate ingestion have not been described in full. It is known that the nitrate–nitrite–NO pathway is gradually activated as the oxygen supply is limited and nitrite is converted to NO under hypoxic and acidic conditions (21). Therefore, as maximal intensity of exercise reproduces these physiological conditions, synthesis of NO could be derived by nitrite oxidation. Interestingly,in the current study, when athletes were supplemented with nitrate prior to exercise it was found that plasma nitrite levels decreased significantly just after finishing maximal workload, suggesting activation of nitrate–nitrite–NO pathway (Figure 1). There is evidence that NO donors which evoke small increase in NO improve muscle metabolism, preventing an excess of calcium release and subsequently modulating the ATP cost of force production (26). In addition, it is known that one of the most energetically costly processes during skeletal muscle contraction is sarcoplasmic reticulum calcium pumping, which may account for up to 50% of the total ATP turnover (3). From this viewpoint, a recent study by Bailey et al. (1) found that a decrease in O_2 cost of exercise after dietary nitrate supplementation was related to a reduction in ATP cost of muscle force production. On the other hand, it is widely accepted that NO is involved in the regulation of mitochondrial O_2 consumption. In mitochondria, a reduction in the O_2 cost of ATP resynthesis would require either more protons to be pumped per O_2 molecule reduced or the use of an alternative terminal electron acceptor. Recent studies have shown that demands of mitochondrial oxygen consumption increase*in vitro* when NO donors are added (5) and decrease*in vivo* when the NOS inhibitor L-NAME is added (27). In relation to these findings, an

interesting study by Larsen et al. (17) indicates that mitochondrial respiration, measured *in vitro* as the amount of oxygen reduced per ATP produced (P/O ratio), is significantly improved after dietary nitrate supplementation in humans. However, all these findings, including the reduction of ATP cost of force production reported by Bailey et al. (1) as well as the improvementin mitochondrial function indicated by Larsen et al. (17) after nitrate supplementation, have been reported only when subjects performed exercise at low to moderate intensity. Currently, it is unclear whether the fall in the VO_{2peak}found in the current study could be explained by these metabolic mechanisms or whether there are other pathways linked to this intriguing physiological response. Further research is needed to elucidate the mechanistic bases of VO_{2peak} reduction in well-trained athletes after dietary nitrate consumption.

*Paragraph Number 28*In conclusion, acute dietary nitrate administration 3 h before an exercise test increases plasma levels of nitrateand nitrite. In contrast with previous studies carried out in moderate trained subjects, we did not find that nitrate supplementation enhances cardiorespiratory adaptation to exercise at low to moderate exercise intensity. However, we found that the VO_{2peak} was significantly reduced when athletes ingested nitrate. These *in vivo* data were found without any changes in cardiorespiratory and performance parameters, which suggests that nitrate and its reaction products could play an important rolein oxygen consumption at maximal intensity of exercise in well-trained athletes.

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Legends

Figure 1. Plasma nitrate and nitrite change (Δ) relative to pre-supplementation baseline in plasma (n= 11).

* Statistical significance between nitrate and placebo ($P< 0.05$).

Statistical significance in nitrate levels between 3 h post-supplementation and after submaximal and maximal workloads of exercise ($P< 0.05$).

‡ Statistical tendency in placebo condition between nitrite levels at 3 h and after maximal test of exercise ($P< 0.10$)

Figure 2. Rate between oxygen consumption and power at ventilatory threshold (VT), at respiratory compensation point (RCP) and at peak of oxygen consumption (VO_{2peak}) (n= 11).

* Statistical significance between nitrate and placebo ($P< 0.05$).

Figure 3. Plasma lactate concentration at rest conditions, after every submaximal workloads equivalents to 2.0, 2.5, 3.0 and 3.5 $W \cdot kg^{-1}$, and at three and five minutes after maximal exercise in both conditions (nitrate and placebo).

Figure 1

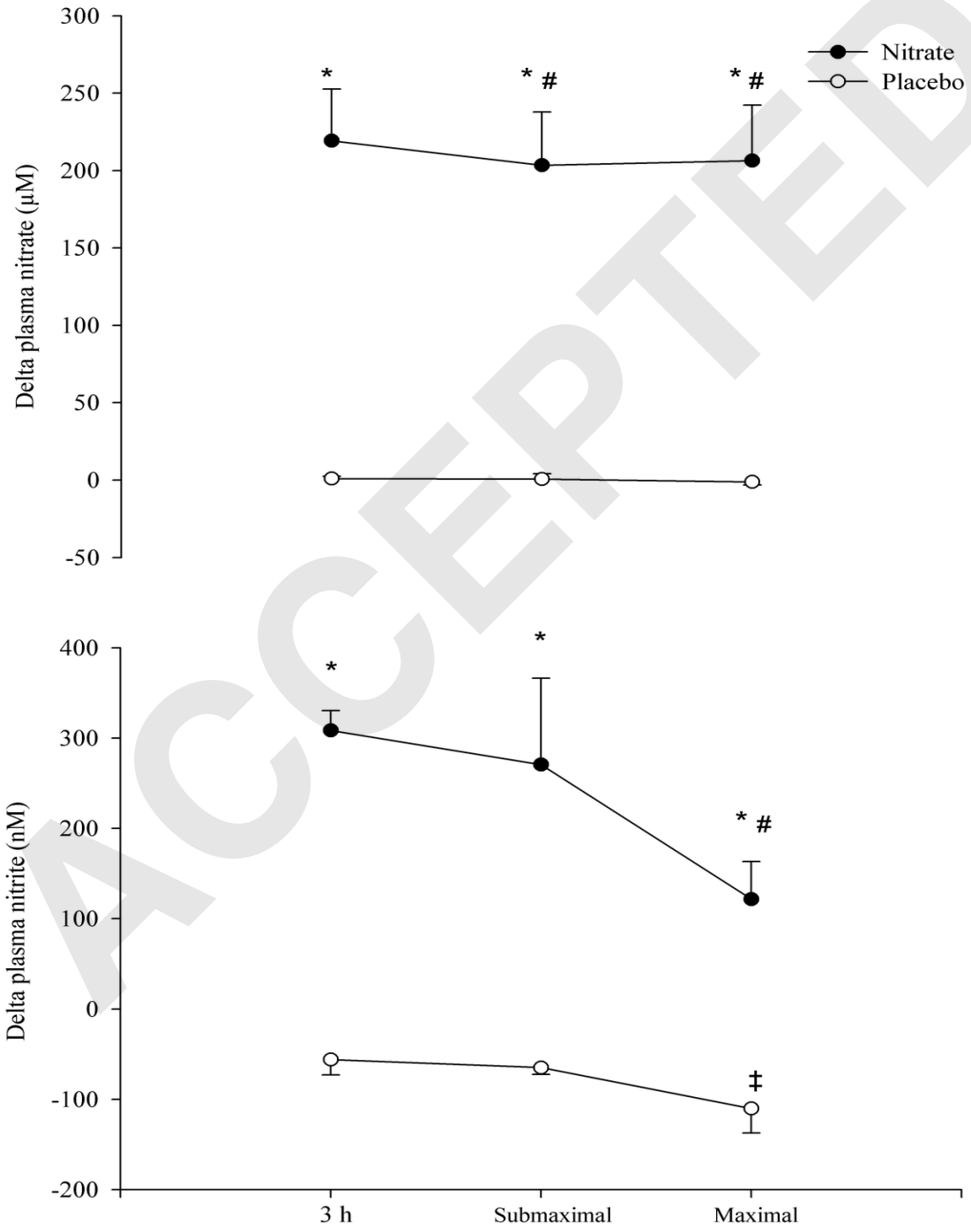

Figure 2

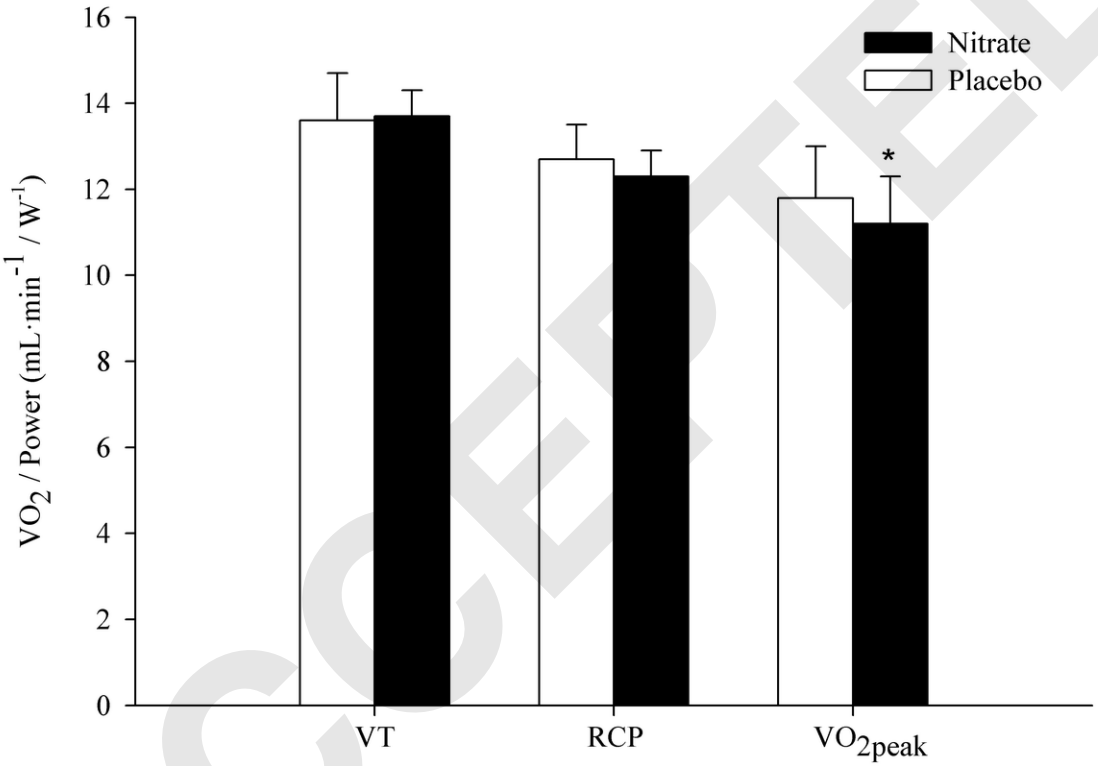

Figure 3

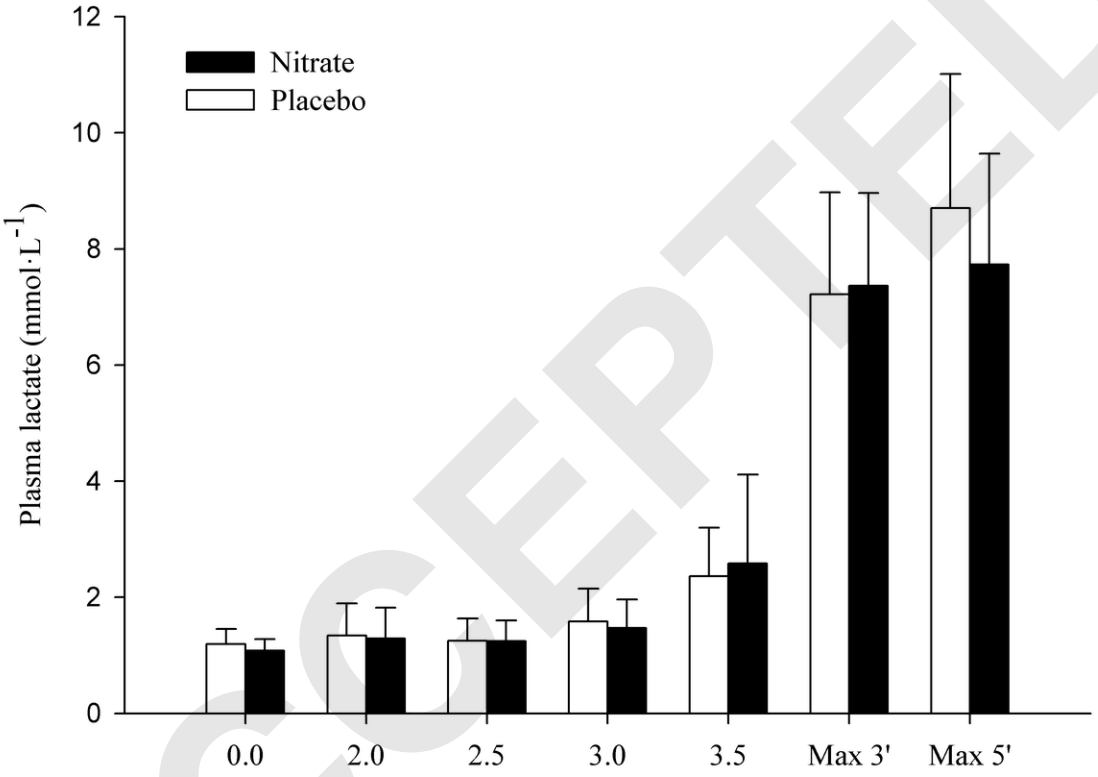

Table 1. Cardiorespiratory dynamics during low-moderate intensity exercise after supplementation with nitrate or placebo (n= 11).

Load	2.0 W·kg^{-1}		2.5 W·kg^{-1}		3.0 W·kg^{-1}		3.5 W·kg^{-1}	
Treatment	Placebo	Nitrate	Placebo	Nitrate	Placebo	Nitrate	Placebo	Nitrate
VO_2								
Baseline (L·min^{-1})	0.42 ± 0.07	0.45 ± 0.09	0.44 ± 0.07	0.41 ± 0.07	0.47 ± 0.08	0.43 ± 0.10	0.53 ± 0.06	0.50 ± 0.11
End exercise (L·min^{-1})	2.37 ± 0.23	2.33 ± 0.26	2.81 ± 0.28	2.74 ± 0.22	3.29 ± 0.29	3.19 ± 0.27	3.74 ± 0.33	3.68 ± 0.32
Time constant, τ (s)	13.4 ± 5.4	14.7 ± 3.5	14.5 ± 5.3	14.3 ± 4.3	16.6 ± 6.2	15.4 ± 4.4	21.5 ± 10.9	22.0 ± 8.6
Primary amplitude (L·min^{-1})	1.98 ± 0.21	1.90 ± 0.23	2.38 ± 0.23	2.30 ± 0.22	2.80 ± 0.27	2.75 ± 0.28	3.22 ± 0.27	3.17 ± 0.26
VCO_2								
Baseline (L·min^{-1})	0.31 ± 0.06	0.35 ± 0.08	0.41 ± 0.08	0.41 ± 0.07	0.44 ± 0.08	0.42 ± 0.10	0.50 ± 0.07	0.46 ± 0.12
End exercise (L·min^{-1})	1.99 ± 0.23	2.01 ± 0.22	2.44 ± 0.30	2.39 ± 0.17	2.77 ± 0.44	2.77 ± 0.24	3.33 ± 0.41	3.34 ± 0.30
VE								
Baseline (L·min^{-1})	10.8 ± 1.9	12.1 ± 2.7	14.2 ± 10.6	14.4 ± 2.8	15.6 ± 2.7	15.1 ± 2.9	18.0 ± 2.6	17.1 ± 4.0
End exercise (L·min^{-1})	49.9 ± 6.4	51.0 ± 6.3	59.8 ± 7.0	59.6 ± 6.6	69.1 ± 10.0	70.5 ± 8.3	84.2 ± 13.7	85.8 ± 12.9
RER								
Baseline	0.73 ± 0.08	0.77 ± 0.08	0.92 ± 0.10	0.98 ± 0.10	0.94 ± 0.06	0.97 ± 0.08	0.96 ± 0.12	0.93 ± 0.13
End exercise	0.84 ± 0.02	0.87 ± 0.07	0.87 ± 0.03	0.87 ± 0.05	0.84 ± 0.09	0.87 ± 0.06	0.89 ± 0.04	0.91 ± 0.04
HR								
Baseline (beats·min^{-1})	59 ± 6	59 ± 7	69 ± 7	70 ± 9	77 ± 7	79 ± 8	86 ± 9	84 ± 11
End exercise (beats·min^{-1})	111 ± 8	110 ± 10	126 ± 10	124 ± 11	142 ± 12	141 ± 14	156 ± 13	156 ± 14
Gross efficiency (%)	18.2 ± 1.3	18.4 ± 1.2	18.7 ± 1.3	19.4 ± 0.9	19.5 ± 1.4	19.9 ± 1.0	19.7 ± 1.4	20.1 ± 1.0

Values are means ± SD. VO_2: oxygen uptake; VCO_2: expired carbon dioxide; VE: minute ventilation; RER: respiratory exchange ratio; HR: heart rate

Table 2. Metabolic and circulatory response to maximal exercise after dietary supplementation with nitrate or placebo (n= 11).

Treatment	Placebo	Nitrate
VO_{2peak} ($L \cdot min^{-1}$)	4.82 ± 0.33	4.64 ± 0.35*
VO_2 at RCP ($L \cdot min^{-1}$)	4.44 ± 0.23	4.31 ± 0.28‡
VO_2 at VT ($L \cdot min^{-1}$)	3.52 ± 0.32	3.45 ± 0.23
VCO_{2peak} ($L \cdot min^{-1}$)	5.25 ± 0.45	5.18 ± 0.50
VE_{max} ($L \cdot min^{-1}$)	156.4 ± 16.5	151.0 ± 22.6
RER	1.09 ± 0.08	1.12 ± 0.07
HR_{max} ($beats \cdot min^{-1}$)	182 ± 14	182 ± 14

Values are means ± SD. VO_{2peak}: peak of oxygen uptake; VO_2 at RCP: oxygen consumption at respiratory compensation point; VO_2 at VT: oxygen consumption at ventilatory threshold; VCO_{2peak}: peak of expired carbon dioxide; VE: maximum minute ventilation; RER: respiratory exchange ratio; HR_{max}: maximum heart rate.
*Statistical significance between nitrate and placebo ($P < 0.05$).
‡ Statistical tendency between nitrate and placebo ($P < 0.10$).