The effect of nitric oxide donors on human performance

Raúl Bescós García

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The effect of nitric oxide donors on human performance

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A mis padres, Dora y Crescencio,

por todo lo que me han dado a lo largo de la vida.

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Bescós R, Rodríguez FA, Iglesias X, Ferrer MD, Iborra E, Pons A. *Acute administration of inorganic nitrate reduces VO_{2peak} in endurance athletes.* Med Sci Sports Exerc 2011; 43: 1979-1986. PMID: 21407132.

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The effect of nitric oxide donors on human performance

This thesis includes two parts. The first part presents an overall summary of the scientific work produced by the Ph.D. candidate structured in eight sections. The second part of this thesis is a compilation of the four published or under review articles where the Ph.D. candidate is the first author. These four selected articles are included in the annex section.

LIST OF ABBREVIATIONS

ADMA	Asymmetric dimethyl-arginine
ANT	Adenine nucleotide translocase
ATP	Adenosine triphosphate
BH4	Tetrahydrobiopterin
CO_2	Carbon dioxide
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
EDTA	Ethylene diamine tetraacetic acid
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GE	Gross efficiency
GH	Growth hormone
GTP	guanosine-5'-triphosphate
HCl	Hydrochloric acid
HR_{max}	Maximum heart rate
iNOS	Inducible nitric oxide synthase
K_m	Michaelis constant is a means of characterizing an enzyme affinity for a substrate
MDA	Malondialdehyde
$NaNO^3$	Sodium nitrate
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NADP	nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
O_2	Oxygen

PGC1α	Peroxisome proliferator-activated receptor-y coactivator 1- α
RCP	Respiratory compensation point
RER	Respiratory exchange ratio
UCP3	Uncoupling protein 3
VCO_2	Carbon dioxide production
Vd-NOS	Vía dependiente de la óxido nítrico sintetasa
VE	Respiratory ventilation
Vi-NOS	Vía independiente de la óxido nítrico sintetasa
VO_2	Oxygen consumption
VO_{2max}	Maximum oxygen consumption
VT	Ventilatory threshold
W	Watts

1. INTRODUCTION

1.1. The molecule: nitric oxide

Nitric oxide or nitrogen monoxide (NO) is a tiny free radical gas which was initially termed endothelium-derived relaxing factor (EDRF). The endogenous formation and biological significance of this intriguing molecule were revealed in a series of studies in the 1980s, and for these seminal discoveries three American researchers – Robert F. Furchgott PhD, Louis J. Ignarro PhD, and Ferid Murad PhD – were subsequently awarded the Nobel Prize in Physiology or Medicine in 1998.

Today, it is known that NO plays an important role in the bodies of all mammals. In the circulation system it has vasodilatory and anti-aggregatory properties. It activates soluble guanylyl cyclase (sGC), with a resulting formation of cyclic guanosine mono phosphate (cGMP). The cGMP then activates intracellular protein kinases ultimately, resulting in vascular smooth muscle relaxation and an increase of blood flow to tissue (1). In addition, NO has other important functions independent of cGMP. For instance, it is known that NO modulates mitochondrial respiration via cytochrome *c* oxidase (COX), the terminal enzyme in the mitochondrial electron transport system (2). Indeed, when the endogenous NO synthesis is blocked, blood pressure and tissue oxygen consumption increase (3). Furthermore, the chemical characteristics of NO also make this gas very reactive towards other free radical species, in particular superoxide (O_2^-), thereby forming peroxynitrite ($ONOO^-$) (4). Peroxynitrite itself is also reactive and when protonated it can decompose to form nitrogen dioxide ($NO_2^{\cdot}$) and hydroxyl radicals ($OH^{\cdot}$), both potent oxidants with potentially pathological consequences (5). The cytotoxic properties of NO and its reaction products are also utilized by white blood cells and other cells in response to infections from bacteria, viruses or parasites (6). The ability to stimulate sGC, inhibit mitochondrial respiration and the reactivity towards superoxide make NO very sensitive to the metabolic condition redox state of the tissue thus allowing it to act as a metabolic sensor and signaling molecule. All this evidence has led a revolution in physiology and pharmacology research during the last few decades.

1.2. Synthesis of nitric oxide

Levels of NO in the tissues are tightly regulated and determined by the balance between its production and consumption. When NO forms, it has a half-life of only a few seconds, depending on the surrounding chemical milieu and the metabolic state of the tissue where it is produced. Currently there is evidence of two endogenous NO synthesis mechanisms in the human body: the synthase-dependent pathway and the synthase-independent pathway.

1.2.1. The nitric oxide synthase-dependent pathway

The nitric oxide synthase-dependent pathway was the first metabolic route discovered (7). In this pathway the amino acid L-arginine is the main donor of NO. L-arginine is degraded, in the presence of NADPH and O_2, to L-citrulline and NO in a series of redox reactions which are catalyzed by nitric oxide synthases (NOS) (8). Three isoforms of NOS are recognized: type I (neuronal NOS, nNOS), type II (inducible NOS, iNOS) and type III (endothelial NOS, eNOS). The eNOS and iNOS are constitutive enzymes which are controlled by intracellular Ca^{2+}/calmodulin. In addition, iNOS expression has been linked to the expression of antioxidant enzymes such as superoxide dismutase (SOD) (9). The nNOS is inducible at the level of gene transcription, Ca^{2+} independent and expressed by muscle activity (10), the ageing process (11), and, by macrophages and other tissues in response to inflammatory mediators (12).

The activity of the various NOS enzymes can be affected by factors that influence the concentration of NOS proteins and cofactors or alter NOS expression and kinetic properties. One of the most important cofactors is tetrahydrobiopterin (BH4), which is synthesized from GTP via the GTP-cyclohydrolase-I (GTP-CH) pathway. Other cofactors in the synthesis of NO via the NOS-dependent pathway are flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and heme (1,13). Furthermore, in the human body there is also an endogenous inhibitor of NOS - asymmetric dimethyl-arginine (ADMA) - which impairs NO formation in certain pathological conditions. This compound competes with L-arginine for binding to NOS and thus competitively antagonizes the enzyme's catalytic activity, giving rise to the hypothesis that L-arginine may be beneficial in patients with elevated ADMA (14).

Diet also plays an important role in the regulation of NO production. It has been demonstrated that a high-fat diet with high levels of free fatty acids results in lower arterial eNOS phosphorylation, hypertension and vascular dysfunction as a result of free-fatty acid-mediated impairment of eNOS phosphorylation and NO production (1). The addition of high amounts of sucrose to a high-fat diet can also decrease whole body NO production and NO-mediated vascular response (15). In contrast, an increase in NOS activity and cofactors of NOS in brain, liver and muscle have been shown after dietary docosahexanoic acid supplementation (16). Diet-induced insulin release can also stimulate NO synthesis in endothelial cells by increasing the production of NADPH and BH4 in endothelial cells, which may modulate tissue blood flow (17). This insulin effect may be the result of a larger fraction of arginine flux converted to NO, as arginine flux is more reduced during acute hyperinsulinemia [for a recent review see (1)].

1.2.2. Sources and metabolism of L-Arginine

As mentioned above, L-arginine is the main donor for NO synthesis via the NOS-dependent pathway. This amino acid is considered a conditional essential proteinogenic amino acid that is a natural constituent of dietary proteins. L-arginine is relatively high in seafood, watermelon juice, nuts, seeds, algae, meat, rice protein concentrate and soy protein isolate (18). The typical dietary intake of L-arginine is approximately of 4–5 grams per day (19). Dietary L-arginine is absorbed in the small intestine and transported to the liver, where the major portion of plasma L-arginine (~ 70 %) is taken up by arginases enzymes. These liver enzymes participate in the fifth and final step of the urea cycle and regulate NO levels in endothelial cells by competing with NOS for the substrate L-arginine (20). Thus L-arginine also plays an important role in the metabolism of the urea cycle. The intracellular transport of L-arginine by the cationic amino acid transporter and the competition with lysine also determine its availability as a precursor of NO. A small part (~ 30 %) of dietary L-arginine bypasses the liver and is utilized as a substrate for NO production, as evidenced by animal and human studies that used ^{15}N-labeled L-arginine as a precursor (21).

L-arginine could also be endogenously synthesized. The main tissue in which endogenous L-arginine synthesis occurs is the kidney, where L-arginine is formed from L-citrulline (22). The liver is also able to synthesize considerable amounts of L-

arginine, although this is completely reutilized in the urea cycle. Thus the liver contributes little or not at all to plasma L-arginine flux (22). Normal plasma L-arginine concentrations depend upon the age of the individual and its homeostasis is primarily achieved via its catabolism (23). The usual range of L-arginine in plasma has been determined as 81.6 ± 7.3 $\mu mol \cdot L^{-1}$ in young males and 113.7 ± 19.8 $\mu mol \cdot L^{-1}$ in elderly males, as compared with 72.4 ± 6.7 $\mu mol \cdot L^{-1}$ in young females and 88.0 ± 7.8 $\mu mol \cdot L^{-1}$ in elderly females (24). Intracellular L-arginine levels have been demonstrated to be considerably higher than L-arginine levels in extracellular fluid or plasma. However, extracellular L-arginine can quickly be taken up by endothelial cells and produce NO.

L-arginine participates in several different metabolic pathways which were discovered during the 20th century. As indicated above, L-arginine participates in the urea cycle, in which ammonia is detoxified through its metabolism into urea (25). This reaction is catalyzed by arginase enzymes in the liver and subsequent L-arginine disintegration produces the amino acid L-ornithine, which is a precursor of polyamines, molecules essential for cell proliferation and differentiation (22). L-arginine is also required for the synthesis of creatine (22). In its phosphorylated form (creatine phosphate), creatine is an essential energy source for muscle contraction in short (< 10 seconds) and high intensity physical efforts. Furthermore, it has been suggested that dietary L-arginine at high doses stimulates the release of several hormones such as plasma insulin, glucagon, growth hormone (GH), prolactin and catecholamine concentrations (26). Finally, in the 1980s it was discovered that L-arginine is a precursor of NO by NOS isoenzymes. Although only a minor portion of L-arginine is metabolized via this pathway *in vivo*, it has attracted much interest in recent years because of the prominent role that NO plays in vascular physiology (22). This evidence has not gone unnoticed in exercise physiology, and interest in L-arginine supplementation has increased during the last decade to the point where it is, currently the most common substrate used in sport supplements promoted as NO donors.

1.2.3. The nitric oxide synthase-independent pathway

This is a new pathway which was discovered by two independent research groups during the 1990s (27-28). Nitrate (NO_3) and nitrite (NO_2) are the main precursors for NO synthesis in this alternative pathway. These compounds have long been considered inert oxidation products and have merely been used by researchers as markers of NOS

activity. However, new research evidence has seriously challenged this view, suggesting that a reverse pathway in which nitrate and nitrite are reduced back to NO exists in the human body (29-30). A number of enzymatic pathways exist in blood and tissues for the further one-electron reduction of nitrite to NO (30). These include deoxyhemoglobin in blood and also tissue proteins including deoxymyoglobin (31), neuroglobin (32), cyctochrome *c* oxidase (2), xanthine oxidoreductase (33), aldehyde oxidase (34) and even NOS itself (33). In circulation the nitrite reductase capacity of deoxyhemoglobin has been proposed as an allosteric-dependent and hypoxia-sensitive regulating mechanism of microcirculatory blood flow (35). The NOS-independent pathway might seem redundant when there are already three isoforms of NOS with high capacity for NO production. However, a fundamental difference between the two pathways is that the NOS-dependent pathway is oxygen dependent (36), while the nitrate-nitrite-NO pathway is greatly facilitated under hypoxia instead (37). Thus the latter pathway can be viewed as complementary to the NOS-dependent pathway and can also operate under conditions with low tissue oxygen tension, such as during exercise or tissue ischemia. In fact the low physiological oxygen tensions during muscular contractions and the relatively high K_m of the NOS for oxygen suggests that enzymatic production of NO will be compromised during exercise. Under these conditions nitrite could possibly act as an alternative source of NO.

1.2.4. Source and metabolism of inorganic nitrate

Dietary nitrate has traditionally been considered a carcinogenic substance and a toxic residue in our food and water. For this reason the amount of nitrate in food has been regulated for a long time and there currently exists an Acceptable Daily Intake (ADI) for humans of 5 mg sodium nitrate or 3.7 mg nitrate·kg^{-1} of body weight, which equals 222 mg for a 60-kg adult. The supposed carcinogenic mechanism is a nitrite-dependent formation of nitrosating agents which can react with dietary amines, forming nitrosamine substances with known carcinogenic properties (38). However, despite extensive research no casual link between dietary intake and gastric cancer in humans has been found (39). In fact this viewpoint is changing due to research performed over the past decade (30). It is now apparent that nitrate and nitrite are physiologically recycled in blood and tissues to form NO and other bioactive nitrogen oxides. Therefore

they should now be viewed as storage pools for NO-like bioactivity, thereby complementing the NOS–dependent pathway.

The main providers of nitrate in human diet are certain vegetables such as lettuce, spinach and beetroot, containing up to 740 mg·100 g^{-1} fresh weight (40). Drinking water and cured meats (bacon and ham), where nitrate is added as a preservative, can also contain considerable amounts of nitrate (41). In contrast, most fruits are low in nitrate. It has been estimated that nitrate consumption derived from food and beverages is on average 100-150 mg·day^{-1} in adults (42). Apart from diet, nitrate and nitrite are generated endogenously in our bodies. The NO generated by L-arginine and NOS enzymes is oxidized in the blood and tissues to form nitrate and nitrite (7). Thus the NOS-dependent pathway of NO synthesis significantly contributes to overall nitrate and nitrite production, which indicates an active recycling pathway for generating NO in the human body. Under normal conditions the contribution of nitrate and nitrite from dietary vs endogenous sources is roughly equal, but with a diet rich in vegetables the dietary source becomes dominant (41). Conversely, with massive activation of NOS as in systemic inflammation, for example, the contribution of nitrate from the NOS-dependent pathway increases dramatically (43). Therefore, although normal plasma levels of nitrate have been reported to be in the 20-40 μM range while nitrite levels are substantially lower (50-1000 nM), these values can be influenced by the activity of NOS or by adjusting the dietary intake of these two anions, as well as physical training (44-45). Additionally, another possible mechanism for manipulating plasma nitrite levels is to disrupt the enterosalivary pathway, either by using an antibacterial mouthwash or by spitting. Both these procedures have been used experimentally and they strongly attenuate the increase in nitrite seen after nitrate intake (46).

The bioactivation of nitrate to NO involves a peculiar enterosalivary pathway. By not yet fully defined mechanisms, circulating nitrate is actively taken up by the salivary glands and concentrated in the saliva (10 – 20 fold higher than in blood) (47). This massive concentration of nitrate can result in salivary nitrate levels of several millimolar. Up to 25% of all circulating nitrate enters this peculiar enterosalivary cycle, while the rest is excreted in the urine. In the oral cavity, facultative anaerobic bacteria on the surface of the tongue reduce nitrate to nitrite by the action of nitrate reductase enzymes (48). In the absence of oxygen, these bacteria use nitrate as an alternative

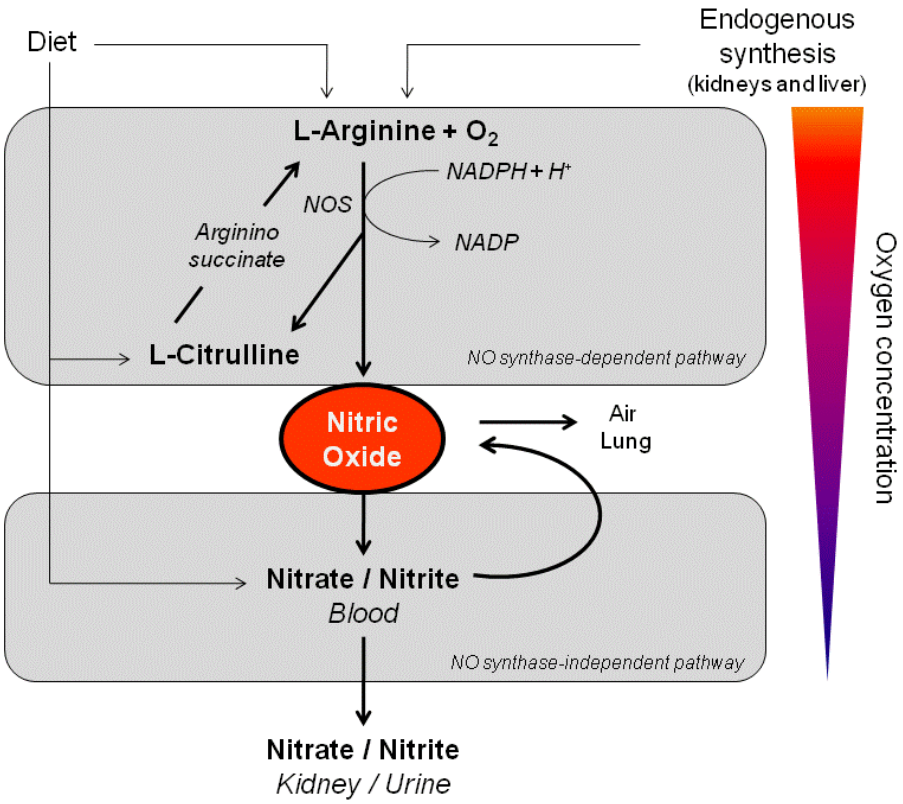


Figure I. Overview of the nitric oxide (NO) synthesis pathways. L-arginine is the main donor of NO via the synthase-dependent pathway. L-arginine originating from both dietary and endogenous sources can be reduced to NO and L-citrulline in the presence of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) and oxygen (O_2). This reaction is catalyzed by nitric oxide synthase enzymes (NOS). Once formed, one part of NO is exhaled via lungs and another part is oxidized to nitrate and nitrite, which are the main donors of NO via the synthase-independent pathway. Blood levels of nitrate and nitrite are regulated from oxidation of NO and by dietary consumption of food rich in these anions, such as green leafy vegetables. Around 75% of all circulating nitrate and nitrite are excreted in the urine, but the rest can be reduced back to NO. This mechanism is mainly activated under low tissue oxygen tension and acidosis conditions.

electron acceptor to gain adenosine triphosphate (ATP). When swallowed, one part of nitrite in the saliva is metabolized to NO locally in the acidic environment of the stomach, but the other part of swallowed nitrite is absorbed intact to increase circulating plasma nitrite (44). Such nitrite can be converted to NO and other bioactive nitrogen oxides in blood and tissues under appropriate physiological conditions (27). These findings demonstrate that a complete reverse pathway (nitrate–nitrite–NO) exists in mammals. This was very surprising as nitrate has been universally considered an inert end-product ever since NO formation in mammals was discovered 25 years ago. The

recognition of this mammalian nitrogen cycle has led researchers to explore the role of nitrate and nitrite in physiological processes involved in the regulation of the cardiorespiratory response during physical exercise.

1.3. Nitric oxide and exercise

Physical exercise can stimulate NO synthesis from endothelial cells by two mechanisms: chemical and physical. The chemical stimulus originates from the interaction of endogenous/exogenous agonists with specific receptors present in the endothelial cells, such as acetylcholine, ATP and bradykinin. Interestingly, physical exercise stimulates the release of all these molecules. For instance neuromuscular junction of motor nerves, which synthesize, store and release acetylcholine, is a physiological source of these compounds during exercise (49). In addition it is known that red blood cells can release ATP (49) and muscular activity increases the interstitial concentrations of bradykinin during high intensity exercise (50).

The physical stimulation is performed by the force that the blood applies to the artery walls, called shear stress. The mechanism by which shear stress promotes the formation of NO is yet to be clarified. It is known that endothelial cells have mechanoreceptors, which can directly activate G proteins, the ionic channels and enzymes from the protein-kinase and phosphatase group that will promote the formation of second messengers (cGMP). Studies in humans and animals have shown that the shear stress induced by physical exercise is a powerful stimulant for the release of vasorelaxing factors produced by the vascular endothelium (NO) and increases the expression of endothelial and neuronal NOS (51). For this reason, the beneficial effects of regular exercise on cardiovascular diseases have been mainly associated with a higher production of endothelium-derived vasodilating agents (NO).

It is therefore worth mentioning that physical exercise seems to have a protective effect on the endothelial integrity, either by increasing the NO production in vessels with preserved endothelium or by repairing the endothelial dysfunction. The magnitude of benefit of physical exercise depends on the intensity or volume of training. While short-term training rapidly increases NO bioactivity, if training is maintained, the short-term functional adaptation is succeeded by NO-dependent structural changes leading to

arterial remodeling and structural normalization of shear (52). This structural remodeling and consequent normalization of shear obviates the need for ongoing functional dilatation, including enhanced NO dilator system function. Apart from volume, it seems that a moderate-to-higher intensity of exercise may be required to impact endothelial function in healthy asymptomatic subjects who demonstrated preserved endothelial function *a priori* (53). All this evidence together raises the intriguing possibility of a threshold effect, volume and intensity, for physical training and mechanisms associated with NO production. However, at the present time it is not known where this limit of physical exercise lies.

1.4. Sport supplements related to nitric oxide

As regards sport supplementation, it has been suggested that dietary compounds related to NO such as L-arginine and inorganic nitrate are ergogenic aids. This claim is based on the potential effects on regulation of blood flow and mitochondrial respiration of NO during physical exercise (3, 54). It has been suggested that the increased blood flow derived from dietary NO donors such as L-arginine or nitrate may improve blood delivery and substrates of the activated tissues (55). This physiological response can enhance muscle work capacity and exercise performance. In addition, an increase in the overall workload stimulates stress on exercised muscle tissues, resulting in muscle hypertrophy of the activated muscles (**Figure II**). These supposed benefits have been claimed in many sport supplements which are currently sold on the market and linked with stimulation of NO production. However, a careful examination of the composition of NO-stimulating supplements shows that, in many cases, they are "cocktails" of a great variety of ingredients such as creatine, carbohydrates, amino acids, vitamins, minerals, etc. It is known that some of these components (creatine, carbohydrates and amino acids) may have an ergogenic effect in themselves (56-58). For this reason, only pharmaceutical products with the "key" component alone were used as supplementation in this thesis to avoid interference between dietary compounds.

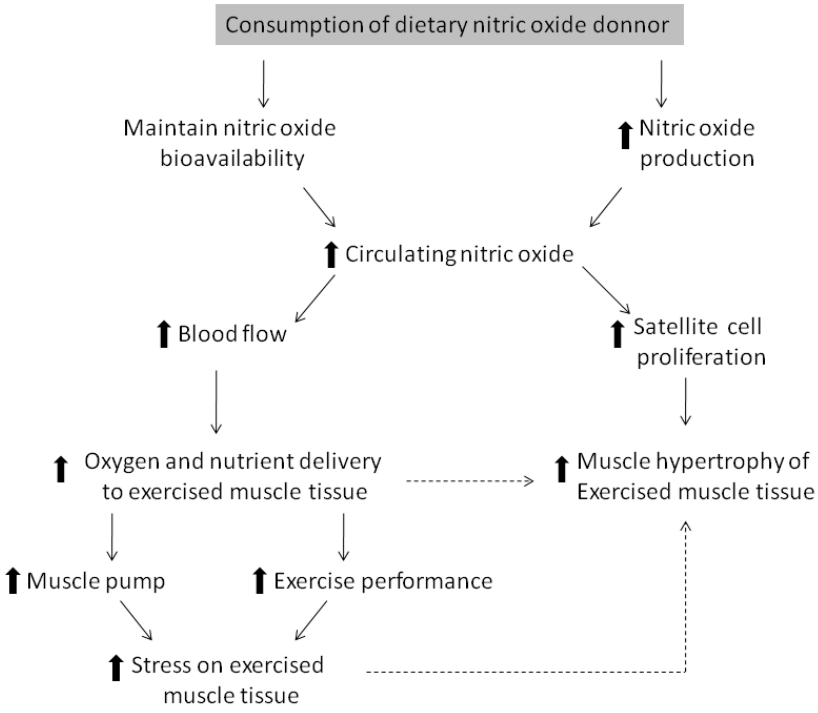


Figure II. Theoretical model for the proposed benefits of dietary nitric oxide supplements [adopted from Bloomer (55)]. It is suggested that dietary supplementation of NO donors can increase the bioavailability of NO in the body, increasing blood flow of the activated muscles and reducing intracellular perturbation (e.g. increasing delivery of oxygen and nutrient substrates, reducing levels of lactic acid, etc.). This physiological response could favor an increase in muscle work capacity and exercise performance. Furthermore, while NO donors may enhance workload capacity and performance, they may induce greater stress on exercised muscle tissue which will stimulate mechanisms such as satellite cell proliferation and hypertrophy to compensate this fact.

2. AIMS

The overall objective of this thesis is to examine the effect of nitric oxide supplements on human performance. More specific aims developed over time were:

1. To investigate the effect of dietary L-arginine on plasma NO markers and on cardio-respiratory response at low-to-moderate intensities of exercise in trained subjects *(Study I)*.
2. To investigate metabolic and cardiorespiratory response to exercise after an acute dietary nitrate supplementation in endurance-trained subjects *(Study II)*.
3. To examine whether dietary nitrate can enhance the endurance performance of trained subjects *(Studies II and III)*.
4. To analyze the effect of dietary nitrate ingestion in other molecules (endothelin-1) which participate in the regulation of blood flow during exercise (*Study III*).
5. To analyze the main conclusions of studies related to nitric oxide donors and human performance in healthy population *(Study-review IV*)

3. METHODS

A summary of the methods is given here. For more details the reader is referred to the individual articles.

3.1. Subjects

Thirty three healthy subjects volunteered to take part in studies in the present thesis. The characteristics of the subjects are summarized in **Table 1**. *Study I* comprised subjects who were competitive tennis players at a national level, while *Studies II* and *III* included current competitive cyclists and triathletes at a national level. The procedures were approved by the Ethics Committee of the Catalonian Sports Council.

Table I. Subject characteristics in the three studies which compose thesis (mean ± SD).

Study	*n*	Age (yr)	Body mass (kg)	Height (cm)	Body mass index ($kg \cdot m^{-2}$)	VO_{2max} ($mL \cdot kg^{-1} \cdot min^{-1}$)
Study I	9	18.2 ± 3.7	67.7 ± 8.7	175 ± 11	22.0 ± 1.9	57.4 ± 3.9
Study II	11	34.3 ± 4.8	73.3 ± 5.6	176 ± 6	23.7 ± 1.5	65.1 ± 6.2
Study III	13	32.6 ± 5.6	72.4 ± 9.7	175 ± 5	23.4 ± 2.0	59.7 ± 7.0

3.2. L-arginine supplementation

In *Study I* three randomized diets were designed. Control Diet (CD): a balanced composition of macronutrients (5.5 ± 0.3 g L-arginine); Diet 1: the same as CD but enriched as much as possible without unbalancing the composition of macronutrients with high L-arginine foods (9.1 ± 1.1 g L-arginine); and Diet 2: the same as CD but further including a oral supplement of 5 g of L-arginine (Arginaid, Novartis, ref. 40285, Barcelona, Spain) three times per day (20.5 ± 0.3 g L-arginine). Sodium intake was set at a constant level (about 160 $mol \cdot day^{-1}$). Each treatment involved three days of controlled diet consumption. A 4-day washout period was scheduled prior to the following stage of the experiment while subjects continued with their normal training. This time has been shown to be sufficient for metabolized oral L-arginine intake (59). To control that dietary guidelines were followed by athletes, all of them were living in the residence of the High Performance Center of Barcelona during the study.

3.3. Inorganic nitrate supplementation

In *Study II* the 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, Barcelona, 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 hours 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 (46). 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.

Similar than in the previous study, in *Study III* the subjects were randomly assigned in a double-blind, crossover design to follow three days of supplementation of either sodium nitrate (10 $mg \cdot kg^{-1}$ of body mass; Acofarma, code 18211, Barcelona, Spain) or the placebo (sodium chloride) dissolved in water. Supplementation was ingested each morning before breakfast. The last day it was ingested among two and three hours before to exercise test. In this study, low nitrate diet was followed two days prior to the tests. Similar than in *Study II*, all subjects received nutritional guidelines and were encouraged to follow a high carbohydrate diet to optimize glycogen deposition, as well as, to avoid alcohol, caffeine products and dietary supplements. Between both treatments (placebo and nitrate) it was included a 4-day washout period.

3.4. Respiratory analysis

In *Study I* a respiratory analyser Jaeger Oxycon Champion (Hoechberg, Germany) was used to measure fraction of CO_2 exhaled (VCO_2), ventilatory volume (VE), rate of ventilatory exchange (RER) and oxygen uptake (VO_2). To assess the same parameters, a respiratory gas analyser Cosmed Quark PFT-Ergo (Rome, Italy) and Jaeger Oxycon

Mobile (Hoechberg, Germany) were used in S*tudies II* and *III*, respectively. Before each test the gas analysers were calibrated with a high-precision two-component gas mixture (16.09 % O_2 and 5.08 % CO_2, Linde Abelló S.A, Barcelona, Spain in *Studies I* and *III*; 16.00 ± 0.01% O_2 and 5.00 ± 0.01% CO_2, Scott Medical Products, Boston, USA in *Study II*).

3.5. Heart rate

Heart rate was measured using the heart rate monitor Polar XtrainerPlus in *Study I*. In *Studies II* and *III* a heart rate monitor Polar RS800SD (Kempele, Finland) was used. Polar Precision Performance SW. v4 *(Study I)* and Polar Protrainer v5 (Kempele, Finland) *(Studies II and III)* software were used to transfer heart rate from monitor to computer.

3.6. Blood lactate

In *Study I* blood lactate concentration was measured through the use of the micro method Lactate Pro LT1710 (Mannheim, Germany). Diaglobal Lactate Photometer plus DP100 (Berlin, Germany) and Lange Miniphotometer LP2 (Berlin, Germany) were used in *Studies II* and *III*, respectively. In all studies capillary blood (10 μL) were collected from the ear lobe.

3.7. Ergometers

In *Study I* the subjects performed an exercise test on designed motor-driven running treadmill Laufergotest LE/6 (Hoechberg, Germany), while in *Studies II* and *III,* the tests were carried out in an electronically braked cycle ergometer Lode Excalibur Sport (Groningen, Netherlands) and Schoberer Rad Messtechnik–SRM (Jülich, Germany), respectively.

3.8. Exercise protocols

In *Study I* an incremental test according to Kuipers et al. (60) was followed to establish the VO_{2max}. It started with a warming up of 5 min at 8 $km \cdot h^{-1}$, and thereafter the exercise intensity was increased by 1 $km \cdot h^{-1}$ every minute until the subject was unable to continue. The results of this test were used to establish submaximal workload at which athletes testing during the experimental intervention. They performed four workloads with every load lasting for 4 min at an initial speed of 10 $km \cdot h^{-1}$ for 3 athletes and 11 $km \cdot h^{1}$ for the remainder. Each level was followed by a 2-min rest period, after which the speed was increased by 1 $km \cdot h^{-1}$ until all subjects reached an intensity between 85 and 90% of VO_{2max} or a RER = 1.

In *Study II* the subjects were required to report to the laboratory on three occasions. The first test was carried out to familiarize the subject with the cycle ergometer, gas analyzer and the testing procedure. 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.

In *Study III* the subjects visited the laboratory on four occasions separated each by one week. The first week subjects performed an anthropometrical evaluation and VO_{2max} test under laboratory controlled conditions. The exercise protocol started at 50 W and increased 25 W every minute until voluntary exhaustion using an electronically braked cycle ergometer. In the second week subjects carried out time trial test to familiarize them with the cycle ergometer, gas analyzer and the testing procedure of time trials. The next two weeks the subjects performed two time trials in both treatments (placebo and nitrate) and under laboratory conditions. They were asked to perform the maximum distance as possible during 40 min. Before to the test, athletes performed 15 min of warm up at 60% of VO_{2max}. Both time trials were carried out at the same time of day (± 1 h). The ergometer was programmed in the mode “open end test”. The subjects started the test in “gear 9” and were allowed to change gear. In this mode, the power

output is changed if either pedal rate or the gear is changed at a constant pedal rate. For each trial, time, distance, power and torque was recorded every second by SRM software. Temperature condition of the lab was controlled in each session (23.9 ± 1 ºC). During the test cyclists were blind of power, speed, cadence and heart rate data. They had only information of time to finish the test. All athletes were strongly verbally encouraged during both time trials. During the test food and fluid ingestion was forbidden.

3.9. Treatment of cardio-respiratory data

In *Study I* data obtained from gas analyzer device such as VO2, VCO2, VE and RER were measured breath-by-breath. All these respiratory data were analyzed in order to exclude errant breaths caused by coughing, swallowing, sighing, etc. Values lying more than 4 SDs from the local mean were removed. Afterwards data were averaged every 5 s.

In *Study II* data of submaximal bouts of exercise were also initially examined to exclude errant breaths. Following the same criteria of *Study I*, values lying more than 4 SDs from the local mean were removed. In this case VO_2 kinetics was analyzed to assess more accurately the effect of nitrate supplementation in respiratory response. 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., Chicago 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:

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

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 (61):

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

In *Study III* the respiratory response of subjects was not recorded continuously. Three samples of respiratory gas exchange were taken during the test: 1) between 12 and 15 min; 2) between 22 and 25 min, and 3) between 32 and 35 min. Data of VO_2, VE, VCO_2 and RER were recorded breath-by-breath and values of the last minute were averaged and used to assess the respiratory response during exercise.

Additionally, in *Studies II* and *III*, HR was continuously recorded (beat-by-beat) with a portable heart rate monitor.

3.10. Analysis of nitric oxide metabolites

The athletes of *Study I* were required to report to the laboratory at baseline on the fourth morning of each treatment before breakfast. In the antecubital vein 2 mL of blood was collected in vacutainer tubes with heparin (BD Vacutainer, New Jersey, USA) and was centrifuged immediately 3000 x *g* for 10 min; the plasma was stored at -20 ºC until analyzed.

Plasma nitrate concentrations were determined by colorimetric fixing of nitrite with the Griess reagent as described by Moshage et al (62). Nitrate was reduced to nitrite by incubating 50 µL of plasma in a final volume of 0.5 mL reaction mixture containing 50 mU nitrate reductase, 50 µM NADPH, 5 µM FAD and 50 mM potassium phosphate buffer pH 7.5 for 20 min at 37ºC. After this period, 30 µL of lactate dehydrogenase (0.2 $mg \cdot mL^{-1}$ in potassium phosphate buffer 0.15 M pH 7.5) and 30 µL of sodium pyruvate 0.2 M were added, and incubation was continued for 5 additional min at 37ºC to oxidize the remaining NADPH. Finally, the samples were deproteinized by addition of 30 µL of 300 $g \cdot L^{-1}$ zinc sulphate, stored at 4ºC for 15 min, and

centrifuged at 15000 g for 5 min at 4ºC. 500 µL of the supernatants were then mixed in Eppendorf tubes with 500 µL of the Griess reagent (1 $g \cdot L^{-1}$ sulphanilamide and 0.1 $g \cdot L^{-1}$ N-(1 naphthyl) ethylenediamine in 25 g/L phosphoric acid) and, after incubation for 15 min at room temperature, absorbance at 540 nm was measured in a Shimadzu UV-160 (Kyoto, Japan) spectrophotometer. Results were compared with a standard curve with known concentrations of nitrite.

In addition, in *Study I* was estimated the levels of malondialdehyde (MDA) which is an indicator of free radical generation using the method of Santos et al (63). The principle of the method is the spectrophotometric measurement of the colour generated by reaction of thiobarbituric acid (TBA) with MDA. For this purpose, 50 µL of plasma samples was mixed with 250 µL of physiological serum, 500 µL of trichloroacetic acid (100% p/v; in HCl 6 N) and 100 µL of TBA 0.12 M (in Tris-HCl 0.26 M, pH 7.0). After incubation for 30 minutes at 100 ºC, 1.1 mL of distilled water was added and centrifugation was performed for 5 min at 3000 g and 4ºC. Absorbance of the supernatants was measured at 532 nm using a spectrophotometer (Shimadzu UV-160, Kyoto, Japan). The concentration of MDA was calculated by the absorbance coefficient of the MDA–TBA complex.

In *Study II* a small catheter was inserted into an antecubital vein for venous blood sampling. Four blood samples were collected to analyze nitrate and nitrite: 1) during resting conditions; 2) 3 hours 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 (Millipore Amicon Ultra, Billerica, USA) 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 (GE Analytical Instruments, NOA 280i Sievers, Boulder, USA).

In *Study III*, two blood samples were collected from the antecubital vein to analyze nitrate and nitrite: 1) after three days of nitrate supplementation or placebo in resting conditions before exercise test; 2) during the first three minutes after time trials (placebo and nitrate). The same procedures of *Study II* were followed to prepare blood

samples and determine plasma nitrate and nitrite levels. For nitrate an adaptation of the method described by Braman (64) was employed. 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).

Nitrite levels were determined following an adaptation of the method described by Castegnaro (65). 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).

In addition, urinary concentration of nitrate and nitrites were analyzed in *Study III* following the same method above described for blood samples. In this study Endothelin-1 levels in plasma were also measured using commercially available immunoassay kits (Assay Designs Inc., Ann Arbor, USA) following the manufacturer's instructions. Assays were performed in duplicate and optical density was determined using a microplate reader set to 450 nm.

3.11. Search of data in Study IV

Scientific articles were retrieved based on an extensive search in MEDLINE (1980-2011) and Google Scholar (1990-2011) databases. Computer search engines used the following combined keywords: 'L-arginine', 'L-citrulline', 'nitrate', 'glycine-propionyl-L-carnitine', 'supplementation', 'nitric oxide', 'exercise', 'performance'. After using these initial keywords, the search engines were limited to human studies excluding research with animals, as well as in humans in pathological states. As a result, 42 articles related to the effects of dietary ingredients linked with NO and performance in

response to exercise were considered. References cited in the retrieved articles were also considered in this review.

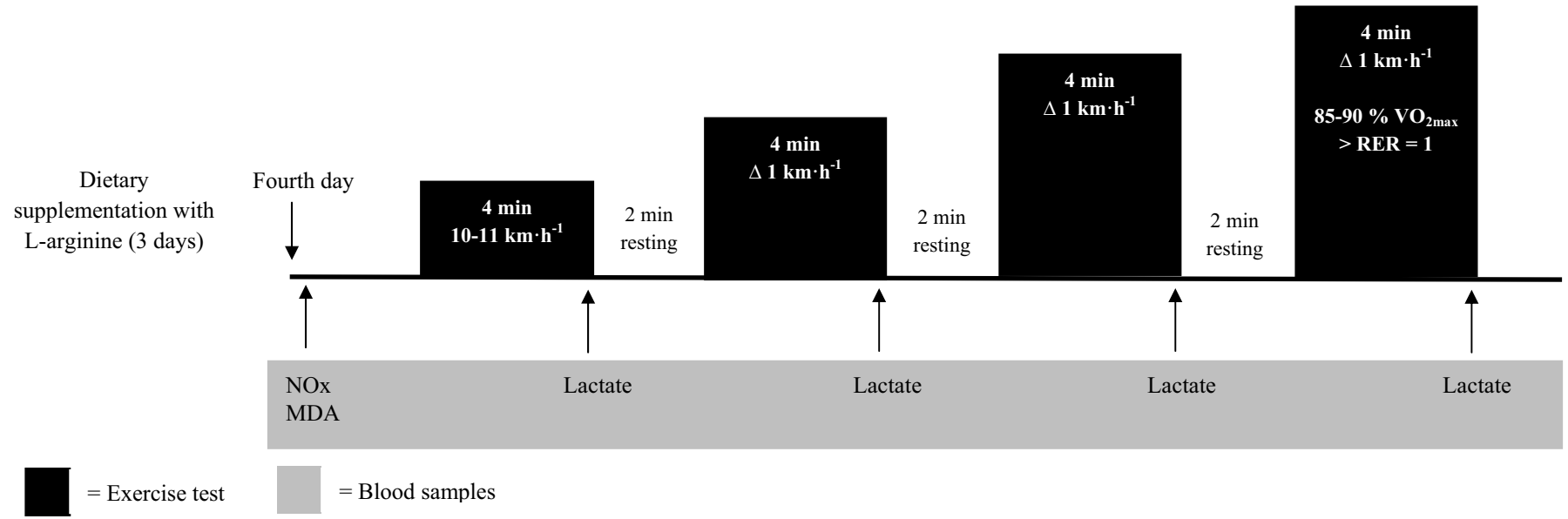
3.12. Statistical analysis

The significance level was set at $P < 0.05$ in all studies, while a trend was noted when $P < 0.10$. All statistical analyses were carried out with SPSS software (SPSS Inc, Chicago, IL, USA, version 12.0 to 15.0). Standard statistical methods were employed to calculate mean and standard deviation (SD). Normally distribution was assessed with Shapiro Wilk's.

Paired Student t-test was used for comparisons between variables obtained from the same subjects on two occasions (*Studies I, II and III*). For repeated measures (> 2) a one way ANOVA with a Tukey's honestly post-hoc test was used in *Study I*. Additionally, two-factorial ANOVA with repeated measures was used to compare cardiovascular, metabolic and biochemical variables between the diets at different task intensities in *Study I*, as well as, the influence of time and treatment in *Study II*. In *Study III* the same statistical analysis was used to investigate the influence of time and treatment. Post-hoc analyses were performed via Tukey's HSD.

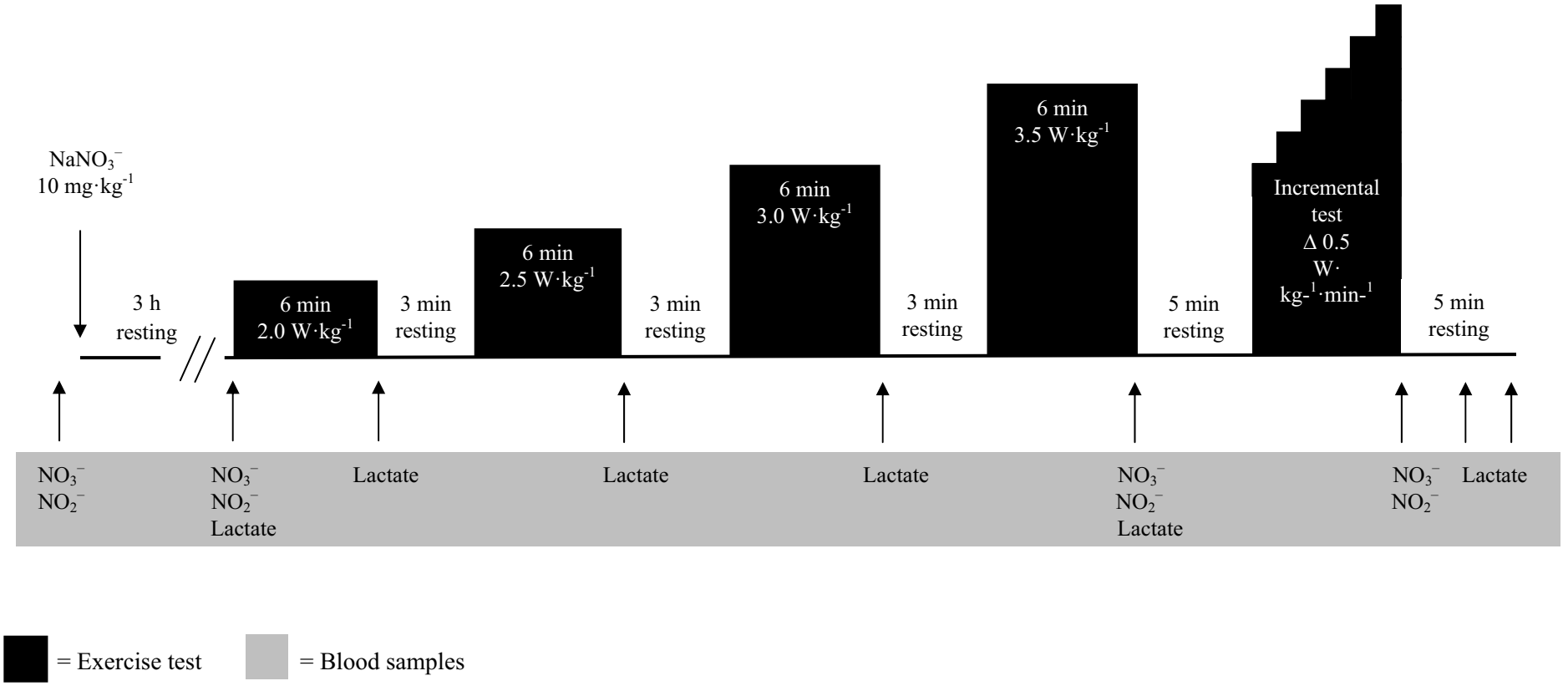
Additionally, *in Study III*, the coefficient of variation (CV) for distance and power output among the third and fourth time trial was calculated by dividing each subject standard deviation (SD) by his mean. A spreadsheet that analyzes validity by linear regression proposed by Hopkins was used for calculations. (HopkinsWG. Analysis of validity by linear regression (Excel spreadsheet). *In: A new view of statistics. sportsci.org: Internet Society for Sport Science, sportsci.org/resource/stats/xvalid.xls. 2000)*. In addition, an intraclass correlation coefficient (ICC) for the same variables was also computed.

Figure III: Procedures of supplementation and exercise test in *Study I*.



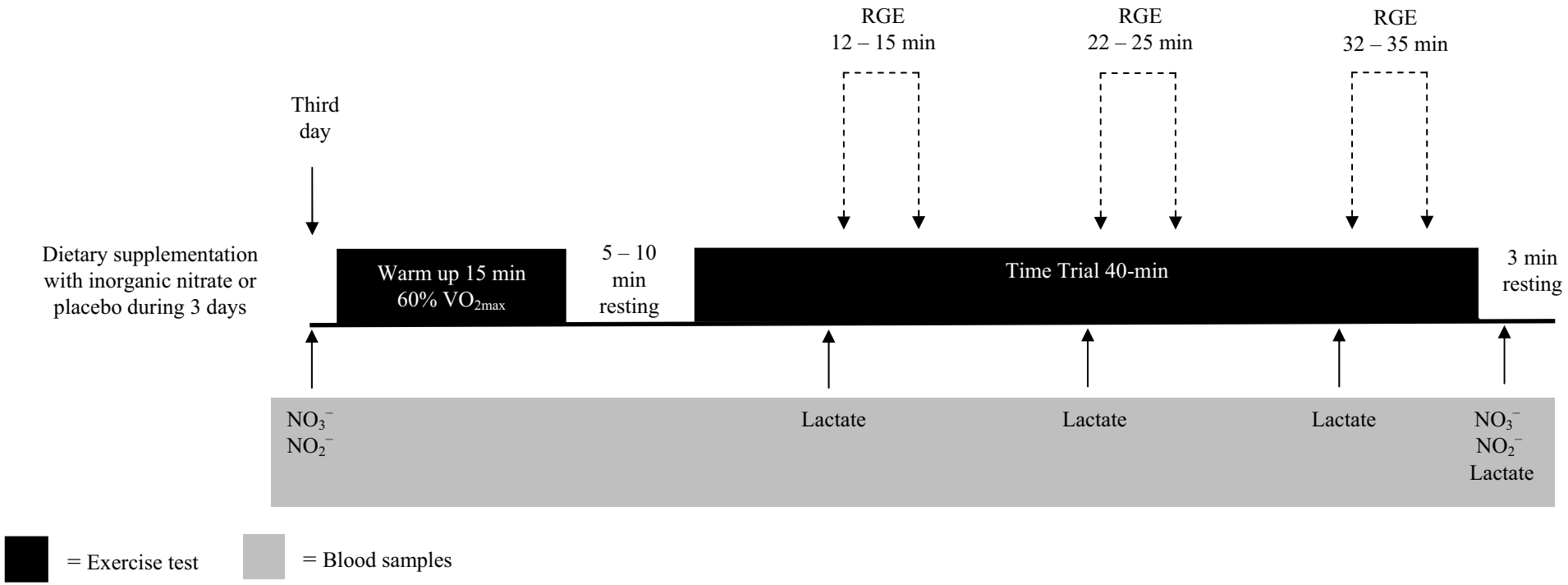
NO_3^- : concentration of plasma nitrate; MDA: Concentration of plasma malondialdheyde ; Δ : increment of speed

Figure IV: Procedures of supplementation and exercise test in *Study II*.



$NaNO_3^-$: Inorganic sodium nitrate ; NO_3^- : concentration of plasma nitrate; NO_2^- : concentration of plasma nitrite ; Δ : increment of power

Figure V: Procedures of supplementation and exercise test in *Study III*.



RGE : respiratory gas exchange analysis ; NO_3^- : concentration of plasma nitrate; NO_2^- : concentration of plasma nitrite

4. RESULTS

4.1 Plasma levels of nitrate and nitrite

Plasma levels of nitrate remained unchanged after every treatment in *Study I* (Control diet: 30.4 ± 5.8 $\mu M \cdot L^{-1}$; Diet 1: 31.9 ± 7.2 $\mu M \cdot L^{-1}$; Diet 2: 31.3 ± 7.9 $\mu M \cdot L^{-1}$) in basal conditions. On the contrary, in *Studies II and III*, the concentration of nitrate increased significantly after 3 hours and 3 days of dietary nitrate supplementation (**Table II**), respectively. In *Study II*, after submaximal (234 ± 82 $\mu M \cdot L^{-1}$; $P = 0.027$) and maximal (237 ± 85 $\mu M \cdot L^{-1}$; $P = 0.045$) workloads, plasma nitrate concentration decreased significantly with the peak value reached 3 hours post-supplementation (250 ± 80 $\mu M \cdot L^{-1}$). In contrast, in *Study III*, plasma nitrate remained unchanged after 40-min time trial (272 ± 60 μm) compared with resting conditions (256 ± 53 μm) after dietary nitrate supplementation (**Table II**).

Plasma nitrite concentration increased significantly 3 hours after nitrate supplementation compared with placebo in *Study II* (placebo: 1,998 ± 206 $nM \cdot L^{-1}$; nitrate: 2,313 ± 157 $nM \cdot L^{-1}$; $P = 0.017$) (**Table II**). In *Study III*, although a slight increase of plasma nitrite levels was found, it was not statistically significant (placebo: 4.2 ± 0.4 μm; nitrate: 4.5 ± 0.5 μm; $P = 0.124$). After submaximal workloads of exercise, plasma nitrite levels were not altered in *Studies II* and *III* (**Table III**). However, in *Study II*, just after an incremental cycle ergometer test until exhaustion, plasma nitrite levels were significantly decreased (2,126 ± 251 $nM \cdot L^{-1}$; $P = 0.044$) compared with the peak value reached 3 hours post-supplementation (2,313 ± 157 $nM \cdot L^{-1}$) (**Table III**). In *Study II*, nitrite also tended to be lower after the placebo treatment and maximal exercise (1,916 ± 168 nM) than under fasting conditions (2,053 ± 278 $nM \cdot L^{-1}$; $P = 0.056$).

4.2. Urinary levels of nitrate and nitrite

In *Study III* urinary levels of nitrate and nitrite were also measured. Urinary nitrate excretion increased significantly after dietary nitrate supplementation compared with placebo in resting conditions and after exercise (placebo: 1,299 ± 90 μM; nitrate: 7,624 ± 468 μM; $P < 0.001$). After nitrate treatment, the losses of nitrate in the urine corresponded to 65 ± 30 % (473 ± 189 mg) of the total amount of nitrate supplemented

(724 ± 97 mg). Additionally, 40-min time trial did not alter urinary excretion levels of nitrate and nitrite after exercise. The nitrate plasma/urinary ratio in resting conditions was 0.07 ± 0.05 and 0.05 ± 0.03 after placebo and nitrate treatment, respectively. After exercise, this ratio did not show significant changes (placebo: 0.09 ± 0.05; nitrate: 0.06 ± 0.04). For nitrite, plasma/urinary ratio in resting conditions was 1.32 ± 0.41 and 1.41 ± 0.53 for placebo and nitrate condition, respectively. This ratio was not significant modified after exercise (placebo: 1.49 ± 0.57; nitrate: 1.31 ± 0.40).

Table II. Plasma nitrate and nitrite levels in resting conditions (mean ± standard deviation).

Study	**Nitrate (µM)**	**Nitrite (µM)**
Study I		
Control Diet	30 ± 6	
Diet 1	32 ± 7	
Diet 2	31 ± 8	
Study II		
Placebo	28 ± 10	2.1 ± 0.3
Nitrate supplementation	250 ± 80*	2.3 ± 0.2*
Study III		
Placebo	44 ± 9	4.2 ± 0.4
Nitrate supplementation	256 ± 53*	4.5 ± 0.5

In *Study I* control diet contained 5.5 ± 0.3 g of L-Arginine, diet 1 9.1 ± 1.1 g and diet 2 20.5 ± 0.3 g. Additionally, in *Study I* plasma nitrate levels were measured by Griess reagent (nitrate + nitrite [NOx]). In *Studies II* and *III* plasma levels of these anions were analyzed by chemiluminescence reaction with ozone. In *Study II*, subjects ingested an acute dose (10 $mg \cdot kg^{-1}$ of body mass) of inorganic nitrate 3 hours before exercise test. In *Study III*, subjects followed the same supplementation (10 $mg \cdot kg^{-1}$ of body mass) during 3 days. * Statistical significance between nitrate and placebo conditions ($P < 0.05$).

Table III. Plasma nitrate and nitrite levels after physical exercise (mean ± standard deviation).

Study	Nitrate (μM)	Nitrite (μM)
Study II		
Placebo[(a)]	29 ± 6	2.0 ± 0.3
Nitrate supplementation[(a)]	234 ± 82*	2.3 ± 0.4
Placebo[(b)]	29 ± 7	1.9 ± 0.2
Nitrate supplementation[(b)]	237 ± 85*	2.1 ± 0.2**
Study III		
Placebo[(a)]	52 ± 7	4.2 ± 0.6
Nitrate supplementation[(a)]	272 ± 107*	4.3 ± 0.5

[(a)] Plasma nitrate and nitrite levels after submaximal workloads of exercise. [(b)] Plasma nitrate and nitrite levels after an incremental test until exhaustion. * Statistically significance between nitrate and placebo conditions ($P < 0.05$). ** Statistical significance between nitrite values after submaximal and maximal workloads following nitrate supplementation.

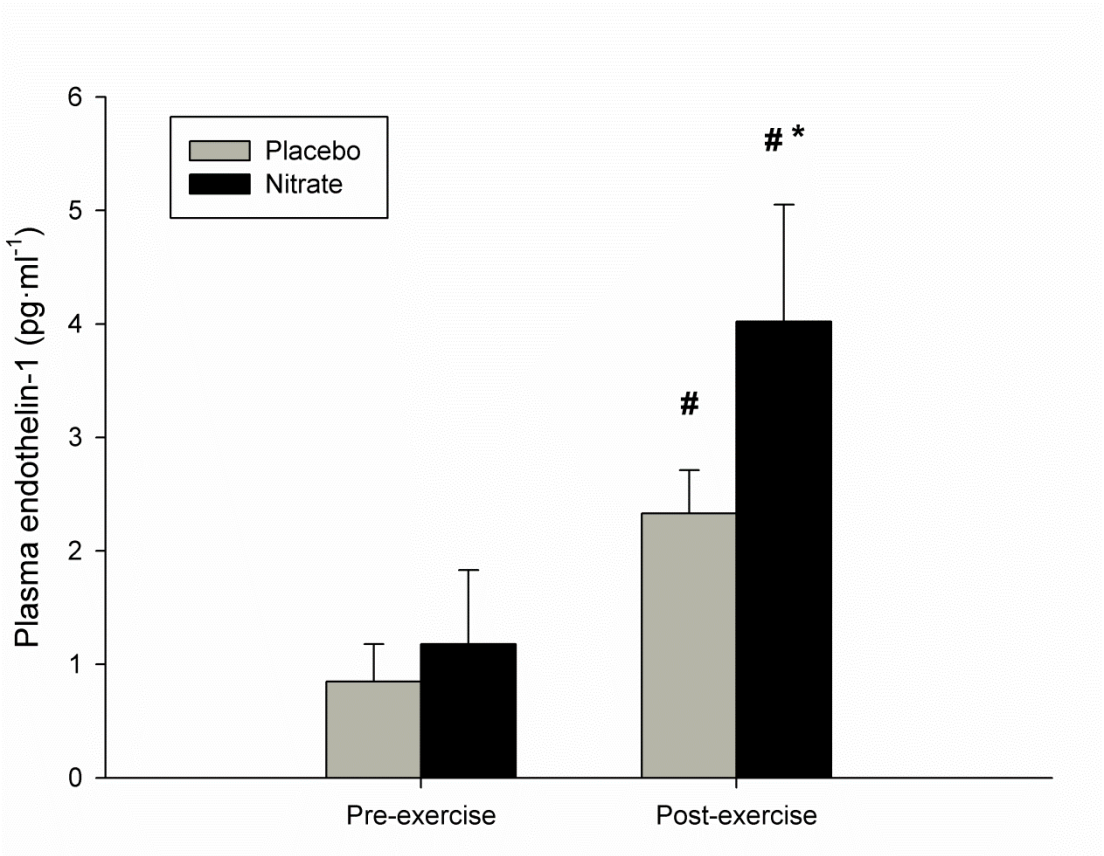
4.3. Plasma levels of malondialdehyde (MDA)

In *Study I*, the concentration of plasma malondialdehydes did not changed between treatments (Control diet: 41.3 ± 2.6 $\mu M \cdot L^{-1}$; Diet 1: 37.7 ± 3.3 $\mu M \cdot L^{-1}$; Diet 2: 42.6 ± 3.7 $\mu M \cdot L^{-1}$).

4.4. Plasma levels of endothelin-1 (ET-1)

In *Study III*, plasma levels of ET-1 were measured before and after exercise. In resting conditions, plasma ET-1 levels did not differ between nitrate and placebo conditions (**Figure VI**). However, a significant increase was showed just after exercise in placebo ($P = 0.030$) and nitrate ($P < 0.001$) groups compared with resting values. In addition, this effect was significantly ($P = 0.010$) greater in nitrate group compared with placebo (**Figure VI**).

Figure VI. Plasma levels of endothelin-1 (ET-1) before and after 40-min time trial in *Study III* (means ± standard deviation).



* Statistical significance between nitrate and placebo ($P < 0.05$).
# Statistical significance between pre-exercise and post-exercise values ($P < 0.05$).

4.5. Effects of dietary L-arginine and inorganic nitrate supplementation on respiratory response to exercise

In *Study I* was not showed differences of the VO_2 response at any workload of exercise and with any dietary intake of L-arginine (**Table IV**). Additionally, in *Studies II* and *III*, VO_2 consumption was not altered at workloads close and below to the respiratory compensation point (RCP) between nitrate supplementation and placebo (**Tables V and VI**). However, in *Study II* was found that at maximal exercise intensity, VO_{peak} dropped significantly after dietary nitrate ingestion (**Figure VII**). Interestingly, this decrease of VO_{peak} did not reduce the overall performance during an incremental cycle ergometer test. On the contrary, it was found a decrease of VO_2/power output (watts) ratio after nitrate supplementation (**Figure VIII**).

In *Study III* the VO_2 consumption was not modified at any point of the time trials when athletes ingested inorganic nitrate compared with placebo (**Table VI**). In addition, other respiratory parameters such as VCO_2, VE and RER were not modified by dietary L-arginine (*Study I*) and inorganic nitrate ingestion (*Studies II and III*).

Table IV. Mean values of oxygen consumption (VO_2) of athletes in the last minute at each submaximal workload of exercise in *Study I* (mean ± standard deviation).

Time (VO_2)	**Control diet**	**Diet 1**	**Diet 2**
5 min ($L \cdot min^{-1}$)	3.04 ± 0.31	3.00 ± 0.42	3.01 ± 0.35
11 min ($L \cdot min^{-1}$)	3.29 ± 0.34	3.26 ± 0.43	3.32 ± 0.37
17 min ($L \cdot min^{-1}$)	3.51 ± 0.34	3.48 ± 0.42	3.56 ± 0.39
23 min ($L \cdot min^{-1}$)	3.68 ± 0.36	3.67 ± 0.39	3.76 ± 0.46

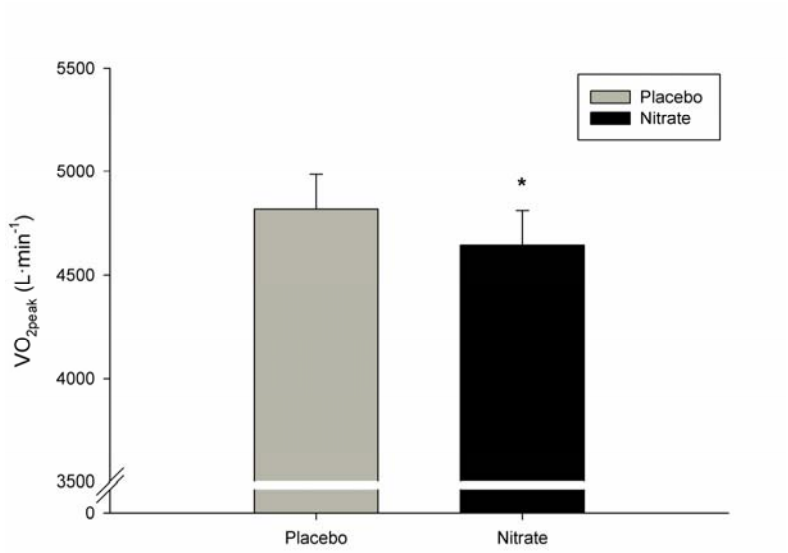
Table V. Average oxygen consumption in the last minute at each submaximal workload of exercise in *Study II* (mean ± standard deviation).

Load	**2.0 $W \cdot kg^{-1}$**		**2.5 $W \cdot kg^{-1}$**		**3.0 $W \cdot kg^{-1}$**		**3.5 $W \cdot kg^{-1}$**	
	Placebo	**Nitrate**	**Placebo**	**Nitrate**	**Placebo**	**Nitrate**	**Placebo**	**Nitrate**
VO_2	2.37	2.33	2.81	2.74	3.29	3.19	3.74	3.68
($L \cdot min^{-1}$)	± 0.23	± 0.26	± 0.28	± 0.22	± 0.29	± 0.27	± 0.33	± 0.62

Table VI. Oxygen consumption at three different points during 40-min time trials in *Study III* (mean ± standard deviation).

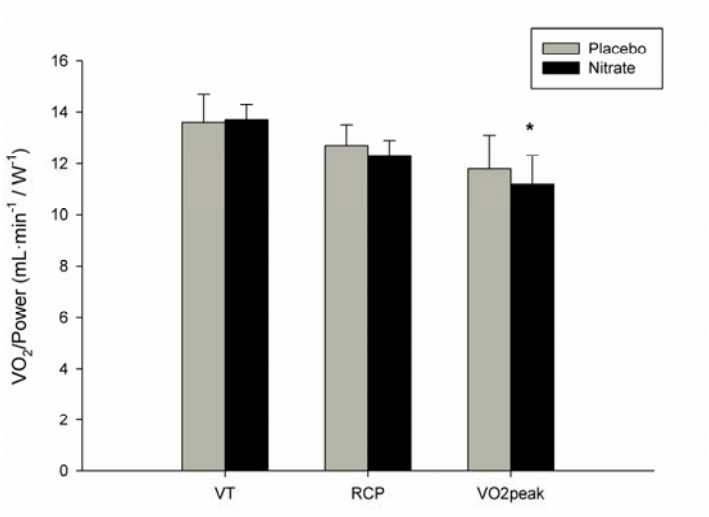
Time	**15 min**		**25 min**		**35 min**		**Average**	
	Placebo	**Nitrate**	**Placebo**	**Nitrate**	**Placebo**	**Nitrate**	**Placebo**	**Nitrate**
VO_2	3.64	3.70	3.64	3.57	3.62	3.63	3.63	3.63
($L \cdot min^{-1}$)	± 0.31	± 0.32	± 0.28	± 0.32	± 0.31	± 0.42	± 0.26	± 0.33

Figure VII: Maximal oxygen consumption during the incremental test in placebo and nitrate conditions in *Study II* (mean ± standard deviation).



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

Figure VIII. Rate between oxygen consumption (VO_2) and power (W) at ventilatory threshold (VT), at respiratory compensation point (RCP) and at peak of oxygen consumption (VO_{2peak}) after placebo and nitrate supplementation in *Study II.*



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

4.6. Gross efficiency (GE)

GE was measured in *Study II* during submaximal workloads. In the **Table VII** is showed data of GE indicating that inorganic dietary nitrate did not alter exercise efficiency compared with placebo.

Table VII. Values of Gross Efficiency (GE) during low-moderate intensity exercise after supplementation with nitrate or placebo in *Study II* (mean ± standard deviation).

Load	**2.0 W·kg^{-1}**		**2.5 W·kg^{-1}**		**3.0 W·kg^{-1}**		**3.5 W·kg^{-1}**	
	Placebo	**Nitrate**	**Placebo**	**Nitrate**	**Placebo**	**Nitrate**	**Placebo**	**Nitrate**
GE (%)	18.2	18.4	18.7	19.4	19.5	19.9	19.7	20.1
	± 1.3	± 1.2	± 1.3	± 0.9	± 1.4	± 1.0	± 1.4	± 1.0

4.7. Heart rate

Data of heart rate of *Studies I and II* is summarized in **Tables VIII** and **IX** respectively. L-arginine and nitrate did not modify heart rate response during exercise at submaximal, as well as maximal intensity of exercise. In *Study III,* it was not found differences between nitrate and placebo in heart rate data. In both treatments heart rate increased significantly during the first period of time trial compared with second and the third period ($P < 0.05$) (**Table X**).

Table VIII: Values of heart rate during the last minute at each submaximal workload in *Study I* (mean ± standard deviation).

	Control diet	**Diet 1**	**Diet 2**
5 min (beats-min^{-1})	158 ± 12	157 ± 12	156 ± 11
11 min (beats-min^{-1})	171 ± 12	170 ± 12	169 ± 12
17 min (beats-min^{-1})	179 ± 12	178 ± 12	177 ± 12
23 min (beats-min^{-1})	185 ± 12	184 ± 11	184 ± 12

Table IX. Values of heart rate during the last minute at each submaximal workload of exercise in *Study II* (mean ± standard deviation).

Load	**2.0 W·kg^{-1}**		**2.5 W·kg^{-1}**		**3.0 W·kg^{-1}**		**3.5 W·kg^{-1}**	
	Placebo	**Nitrate**	**Placebo**	**Nitrate**	**Placebo**	**Nitrate**	**Placebo**	**Nitrate**
Heart rate (beats-min^{-1})	111 ± 8	110 ± 10	126 ± 10	124 ± 11	142 ± 12	141 ± 14	156 ± 13	156 ± 14

Table X. Values of heart rate during 40-min time trials in *Study III* (mean ± standard deviation).

Time	**15 min**		**25 min**		**35 min**		**Average**	
Treatment	**Placebo**	**Nitrate**	**Placebo**	**Nitrate**	**Placebo**	**Nitrate**	**Placebo**	**Nitrate**
Heart rate (beats-min^{-1})	160 ± 11	160 ± 11	166* ± 12	165* ± 11	167* ± 10	167*# ± 12	164 ± 11	165 ± 11

* significant difference with respect to 15 min values
# significant difference with respect to 25 min values

4.8. Blood lactate

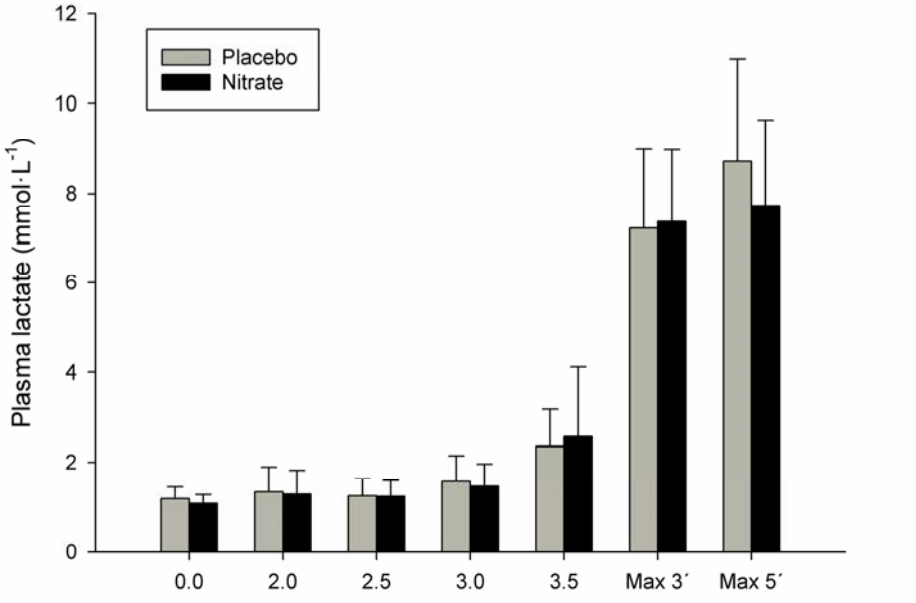
In *Study I* blood lactate decreased significantly ($P = 0.03$) at the end of the first work out of the test when control diet and diet 2 were compared (**Table XI**). However this tendency was not followed when exercise intensity increased. In *Studies II* no differences were found in blood lactate accumulation between conditions (nitrate and placebo) at any point of tests (**Figure IX)**. In *Study III* blood lactate accumulation increased significantly at three minutes after exercise in respect values at 30 min in placebo group ($P < 0.017$) and at 10 min in nitrate group ($P < 0.024$). However, there were no differences in average blood lactate concentration at any point of the test between placebo and nitrate groups (**Figure X)**.

Table XI. Blood lactate concentration during the last minute of each workload in *Study I* (mean ± standard deviation).

	Control diet	Diet 1	Diet 2
5 min (mmol·L^{-1})	3.0 ± 0.5	2.9 ± 0.5	2.5 ± 0.5*
11 min (mmol·L^{-1})	3.2 ± 0.8	3.0 ± 0.7	3.2 ± 1.0
17 min (mmol·L^{-1})	4.2 ± 0.9	4.1 ± 0.8	4.3 ± 0.7
23 min (mmol·L^{-1})	6.0 ± 1.4	5.7 ± 1.3	5.7 ± 0.8

* Statistical significance between control diet and diet 2 ($P < 0.05$).

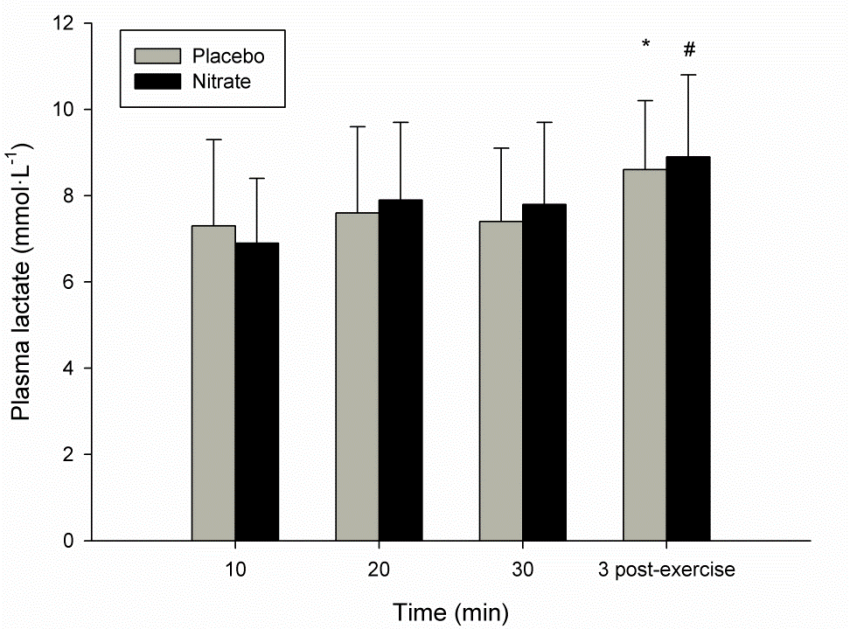
Figure IX: Plasma lactate concentration at rest conditions, after every submaximal workloads equivalents to 2.0, 2.5, 3.0 and 3.5 W·kg^{-1}, and at three and five minutes after maximal exercise in both conditions (nitrate and placebo) in *Study II* (mean ± standard deviation).



4.9. Performance

No changes in the tolerance of exercise measured as time to exhaustion during an incremental exercise test (**Figure XI**) was showed in the *Study II* after an acute dose of nitrate supplementation compared with placebo. Additionally, 3 days of nitrate supplementation did not enhance performance measured as mean distance and power performed during a time trial of 40-min in the *Study III* (**Figure XII**).

Figure X: Plasma lactate concentration at minute 10, 20, 30 during 40-min time trial, as well as after 3 minutes post-exercise in both conditions (nitrate and placebo) in *Study III* (mean ± standard deviation).



* Statistical significance between mean values at 30 minutes and 3 minutes post-exercise in placebo group. ($P < 0.05$).
# Statistical significance between mean values at 10 minutes and 3 minutes post-exercise in nitrate group. ($P < 0.05$).

Figure XI: Time to exhaustion performed during an incremental cycle ergometer test after placebo and nitrate supplementation in *Study II* (mean ± standard deviation).

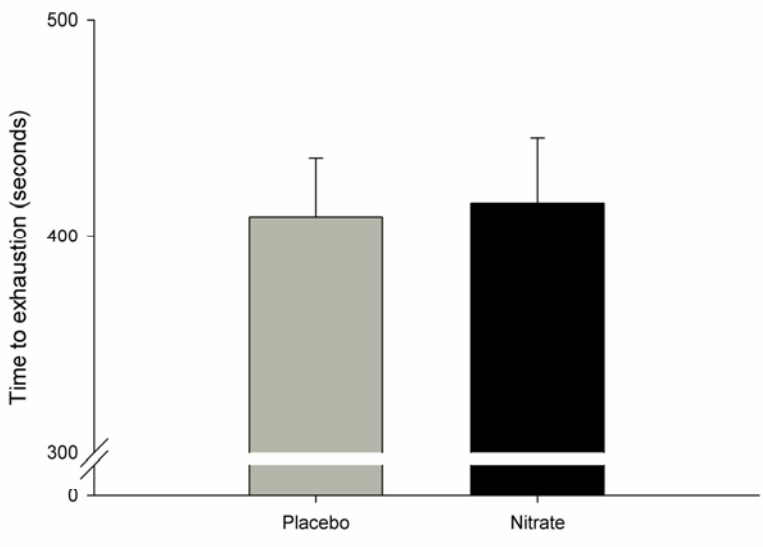
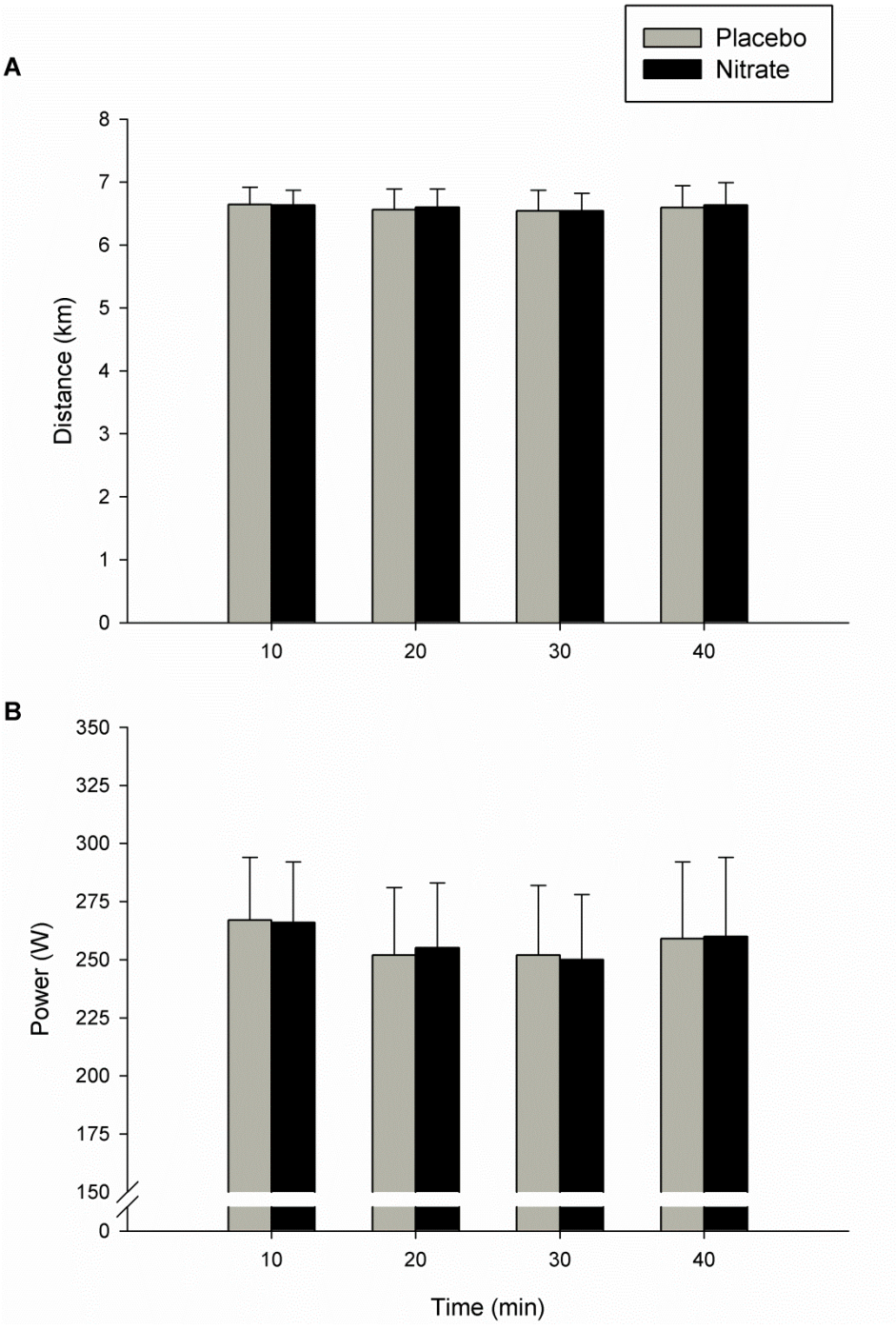


Figure XII: Profile of distance (A) and power output (B) performed by subjects during the 40-min time trials after dietary inorganic nitrate and placebo supplementation.



5. GENERAL DISCUSSION

5.1. The effect of dietary L-arginine supplementation on plasma levels of nitrate and nitrite

In *Study I* of this thesis we saw that, despite enriching the diet of athletes with L-arginine at different doses, no increase in NO markers measured as nitrate and nitrite (NOx) was found. As shown in *Study IV*, these results were in line with other previous studies in well-trained athletes which found no increase in plasma NO markers after dietary supplementation of L-arginine (66-67). One explanation suggested for this effect is the low bioavailability of this amino acid in humans, since dietary L-arginine bioavailability is only about 30%. Another 40% is metabolized in the liver by arginase enzyme which participates in the fifth and final step in the urea cycle competing with NOS for L-arginine (20, 59). Furthermore, in the athletic population there is evidence that exhaustive exercise increases arginase activity in lymphocites nearly 6-fold, limiting L-arginine availability for lymphocyte iNOS activity (68). Apart from the activity of arginase enzyme in the liver, there are several other factors which can limit the bioavailability of L-arginine in humans. For instance amino acid lysine competes with L-arginine for entry into the cells also inhibiting arginase activity (69). The dietary L-arginine:lysine ratio may be a critical factor influencing the effect of L-arginine supplementation. Under normal feeding conditions, the total amount of L-arginine in the diet should not be 150% greater than that of lysine (namely, L-arginine:lysine < 2.5) (70). However, despite these factors which can reduce the bioavailability of L-arginine, it seems that low bioavailability is not the keypoint related to the scarce ergogenic effect of L-arginine since other studies showed that intravenous infusion of L-arginine was also ineffective in increasing NO markers and enhancing exercise performance (71-72) (*Study IV*).

In addition, despite the fact that plasma nitrate and nitrite levels have been used extensively as the main NO markers, there are other molecules which can also be used as indirect markers of NO synthesis. For example, as explained in *Study IV*, L-citrulline has been indicated to be an alternative NO marker via the NOS-dependent pathway. L-citrulline is produced endogenously in this pathway when L-arginine is oxidized to NO by the NOS enzymes. Therefore it is suggested that an increase in plasma levels of L-citrulline can also indicate an increase in NO synthesis (73). Accordingly, Liu et al (66) assessed plasma levels of L-citrulline after dietary L-arginine supplementation. They

concluded that L-arginine did not increase levels of L-citrulline in comparison with a placebo. Therefore these findings corroborate previous data showing that L-arginine supplementation has a limited effect on to increasing NO bioavailability in well-trained subjects (*Study IV*).

Nevertheless, in contrast with the above data, a recent study by Bailey et al (74) showed that dietary L-arginine supplementation significantly increased plasma nitrite levels in healthy subjects. In an attempt to explain this controversial finding, in *Study IV* of this thesis it is suggested that training status is an important factor related to the capacity of NO donors to increase NO synthesis in humans. While some studies have reported an increase in NO markers after L-arginine supplementation in patients with cardiovascular disease (24, 75-76), as well as in the sedentary healthy population (77), there is a lack of data showing a significant change of NO markers in well-trained athletes after dietary L-arginine supplementation. In *Study IV* it is explained that chronic training has an important effect on the regulation of NO metabolism. Studies have shown that short-term training rapidly increases NO bioactivity, but, if training is maintained the short-term functional adaptation is succeeded by NO-dependent structural changes, leading to arterial remodeling and structural normalization of shear (52-53, 78). Nevertheless, exercise not only has an effect on NO metabolism; chronic training induces many adaptations in other systems of the human body such as muscle fibre type transformation (79), aerobic enzyme capacity within the muscle (80) and the expression of some proteins (PGC1α, and ANT) (81). All these exercise benefits together may overcome any potential effect of dietary L-arginine supplementation.

5.2. The effect of dietary inorganic nitrate supplementation on plasma levels of nitrate and nitrite

In *Studies II* and *III* we found a significant increase in plasma nitrate levels. However, this finding was expected since athletes were supplemented with nitrate supplementation for one and three days, respectively. In addition, an interesting finding in *Study II* was that only one dose of inorganic nitrate increased plasma levels up to values which were previously shown in studies following several days of supplementation (82-83). This fact was also corroborated in *Study III*.

In *Study II* plasma nitrate levels decreased significantly after submaximal workload of exercise compared with values reached at 3 hours post-supplementation. This fact was not consistent with another previous study (82). However, such a response was not attributed to the effect of exercise as the level of nitrate was no different after both exercise conditions (submaximal and maximal workloads). This finding was related to the pharmacokinetics of nitrate after dietary ingestion. It is known that the half-life of plasma nitrate in humans is approximately 5 hours and there is a substantial decrease after 4 hours of ingestion (46). In *Study II*, the timing was at the borderline of the nitrate half-life. Athletes completed submaximal and maximal workloads at 3 hours 45 min (± 10 min) and 4 hours 5 min (± 14 min) respectively. This hypothesis of the pharmacokinetics effect after an acute dose of nitrate ingestion 3 hours before exercise was corroborated in *Study III.* In this study, plasma nitrate levels remained unchanged after a 40-min time trial following 3 days of dietary nitrate supplementation.

There is some controversy about plasma nitrite levels between *Studies II* and *III*. In resting conditions, plasma nitrite levels increased significantly in *Study II* and this finding was consistent with other previous studies (82, 84-90). However, in *Study III* plasma levels of nitrite were not significantly modified after dietary nitrate ingestion. This finding was not expected and was carefully analyzed. It is known, for instance, that some disruption on the enterosalivary circuit of nitrate can alter plasma nitrite levels. Studies by Petersson et al (91) and Govoni et al (92) showed that daily use of a commercial antibacterial mouthwash can eliminate the oral microflora and significantly attenuate plasma nitrite increase after the ingestion of food rich in nitrate. For this reason we asked the subjects about the use of antibacterial mouthwash, but none of them used such products. Therefore, discarding this possibility, it is difficult to explain the lower response of some subjects in *Study III* to increase plasma nitrite after dietary nitrate ingestion. While seven subjects showed a significant increase in plasma nitrite after dietary nitrate supplementation, the other subjects (6 athletes) did not respond in this way, showing similar or lower plasma nitrite values compared with the placebo condition. In addition, like in *Study II*, we found high plasma nitrite levels compared with other studies (82, 84-89). We suggested that these levels were related to methodological issues or training levels since there is evidence that plasma nitrite can increase with exercise capacity (90). However, this is only speculation and further in-

depth studies are needed to analyze the lower response to increasing plasma nitrite levels as well as the high plasma values of nitrite in endurance-trained athletes.

In *Study II* it was shown that plasma nitrite levels decreased significantly just after finishing an incremental exercise test until exhaustion. This finding was in agreement with another previous study by Larsen et al (84) suggesting that a decrease in plasma nitrite could be caused by an activation of the nitrate-nitrite-NO pathway. It has been indicated that this pathway is mainly activated under acidosis conditions and at low oxygen tensions (30, 84). This physiology situation could be common at higher intensities of exercise under anaerobic conditions. In *Study III* athletes performed 40-min time trials at a mean intensity of 91 ± 3% of HR_{max} which was equivalent to 85 ± 6% of VO_{2max}. Although this intensity should be considered as submaximal, the mean level of acidosis measured as blood lactate concentration was high throughout the test in athletes (> 7 $mmol \cdot L^{-1}$). Nevertheless, plasma nitrite levels remained unchanged, suggesting that NO synthesis via the NOS-independent pathway was not activated. Therefore all these findings together seem to indicate that in endurance-trained athletes the NOS-independent pathway is only activated at maximal intensities of exercise, when low oxygen tension occurs. Future studies assessing the effect of dietary nitrate ingestion in hypoxic conditions could be very interesting if they carefully analyze the role played by this intriguing compound in our diet.

5.3. Cardiopulmonary and metabolic response to exercise after L-arginine and inorganic nitrate supplementation

In *Study I* it was found that blood lactate concentration decreased significantly at low workloads of exercise following a diet enriched with dietary sources of L-arginine (Diet 1) compared with the control diet, but this effect was not shown at higher effort intensities. In *Studies II* and *III* of this thesis it was shown that inorganic nitrate supplementation did not alter heart rate response and blood lactate concentration during exercise tests. These results were consistent with other previous studies indicating that dietary L-arginine and inorganic nitrate have no effect on heart response and blood lactate during exercise (66, 74, 82, 84-89).

Focusing our attention on respiratory response, the results from *Study I* showed that at submaximal intensity of exercise, pulmonary response was not changed when diet was enriched with different amounts of L-arginine. As shown in *Study IV* of this thesis, these results were in agreement with other previous investigations (66, 93). However, there are other studies which have reported benefits in the pulmonary response to exercise after L-arginine supplementation (74, 94). A careful analysis of such studies included in *Study IV* shows that in these cases L-arginine supplementation was combined with other ingredients such as creatine, carbohydrates, amino acids, vitamins, minerals, etc., in an attempt to increase L-arginine bioavailability. This makes it more difficult to elucidate specifically the effect of L-arginine related to NO synthesis and performance since some of these ingredients, such as creatine and carbohydrates, may have an ergogenic effect in themselves (56-58). Additionally, only the study by Bailey et al (74) related their findings to an increase in NO markers measured as plasma nitrite level. However, as mentioned earlier, Bailey et al (74) assessed healthy but not well-trained athletes. From this viewpoint, in *Study IV* it is suggested that in moderately-trained subjects L-arginine supplementation may have some positive effect, although these benefits may come from other pathways independent of NO where L-arginine also participates. For instance, this amino acid is essential for the normal functioning of the urea cycle, in which ammonia is detoxified through its metabolism into urea (25). Furthermore, it is an important component for the synthesis of endogenous creatine (25). Additionally, L-arginine is also a potent hormone secretagogue. L-arginine infusion at rest increases plasma insulin, glucagon, growth hormone (GH), prolactin and catecholamines concentrations (26). Therefore, according to current scientific data which is included in *Study IV* of this thesis, there is a lack of evidence indicating that L-arginine supplementation alone has a potential effect to enhance respiratory response in trained subjects.

Studies II and *III* of this thesis evaluated the effect of dietary inorganic nitrate on the respiratory response to exercise. At submaximal loads of exercise, below to the respiratory compensation point (RCP), nitrate supplementation did not alter pulmonary response in athletes. At maximal intensity during an incremental exercise test, the peak of oxygen consumption was significantly reduced after dietary nitrate ingestion compared with the placebo (*Study II*). This effect was consistent with another previous study by Larsen et al (84). On the other hand, in contrast with these results, there are

several studies which have shown a decrease in oxygen demands at submaximal workloads of exercise (82, 85-86, 88-89). Currently it is difficult to explain why some studies found that nitrate supplementation reduced oxygen demands at submaximal workloads and others did not find such an effect. Perhaps, as reported in *Study IV*, differences in the training status of the subjects could explain these discrepancies. All studies which have shown a decrease in oxygen consumption at low-to-moderate intensities of exercise analyzed recreationally active subjects (82, 85-86, 88-89). In contrast to this fact, *Studies II* and *III* of this thesis assessed endurance-trained subjects. Apart from the benefits induced by endurance training on the cardiovascular and NO system mentioned earlier, there is also evidence indicating that training (especially at high intensities) improves energy efficiency (81). Potential mechanisms which might be responsible for training-related increases in efficiency include muscle fiber type transformation (79), aerobic enzyme capacity within the muscle (80) and the expression of proteins such as PGC1α and ANT (81). All these effects together may enhance cellular respiration and improve the overall respiratory response to exercise reported after a period of training.

5.4. Can dietary nitric oxide donors increase endurance performance?

This question is extensively dealt with in *Study IV* of this thesis. In short, results from *Studies I*, *II* and *III* show that dietary L-arginine and inorganic nitrate supplementation were not effective in enhancing exercise performance in trained athletes. These findings are consistent with other studies which have evaluated the ergogenic effect of L-arginine supplementation alone using different types of athletic populations such as judo athletes (66, 95), tennis players (96) and cyclists (84). Despite analyzing supplements for different durations (between 1 and 28 days) and doses (between 6 and 12 g), no benefit was indicated in parameters linked to performance such as power in a cycle ergometer test (66) or VO_2 consumption during a treadmill test (93, 96). As indicated previously, one plausible explanation which has been suggested to explain this lower effect of L-arginine supplementation is derived from the low bioavailability of this amino acid. However, this explanation does not seem feasible since there are other studies which have used intravenous infusion of L-arginine and did not also report a positive effect in parameters of performance such as maximal workload during an

incremental cycle ergometer test or the amount of work completed in a 15-minute test (71-72). In addition, there is also evidence indicating that higher doses of dietary L-arginine were ineffective in increasing blood flow in healthy humans, suggesting that synthesis of NO was not affected (97-98).

For all these reasons, the emerging interest in the amino acid L-citrulline as a secondary NO donor via the NOS-dependent pathway has increased. Unlike L-arginine, L-citrulline bypasses the hepatic metabolism and is not a substrate of arginase enzymes. Thus it has been indicated that systemic administration of L-citrulline could be a more efficient way to elevate extracellular levels of L-arginine by itself (99). However, there is only one study which has analyzed L-citrulline supplementation alone, showing an impairment of exercise performance after dietary L-citrulline ingestion (100). However, the addition of malate to dietary L-citrulline supplements has shown an increase in NO metabolites (68,75), but such findings were not related to exercise performance. In this thesis no study assessed the effect of amino acid L-citrulline, but according to current experience and the scientific data of NO donors reported in *Study IV*, it is at present difficult to suggest that dietary L-citrulline could be an effective ergogenic supplement in athletes.

Inorganic nitrate has also led to increased interest in exercise physiology during the last few years because of its role in NO synthesis. There is current, controversy about the effect of nitrate supplementation and exercise performance. The first research group which analyzed the effect of dietary inorganic nitrate in exercise reported that nitrate ingestion improved Gross Efficiency at low-to-moderate workloads of exercise (82), but it did not indicate a significant improvement in performance measured as time-to-exhaustion during an incremental exercise test (84). Following these studies, another independent research group has published several studies showing that dietary nitrate supplementation in the form of beetroot juice significantly enhances performance measured as time-to-exhaustion both at fixed workload and during incremental exercise tests, as well as time-to-finish time trials (85-89). These results have been related to an attenuation of the VO_2 slow component and an associated blunting of changes in the muscle metabolic milieu (reduction of phosphocreatine degradation and diminution of the ATP cost production) (85). However, *Studies II* and *III* of this thesis did not corroborate these findings. In both studies, using a pharmacological nitrate supplementation, exercise performance was not improved compared with the placebo.

To explain these differences between studies, again it is suggested that the training status of subjects could be an important factor linked to the effectiveness of some dietary products. For instance, in *Study II* all subjects were endurance athletes with high VO_{2peak} (65.1 ± 6.2 $mL \cdot kg^{-1} \cdot min^{-1}$). They trained an average of 18.7 ± 7.1 hours. In *Study III* all subjects were also endurance athletes, although their endurance performance measured as VO_{2peak} was low (59.7 ± 7.0 $mL \cdot kg^{-1} \cdot min^{-1}$) compared with the previous subjects in *Study II*. They trained a considerable amount of hours per week (15.7 ± 5.0 hours). In contrast, in previous studies the endurance capacity was lower (VO_{2peak} between 45 and 56 $mL \cdot kg^{-1} \cdot min^{-1}$) (82-86, 88-89) compared with *Studies II* and *III* of this thesis.

The conclusion extracted from *Study IV* is that training performed by competitive athletes has a greater effect on improving the cardiovascular response to exercise than any dietary NO intervention. This fact raises the intriguing possibility of a threshold effect – volume and intensity – for training and mechanisms associated with NO production. Further investigation is needed to elucidate where this limit of physical exercise lies. This is not only important for athletes and exercise performance, but even so for the sedentary or sick population who need to improve their cardiovascular health and tolerance to exercise.

5.5. New hypothesis derived from this thesis

Despite the fact that this thesis has been focused on analyzing the effect of NO donors on human performance, there are other findings reported in this thesis which it could be very interesting to analyze carefully in future investigations. Personally, I believe the most interesting of these findings was the surprising effect of dietary nitrate supplementation increasing plasma levels of ET-1 just after exercise, which is reported in *Study III*. ET-1 is a potent vasoconstrictor peptide produced by vascular endothelial cells which contributes to the control of basal vascular tonus in humans (101). Thus this molecule has the opposite effect to NO. Although research related to dietary compounds such as L-arginine and inorganic nitrate has been focused on the vasodilator response of NO, it is known that vascular tone and vascular growth largely depend on a delicate balance between dilators and constrictors (102). Perhaps, whereas we are trying to alter the vasodilator response by the use of dietary NO donors, such products can also alter

constrictor mechanisms. This fact has not been analyzed in any previous investigation. Looking at the results of *Study III*, it could be hypothesized that nitrate supplementation may have another role independent of NO synthesis, increasing blood flow in exercising tissues and reducing intracellular perturbation (e.g., increased lactic acid, decreased phosphocreatine). This hypothesis is based on studies by Maeda et al. (103-105) suggesting that an increase in plasma ET-1 in the nonactive tissues may cause the increase in vascular tone and the consequent decrease in blood flow in nonexercising tissues, which could be helpful in increasing blood flow in exercising muscles or the lungs. Further studies are needed in this line of research to elucidate the role that dietary nitrate plays in the release of ET-1 and in the regulation of blood flow during exercise.

Additionally, all the studies in this thesis as well as all those mentioned previosly have been carried out on young male subjects. As vascular function and NO availability are impaired with age (106-107), other studies should be planned in order to assess the effect of NO supplements in adults (> 40 years). Although this thesis elucidates that physical training plays a more important role in improving cardiovascular function than dietary supplementation of L-arginine and inorganic nitrate, such supplements could be helpful in those subjects with limited capacity of movement associated with age or during convalescence periods after illness.

Lastly, gender differences have not been analyzed, although it is recognized that there are structural/morphological differences between adult males and females for many if not all organ systems, which may have a significant impact on physiological function (108). This fact is mainly based on the difficulty in controlling the ovulatory process, which can alter several mechanisms in the female body. However, this point needs further research since it is known that reproductive abnormalities including delayed menarche, primary and secondary amenorrhea and oligomenorrhea occur in 6–79% of women engaged in athletic activity (109). All these disturbances are also associated with altered endothelial function (110). For this reason, NO donors may have more potential effects in females compared with males.

6. CONCLUSIONS

- Dietary L-arginine supplementation at different doses does not increase plasma NO metabolites in competitive tennis players.
- Higher doses of dietary L-arginine does not alter the cardiorespiratory and metabolic response to endurance exercise in trained athletes.
- Acute as well as 3 days of dietary inorganic nitrate supplementation significantly increase blood levels of nitrate in endurance-trained athletes.
- Inorganic dietary nitrate does not induce an increase in plasma levels of nitrite in all endurance-trained athletes.
- Gross Efficiency, defined as the ratio of mechanical work output to the metabolic energy input, is not improved by acute ingestion of inorganic nitrate in well-trained athletes.
- Maximal oxygen uptake during maximal exercise is significantly reduced after ingestion of one dose of nitrate. This physiological response does not alter performance measured as time-to-exhaustion during an incremental test.
- Three days of inorganic nitrate supplementation does not enhance overall performance measured as mean power output and total distance during a 40-min time trial in trained subjects.
- Plasma endothelin-1 levels in the forearms are significantly increased after dietary nitrate supplementation just after a 40-min time trial test.
- Current scientific data indicates that NO donors have no effect on increasing exercise performance in well-trained athletes.
- The benefits reported in some studies analyzing L-arginine and L-citrulline supplementation could be derived from the other ingredients included in supplements as well as from other metabolic pathways, independently of NO synthesis, which these amino acids participate in.

7. SUMMARY IN SPANISH

Introducción

El óxido nítrico (NO) es un gas producido por diferentes células en el organismo humano. Esta molécula que inicialmente fue denominada factor de relajamiento endotelial, se descubrió a partir de una serie de estudios durante la década de 1980. Por la importancia de estos hallazgos y de otros estudios posteriores, los Doctores Robert F. Furchgott, Ferid Murad y Louis J. Ignarro fueron galardonados con el Premio Nobel de Medicina y Fisiología en 1998. Desde entonces, el interés por el NO ha generado una gran revolución en el ámbito de la investigación farmacológica y fisiológica. Actualmente, se sabe que el NO es una molécula clave en la regulación del flujo sanguíneo (13). La producción de pequeñas cantidades de NO en el endotelio vascular provoca una reacción en cadena donde se liberan diversas moléculas como la guanilil ciclasa (sGC), el cGMP y algunas proteínas quinasas intracelulares (13). Estas moléculas favorecen la relajación del músculo liso de los vasos sanguíneos, facilitando la irrigación de los diferentes tejidos y órganos del cuerpo. Además de la función vasodilatadora, el NO posee otras importantes funciones en el cuerpo. Por ejemplo se sabe que es un importante componente para la regulación de los procesos de agregación plaquetaria y neurotransmisión, así como un mecanismo regulador del sistema inmunitario (7).

En el organismo humano el NO se sintetiza mediantes dos vías metabólicas: la via dependendiente de la óxido nítrico sintetasa (vd-NOS) y la vía independiente de la óxido nítrico sintetasa (vi-NOS). En la primera de ellas, un conjunto de enzimas denominadas óxido nítrico sintetasa (NOS) oxidan el aminoácido L-arginina produciendo NO y L-citrulina (**Figura 1**) (69). Esta vía es estimulada principalmente en situaciones aeróbicas. Por el contrario, en situaciones anaeróbicas, la actividad de la vd-NOS decrece drásticamente y se activa la vi-NOS. En esta vía, los nitratos y los nitritos plasmáticos son los principales precursores del NO (30). Estos compuestos son moléculas nitrogenadas, que durante mucho tiempo han sido utilizados como metabolitos y marcardores endógenos de la producción de NO. No obstante, investigaciones realizadas durante la última década han observado que los nitratos y nitritos desempeñan un papel importante en la síntesis de NO en determinadas circunstancias (44, 111). Por ejemplo, se ha demostrado que la reducción de la

oxihemoglobina a deoxihemoglobina favorece también la reducción de los nitratos y nitritos a NO (31, 35). Otras moléculas del cuerpo como las enzimas de la cadena respiratoria mitocondrial pueden producir NO a partir de los nitratos y nitritos (2). La vi-NOS ha sido propuesta como una vía complementaria de la vd-NOS. Cuando en situaciones de hipoxia la capacidad de las enzimas NOS se reduce y se limita la síntesis de NO, la vi-NOS se activa favoreciendo así la síntesis de NO y la vasodilatación en dichas circunstancias. Este mecanismo alternativo de la síntesis de NO ha sido propuesto como un símil de la glucolisis anaeróbica y aeróbica en el metabolismo energético (30).

El interés por los precursores del NO ha aumentado de forma importante durante los últimos años. Debido a que la L-arginina fue el primer precursor de NO descubierto, este aminoácido ha sido investigado más extensamente que los nitratos inorgánicos. Actualmente, se conoce que la L-arginina se encuentra presente en una gran variedad de alimentos que son consumidos con gran frecuencia en las poblaciones occidentales como la carne, el pescado o los huevos (18). Se ha estimado que la ingesta media de L-arginina es de 4-5 gr al día (19). Además la L-Arginina puede ser sintetizada endógenamente a partir de otros aminoácidos como la L-citrulina (112). Por este motivo este aminoácido no es considerado esencial.

Numerosos estudios han analizado los efectos de la suplementación de L-Arginina utilizando diferentes dosis y en diferentes grupos de población (*Estudio IV*). Resumiendo los resultados obtenidos por estas investigaciones, se ha observado que la suplementación con L-Arginina en dosis moderadas (5-10 gr·día^{-1}) puede mejorar la respuesta cardiorrespiratoria durante un ejercicio aeróbico en pacientes con patología cardiovascular (infarto de miocardio) y en personas sanas sedentarias (24, 76, 113-114). No obstante, no existe evidencia suficiente que relacione tales efectos con un aumento de la síntesis de NO. La L-arginina participa en otros procesos en el organismo humano como por ejemplo la eliminación del amonio mediante el ciclo de la urea (25), la segregación de diversas hormonas (insulina, hormona del crecimiento) (26), o la síntesis de creatina (22), que también podrían verse favorecidos por un aumento de la ingesta de este aminoácido. Además, en numerosos estudios la suplementación con L-arginina ha sido combinada con otros componentes nutricionales (aminoácidos, vitaminas, minerales, carbohidratos, creatina, etc.) (*Estudio IV*). La adición de estos compuestos

dificulta establecer sí los efectos observados derivan de la L-arginina por sí misma o por la interacción de los ingredientes adicionales.

Por otra parte, los nitratos y nitritos han sido considerados durante muchos años productos inertes en el organismo humano (30). Durante mucho tiempo han sido utilizados como marcadores indirectos de la producción de NO mediante la vd-NOS. Además, la cantidad de nitratos y nitritos en los alimentos y el agua ha sido rigurosamente controlada debido a su presunta relación con la formación de nitrosaminas y nitrosamidas (115). Estas moléculas resultan muy tóxicas para el organismo y han sido directamente relacionadas con un incremento de la incidencia de cáncer, principalmente de esófago y estómago. No obstante, estudios realizados durante la última década han descartado la relación entre el consumo de nitratos y nitritos (en dosis moderadas) con el desarrollo de cáncer (29). Por el contrario, hallazgos muy recientes indican que el consumo moderado de alimentos ricos en nitratos (lechuga, espinacas, acelgas, remolacha, etc.) puede poseer un efecto beneficioso sobre la función cardiovascular (46, 116). Tales beneficios han sido atribuidos a la capacidad que poseen algunas moléculas como los glóbulos rojos, la mioglobina y algunas enzimas de la cadena respiratoria mitocondrial para reducir los nitritos a NO en situaciones de hipoxia y/o acidosis (30).

En el ámbito deportivo, tanto la L-arginina, como más recientemente los nitratos inorgánicos, han generado un gran interés por la potencial acción ergogénica de estas sustancias (55). Aunque existe una carencia de evidencia científica, se ha extendido de forma popular la hipótesis de que la suplementación nutricional con precursores de NO estimula un aumento de esta molécula en el organismo. Ha sido sugerido que el aumento del NO favorecería un aumento del flujo sanguíneo en los tejidos activos durante un ejercicio físico. Este efecto aumentaría la llegada de moléculas de oxígeno y nutrientes necesarios para la función de las células musculares, además de favorecer la eliminación del ácido láctico y otras sustancias de deshecho. Toda esta respuesta en global, podría mejorar la tolerancia al ejercicio físico (55) (**Figura II**). No obstante, esto es sólo una hipótesis que debe ser corroborada por las pertinentes investigaciones.

Objetivos

Los principales objetivos de esta tesis fueron los siguientes:

1. Investigar el efecto de la ingesta dietética de L-arginina en los marcadores plasmáticos de NO, así como, la respuesta cardiorrespiratoria durante un ejercicio aeróbico de intensidad moderada en sujetos entrenados (*Estudio I*).
2. Investigar el efecto de una ingesta aguda de nitratos inorgánicos en la respuesta metabólica y cardiorrespiratoria durante un ejercicio físico aeróbico en sujetos entrenados (*Estudio II*).
3. Evaluar si una suplementación de nitratos inorgánicos mejora el rendimiento de sujetos entrenados (*Estudios II y III*).
4. Analizar el efecto de la ingesta de nitratos inorgánicos en otras moléculas como la endotelina-1 que también participan en la regulación del flujo sanguíneo durante el ejercicio físico (*Estudio III*).
5. Analizar las principales conclusiones de los estudios relacionados con los precursores de NO y el rendimiento humano en sujetos sanos (*Estudio IV*).

Resultados y discusión

En relación a estos objetivos y los resultados obtenidos en esta tesis, se ha demostrado que la ingesta dietética de L-arginina a diferentes dosis no induce un aumento de los marcadores plasmáticos de NO y tampoco mejora la adaptación cardiorrespiratoria durante un ejercicio físico de resistencia en deportistas (*Estudio I*). Estos resultados coinciden con los obtenidos por otras investigaciones tal y como se recoge en el *Estudio IV*. La baja biodisponibilidad de la L-arginina oral ha sido uno de los argumentos más utilizados para explicar la limitada capacidad ergogénica de este aminoácido (117). Aproximadamente, sólo el 30% de la L-arginina oral llega a la circulación sanguínea debido al gran catabolismo que sufre este aminoácido a su paso por el hígado (118). No obstante, observando los resultados de otras investigaciones que suministraron L-arginina por vía endovenosa (71-72), el argumento de la baja biodisponibilidad ha sido descartado. Dichos estudios concluyeron que tanto los marcadores plasmáticos de NO, como los parámetros relacionados con el rendimiento, no mejoraron tras la suplementación con L-arginina suministrada por vía endovenosa (71-72).

Por otra parte, la ingesta de nitratos inorgánicos aumenta significativamente la concentración de estas moléculas en plasma (*Estudios II y III*). Tal aumento se corresponde con los resultados observados por otros estudios anteriores (46, 82, 84-89). Mientras aproximadamente el 70% del nitrato ingerido oralmente es filtrado en el riñón y eliminado en la orina, el 30% restante es almacenado en las glándulas salivares de la boca por mecanismos que todavía se desconocen (*Estudio III*) (30). Los nitratos se mezclan con la saliva y son excretados en la cavidad bucal donde la presencia de bacterias anaeróbicas favorece la reducción del nitrato a nitrito. Una vez absorbido, en el medio ácido del estómago parte del nitrito es reducido a NO. Otra parte llega al intestino, donde los nitritos son absorbidos y pasan hacía la circulación sanguínea. En situaciones de hipoxia y/o acidosis estas moléculas pueden ser reducidas a NO (30).

En el *Estudio II*, paralelo al aumento de nitratos, se observó también un aumento significativo de la concentración de los nitritos plasmáticos. Por el contrario, este efecto no fue corroborado en el *Estudio III* utilizando la misma dosis de nitratos inorgánicos en un grupo de sujetos con similares características. Es difícil explicar porqué en el *Estudio III* los nitritos plasmáticos no aumentaron tras la suplementación con nitratos inorgánicos. Algunos estudios previos han observado que el uso regular de enjuagues bucales anti-bactericidas disminuyen la flora bacteriana bucal, reduciendo así también, la capacidad de oxidar los nitratos a nitritos (91-92). No obstante, ninguno de los deportistas analizados en el *Estudio III* utilizaban dichos productos, con lo cual, parece poco probable que los niveles de nitritos plasmáticos no aumentaran por este motivo. Según los resultados del *Estudio III* existe una alta variabilidad inter-sujeto en la respuesta al incremento de los nitritos plasmáticos en deportistas. A pesar de que todos los sujetos (n = 13) analizados evidenciaron un incremento significativo de los nitratos en plasma, esta respuesta no fue acompañada por un aumento de los nitritos plasmáticos en seis de los sujetos analizados.

Otro punto a destacar tanto en el *Estudio II* como en el *III* fue la elevada concentración de nitritos plasmáticos en los deportistas analizados en comparación con los valores observados por otros estudios (46, 82, 84-89). Quizás, estas diferencias puedan deberse al nivel físico de los sujetos que fueron analizados. Ha sido demostrado que la concentración de nitritos plasmáticos está directamente relacionada con el nivel de entrenamiento (119). Por tanto, desde este punto de vista, parece lógico que en los *Estudios II* y *III* de esta tesis los valores de nitritos plasmáticos fueran superiores a los

observados por estudios previos en sujetos sanos pero poco entrenados (46, 82, 84-89). Aunque en los *Estudios II* y *III* no participaron deportistas profesionales, los sujetos analizados realizaban un volumen de entrenamiento considerable (> 10.000 km de ciclismo/año). Este volumen no sólo puede mejorar el metabolismo del NO, también favorece las funciones de otros mecanismos (cardiovasculares, nerviosos, metabólicos, etc.) (52, 78) que mejoran la adaptación del organismo al ejercicio físico. Para conocer con más detalle el efecto del entrenamiento en la concentración de nitratos y nitritos plasmáticos son necesarios nuevos estudios que analicen detalladamente estos aspectos.

Teniendo en cuenta que los nitritos son los principales precursores de NO en la vi-NOS (30), el limitado aumento de la concentración plasmática de estas moléculas que fue observado en el *Estudio III*, puede explicar la ausencia de una mejora en el rendimiento físico de los sujetos analizados. En el *Estudio II*, a pesar de que los nitritos plasmáticos aumentaron significativamente tampoco se observaron diferencias significativas ni en la Gross Efficiency, ni en el rendimiento valorado a partir del tiempo de ejercicio hasta la fatiga durante una prueba de esfuerzo progresiva en cicloergómetro. Estos resultados difieren de los obtenidos por otras investigaciones. Por ejemplo, en un estudio de Larsen et al (82) se observó que una suplementación de nitratos inorgánicos inducía una mejora en la Gross Efficiency en intensidades de esfuerzo comprendidas entre el 40 y el 60% del VO_{2max}. Similares resultados han sido descritos por otros estudios utilizando una suplementación de nitratos en forma de jugo de remolacha (86, 88). Además, también ha sido indicado que la ingesta aguda (1 sólo día) de nitratos (500 ml de zumo de remolacha) puede mejorar el rendimiento aeróbico en sujetos sanos (87, 89). En estos momentos es difícil saber porque existen estas divergencias en los resultados de los diferentes estudios. Una posible respuesta que podría explicar parcialmente tales diferencias ha sido sugerida en el *Estudio IV* y hace referencia al nivel de condición física de los sujetos analizados en cada uno de los estudios. Mientras todas aquellas investigaciones que han analizado sujetos sanos pero poco entrenados han observado una mejora del rendimiento asociada a la ingesta de nitratos inorgánicos (VO_{2max} < 56 $ml \cdot kg^{-1} \cdot min^{-1}$), los *Estudios II* y *III* de esta tesis no observaron una mejoría en el rendimiento de sujetos bien entrenados (VO_{2max} > 59 $ml \cdot kg^{-1} \cdot min^{-1}$). Esta tendencia parece ser similar a la observada en los estudios previos con L-arginina (66). Como ha sido indicado anteriormente, este aminoácido puede ser efectivo para mejorar la tolerancia al ejercicio físico de personas poco entrenadas, no obstante, parece ser

poco efectivo como ayuda ergogénica en atletas bien entrenados. Este hecho puede sugerir la existencia de un umbral de entrenamiento –volumen e intensidad– a partir del cual los efectos del ejercicio físico sobrepasan los derivados de cualquier suplementación con L-arginina o nitratos (*Estudio IV*). Por su importancia no sólo en el rendimiento físico, sino también en la salud cardiovascular, este punto debe ser analizado con mucho más detalle en futuras investigaciones.

Por otra parte, los estudios incluidos en esta tesis han aportado algunos resultados interesantes en relación a otros posibles efectos de la L-arginina y los nitratos inorgánicos en el organismo. Quizás, el principal de estos hallazgos fue el aumento significativo de los niveles plasmáticos de la endotelina-1observados en el *Estudio III*. Mientras el NO posee una función principalmente vasodilatadora, la endotelina-1 es una molécula con una potente acción vasoconstrictora, contrarrestando así el efecto del NO (102). Estudios de Maeda et al (103-105) han descrito como los niveles de endotelina-1 aumentan en los tejidos no activos durante un ejercicio físico. Esta respuesta ha sido sugerida como un mecanismo de regulación del flujo sanguíneo. El aumento de endotelina-1 en los órganos y tejidos no activos aumentaría la vasoconstricción en estas zonas del cuerpo, para favorecer una mayor irrigación de los músculos y órganos activos durante el ejercicio. Teniendo en cuenta los resultados obtenidos en el *Estudio III* (la concentración de endotelina-1 aumentaba de forma significativa en la vena cubital del brazo mientras los sujetos realizaban una prueba de esfuerzo en cicloergómetro) puede sugerirse que los nitratos inorgánicos poseen un papel importante en la modulación del flujo sanguíneo durante el ejercicio físico. No obstante, esto es sólo una hipótesis y se requieren nuevas investigaciones que analicen que función desempeñan los nitratos inorgánicos en la modulación del flujo sanguíneo y las moléculas que están detrás de estos efectos.

Por último, prácticamente la totalidad de estudios que han analizado el efecto de la L-arginina y los nitratos inorgánicos en el rendimiento humano se han basado en el análisis de varones jóvenes (< 35 años). En contra de este hecho, existen pocos estudios que hayan valorado el efecto de estos componentes en personas de la tercera edad. Estos estudios pueden ser de gran interés, desde que se conoce que la función cardiovascular sufre un deterioro progresivo paralelo al proceso de envejecimiento. Las principales características de este proceso reflejan cambios anatómicos y estructurales a nivel de la pared de los vasos, la relajación miocárdica, el llenado ventricular y la respuesta a las

catecolaminas. Además también se produce una disminución de la producción de óxido nítrico (106-107). Por esta razón puede ser interesante analizar en futuras investigaciones el efecto de la suplementación con precursores de NO sobre la tolerancia al ejercicio físico en sujetos de edad avanzada. Otro aspecto que debería ser abordado en futuros estudios es el efecto de la ingesta de nitratos inorgánicos o la L-arginina en la población femenina. Existe un elevado porcentaje de mujeres que compiten a alto nivel y padecen trastornos relacionados con el ciclo menstrual (retraso de la menarquía, amenorrea primaria y secundaria, etc.) (109). Tales alteraciones pueden también afectar a diversos sistemas del organismo como el aparato cardiovascular (110). Por este motivo puede ser de gran interés analizar el efecto de los precursores de óxido nítrico en mujeres atletas, así como también en mujeres durante la etapa post-menopáusica.

Conclusiones

- La suplementación dietética con L-arginina a diferentes dosis no aumenta los marcadores plasmáticos de NO en tenistas.
- Altas ingestas de L-arginina no mejoran la respuesta cardiorespiratoria y metabólica en el ejercicio físico de resistencia en sujetos entrenados.
- Una ingesta aguda (1 día), así como, 3 días de suplementación con nitratos inorgánicos aumentan significativamente los niveles plasmáticos de nitratos en sujetos entrenados.
- El incremento de los niveles de nitrato plasmático no induce un aumento de los nitritos en plasma en todos los sujetos entrenados.
- La Gross Efficiency, definida como la relación entre el trabajo mecánico y la producción de energía metabólica, no mejora tras una suplementación con nitratos inorgánicos en sujetos entrenados.
- El consumo máximo de oxígeno se reduce significativamente después de una ingesta aguda de nitratos inorgánicos. Esta respuesta fisiológica no altera el rendimiento valorado como el tiempo hasta la claudicación por fatiga durante una prueba de esfuerzo incremental y máxima.

- Tres días de suplementación con nitratos inorgánicos no mejora el rendimiento valorado mediante la potencia media y la distancia total desarrollada durante una contrarreloj de 40 minutos en cicloergómetro.
- Tres días de suplementación con nitratos aumenta significativamente la concentración plasmática de endotelina-1 en la vena cubital del brazo justo al finalizar una contrarreloj de 40 minutos en cicloergómetro.
- Los estudios científicos actuales sugieren que la suplementación con precursores del NO no aumentan el rendimiento físico en sujetos entrenados.
- Los beneficios relacionados con la ingesta de L-arginina y L-citrulina que han sido indicados en algunos estudios previos pueden derivar de otros ingredientes incluidos en los suplementos, o de otras funciones que estos aminoácidos desempeñan en el organismo.

8. REFERENCES

1. Luiking YC, Engelen MP, Deutz NE. Regulation of nitric oxide production in health and disease. *Curr Opin Clin Nutr Metab Care.* 2010;13:97-104.
2. Castello PR, David PS, McClure T, Crook Z, Poyton RO. Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes. *Cell Metab.* 2006;3:277-87.
3. Shen W, Xu X, Ochoa M, Zhao G, Wolin MS, Hintze TH. Role of nitric oxide in the regulation of oxygen consumption in conscious dogs. *Circ Res.* 1994;75:1086-95.
4. Ghafourifar P, Cadenas E. Mitochondrial nitric oxide synthase. *Trends Pharmacol Sci.* 2005;26:190-5.
5. Dedon PC, Tannenbaum SR. Reactive nitrogen species in the chemical biology of inflammation. *Arch Biochem Biophys.* 2004;423:12-22.
6. Bogdan C. Nitric oxide and the immune response. *Nat Immunol.* 2001;2:907-16.
7. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med.* 1993;329:2002-12.
8. Schmidt HH, Nau H, Wittfoht W, et al. Arginine is a physiological precursor of endothelium-derived nitric oxide. *Eur J Pharmacol.* 1988;154:213-6.
9. Sureda A, Tauler P, Aguiló A, et al. Blood cell NO synthesis in response to exercise. *Nitric Oxide.* 2006;15:5-12.
10. Balon TW, Nadler JL. Evidence that nitric oxide increases glucose transport in skeletal muscle. *J Appl Physiol.* 1997;82:359-63.
11. Capanni C, Squarzoni S, Petrini S, et al. Increase of neuronal nitric oxide synthase in rat skeletal muscle during ageing. *Biochem Biophys Res Commun.* 1998;245:216-9.
12. Shen W, Zhang X, Zhao G, Wolin MS, Sessa W, Hintze TH. Nitric oxide production and NO synthase gene expression contribute to vascular regulation during exercise. *Med Sci Sports Exerc.* 1995;27:1125-34.
13. Chen K, Pittman RN, Popel AS. Nitric oxide in the vasculature: where does it come from and where does it go? A quantitative perspective. *Antioxid Redox Signal.* 2008;10:1185-98.
14. Kao CC, Bandi V, Guntupalli KK, Wu M, Castillo L, Jahoor F. Arginine, citrulline and nitric oxide metabolism in sepsis. *Clin Sci.* 2009;117:23-30.
15. Magne J, Huneau JF, Delemasure S, Rochette L, Tome D, Mariotti F. Whole-body basal nitric oxide production is impaired in postprandial endothelial dysfunction in healthy rats. *Nitric Oxide.* 2009;21:37-43.
16. Li P, Kim SW, Li X, Datta S, Pond WG, Wu G. Dietary supplementation with cholesterol and docosahexaenoic acid increases the activity of the arginine-nitric oxide pathway in tissues of young pigs. *Nitric Oxide.* 2008;19:259-65.
17. Wu G, Meininger CJ. Nitric oxide and vascular insulin resistance. *Biofactors.* 2009;35:21-7.

18. King DE, Mainous AG, Geesey ME. Variation in L-arginine intake follow demographics and lifestyle factors that may impact cardiovascular disease risk. *Nutr Res.* 2008;28:21-4.

19. Venho B, Voutilainen S, Valkonen VP, et al. Arginine intake, blood pressure, and the incidence of acute coronary events in men: the Kuopio Ischaemic Heart Disease Risk Factor Study. *Am J Clin Nutr.* 2002;76:359-64.

20. Mori M, Gotoh T. Regulation of nitric oxide production by arginine metabolic enzymes. *Biochem Biophys Res Commun.* 2000;275:715-9.

21. Boger RH, Tsikas D, Bode-Boger SM, Phivthong-Ngam L, Schwedhelm E, Frolich JC. Hypercholesterolemia impairs basal nitric oxide synthase turnover rate: a study investigating the conversion of L-[guanidino-(15)N(2)]-arginine to (15)N-labeled nitrate by gas chromatography--mass spectrometry. *Nitric Oxide.* 2004;11:1-8.

22. Boger RH, Bode-Boger SM. The clinical pharmacology of L-arginine. *Annu Rev Pharmacol Toxicol.* 2001;41:79-99.

23. Castillo L, Chapman TE, Sanchez M, et al. Plasma arginine and citrulline kinetics in adults given adequate and arginine-free diets. *Proc Natl Acad Sci.* 1993;90:7749-53.

24. Boger RH. L-Arginine therapy in cardiovascular pathologies: beneficial or dangerous? *Curr Opin Clin Nutr Metab Care.* 2008;11:55-61.

25. Cynober L. Pharmacokinetics of arginine and related amino acids. *J Nutr.* 2007 2007;137:1646-9.

26. McConell GK. Effects of L-arginine supplementation on exercise metabolism. *Curr Opin Clin Nutr Metab Care.* 2007;10:46-51.

27. Lundberg JO, Weitzberg E, Lundberg JM, Alving K. Intragastric nitric oxide production in humans: measurements in expelled air. *Gut.* 1994;35:1543-6.

28. Benjamin N, O'Driscoll F, Dougall H, et al. Stomach NO synthesis. *Nature.* 1994;368:502.

29. Lundberg JO, Weitzberg E. The biological role of nitrate and nitrite: the times they are a-changing. *Nitric Oxide.* 2010;22:61-3.

30. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov.* 2008;7:156-67.

31. Shiva S, Huang Z, Grubina R, et al. Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration. *Circ Res.* 2007;100:654-61.

32. Petersen MG, Dewilde S, Fago A. Reactions of ferrous neuroglobin and cytoglobin with nitrite under anaerobic conditions. *J Inorg Biochem.* 2008;102:1777-82.

33. Webb AJ, Milsom AB, Rathod KS, et al. Mechanisms underlying erythrocyte and endothelial nitrite reduction to nitric oxide in hypoxia: role for xanthine oxidoreductase and endothelial nitric oxide synthase. *Circ Res.* 2008;103:957-64.

34. Li H, Cui H, Kundu TK, Alzawahra W, Zweier JL. Nitric oxide production from nitrite occurs primarily in tissues not in the blood: critical role of xanthine oxidase and aldehyde oxidase. *J Biol Chem.* 2008;283:17855-63.

35. Cosby K, Partovi KS, Crawford JH, et al. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med.* 2003;9:1498-505.

36. Stuehr DJ, Santolini J, Wang ZQ, Wei CC, Adak S. Update on mechanism and catalytic regulation in the NO synthases. *J Biol Chem.* 2004;279:36167-70.

37. Feelisch M, Fernandez BO, Bryan NS, et al. Tissue processing of nitrite in hypoxia: an intricate interplay of nitric oxide-generating and -scavenging systems. *J Biol Chem.* 2008;283:33927-34.

38. Tricker AR, Preussmann R. Carcinogenic N-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential. *Mutation Res.* 1991;259:277-89.

39. McKnight GM, Duncan CW, Leifert C, Golden MH. Dietary nitrate in man: friend or foe? *Br J Nutr.* 1999;81:349-58.

40. Lundberg JO, Weitzberg E. NO generation from inorganic nitrate and nitrite: Role in physiology, nutrition and therapeutics. *Arch Pharm Res.* 2009;32:1119-26.

41. Hord NG, Tang Y, Bryan NS. Food sources of nitrates and nitrites: the physiologic context for potential health benefits. *Am J Clin Nutr.* 2009;90(1):1-10.

42. Zeegers MP, Selen RF, Kleinjans JC, Goldbohm RA, van den Brandt PA. Nitrate intake does not influence bladder cancer risk: The netherlands cohort study. *Environ Health Perspect.* 2006;114:1527-31.

43. Kilbourn RG, Gross SS, Jubran A, et al. NG-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: implications for the involvement of nitric oxide. *Proc Natl Acad Sci.* 1990;87:3629-32.

44. Lundberg JO, Giovoni M. Inorganic Nitrate is a possible source for systemic generation of nitric oxide. *Free Rad Bio Med.* 2004;37:395-400.

45. Kingwell BA, Sherrard B, Jennings GL, Dart AM. Four weeks of cycle training increases basal production of nitric oxide from the forearm. *Am J Physiol.* 1997;272:1070-7.

46. Webb AJ, Loukogeorgakis S, Okorie M, et al. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension.* 2008;51:784-90.

47. Tannenbaum SR, Weisman M, Fett D. The effect of nitrate intake on nitrite formation in human saliva. *Food Cosmet Toxicol.* 1976;14:549-52.

48. Duncan C, Dougall H, Johnston P, et al. Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nat Med.* 1995;1:546-51.

49. Kingwell B. Nitric oxide as a metabolic regulator during exercise: effects of training in health and disease. *Clin Exp Pharm Physiol.* 2000;27(4):239-50.

50. Langberg H, Bjorn C, Boushel R, Hellsten Y, Kjaer M. Exercise-induced increase in interstitial bradykinin and adenosine concentrations in skeletal muscle and peritendinous tissue in humans. *J Physiol.* 2002;542:977-83.

51. Whyte JJ, Laughlin MH. The effects of acute and chronic exercise on the vasculature. *Acta Physiol.* 2010;199:441-50.

52. Maiorana A, O'Driscoll G, Taylor R, Green D. Exercise and the nitric oxide vasodilator system. *Sports Med.* 2003;33:1013-35.

53. Green DJ, Spence A, Halliwill JR, Cable NT, Thijssen DH. Exercise and vascular adaptation in asymptomatic humans. *Exp Physiol.* 2011;96:57-70.

54. Petroczi A, Naughton DP. Potentially fatal new trend in performance enhancement: a cautionary note on nitrite. *J Int Soc Sports Nutr.* 2010;7:25.

55. Bloomer RJ. Nitric oxide supplements for sports. *J Strength Cond Res.* 2010;32:14-20.

56. Schoch RD, Willoughby D, Greenwood M. The regulation and expression of the creatine transporter: a brief review of creatine supplementation in humans and animals. *J Int Soc Sports Nutr.* 2006;3:60-6.

57. Burke LM, Kiens B, Ivy JL. Carbohydrates and fat for training and recovery. *J Sports Sci.* 2004;22:15-30.

58. Tipton KD, Ferrando AA. Improving muscle mass: response of muscle metabolism to exercise, nutrition and anabolic agents. *Essays Biochem.* 2008;44:85-98.

59. Bode-Boger SM, Boger RH, Galland A, Tsikas D, Frolich JC. L-arginine-induced vasodilation in healthy humans: pharmacokinetic-pharmacodynamic relationship. *Br J Clin Pharmacol.* 1998;46:489-97.

60. Kuipers H, Rietjens G, Verstappen F, Schoenmakers H, Hofman G. Effects of stage duration in incremental running tests on physiological variables. *Int J Sports Med.* 2003;24:486-91.

61. Brouwer E. On simple formulae for calculating the heat expenditure and the quantities of carbohydrate and fat oxidized in metabolism of men and animals, from gaseous exchange (Oxygen intake and carbonic acid output) and urine-N. *Acta Physiol Pharmacol Neerl.* 1957;6:795-802.

62. Moshage H, Kok B, Huizenga JR, Cansen PLM. Nitrite and nitrate determinations in plasma: A critical evaluation. *Clinical Chemistry.* 1995;41:892-6.

63. Santos MT, Valles J, Aznar J, Vilches J. Determination of plasma malondialdehyde-like material and its clinical application in stroke patients. *J Clin Pathol.* 1980;33:973-6.

64. Braman RS, Hendrix SA. Nanogram nitrite and nitrate determination in environmental and biological materials by vanadium (III) reduction with chemiluminescence detection. *Anal Chem.* 1989;61:2715-8.

65. Castegnaro M, Massey RC, Walters CL. The collaborative evaluation of a procedure for the determination of N-nitroso compounds as a group. *Food Addit Contam.* 1987;4:37-43.

66. Liu TH, Wu CL, Chiang CW, Lo YW, Tseng HF, Chang CK. No effect of short-term arginine supplementation on nitric oxide production, metabolism and performance in intermittent exercise in athletes. *J Nutr Biochem.* 2008;20:462-8.

67. Olek RA, Ziemann E, Grzywacz T, et al. A single oral intake of arginine does not affect performance during repeated Wingate anaerobic test. *J Sports Med Phys Fitness.* 2010 Mar;50(1):52-6.

68. Sureda A, Batle JM, Tauler P, Ferrer MD, Tur JA, Pons A. Vitamin C supplementation influences the antioxidant response and nitric oxide handling of erythrocytes and lymphocytes to diving apnea. *Eur J Clin Nutr.* 2006;60:838-46.

69. Wu G, Morris M. Arginine metabolism: nitric oxide and beyond. *Biochem J.* 1998;336:1-17.

70. Wu GY, Bazer FW, Davis TA, et al. Arginine metabolism and nutrition in growth, health and disease. *Amino Acids.* 2009;37:153-68.

71. McConell GK, Huynh NN, Lee-Young RS, Canny BJ, Wadley GD. L-Arginine infusion increases glucose clearance during prolonged exercise in humans. *Am J Physiol Endocrinol Metab.* 2006;290:60-6.

72. Schaefer A, Piquard F, Geny B, et al. L-arginine reduces exercise-induced increase in plasma lactate and ammonia. *Int J Sports Med.* 2002;23:403-7.

73. Rabier D, Kamoun P. Metabolism of citrulline in man. *Amino Acids.* 1995;9:299-316.

74. Bailey SJ, Winyard PG, Vanhatalo A, et al. Acute L-arginine supplementation reduces the O2 cost of moderate-intensity exercise and enhances high-intensity exercise tolerance. *J Appl Physiol.* 2010;109:1394-403.

75. Boger RH, Ron ES. L-Arginine improves vascular function by overcoming deleterious effects of ADMA, a novel cardiovascular risk factor. *Altern Med Rev.* 2005;10:14-23.

76. Doutreleau S, Mettauer B, Piquard F, et al. Chronic L-arginine supplementation enhances endurance exercise tolerance in heart failure patients. *Int J Sports Med.* 2006;27:567-72.

77. McKnight JR, Satterfield MC, Jobgen WS, et al. Beneficial effects of L-arginine on reducing obesity: potential mechanisms and important implications for human health. *Amino Acids.* 2010;39:349-57.

78. Green D, Maiorana AO, Driscoll G, Taylor R. Effect of exercise training on endothelium-derived nitric oxide function in humans. *J Physiol.* 2004;561:1-25.

79. Jansson E, Kaijser L. Muscle adaptation to extreme endurance training in man. *Acta Physiol Scand.* 1977;100:315-24.

80. Burgomaster KA, Hughes SC, Heigenhauser GJ, Bradwell SN, Gibala MJ. Six sessions of sprint interval training increases muscle oxidative potential and cycle endurance capacity in humans. *J Appl Physiol.* 2005;98:1985-90.

81. Hopker J, Passfield L, Coleman D, Jobson S, Edwards L, Carter H. The effects of training on gross efficiency in cycling: a review. *Int J Sports Med.* 2009;30:845-50.

82. Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B. Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol.* 2007;191:55-66.

83. Larsen FJ, Schiffer TA, Borniquel S, et al. Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell Metab.* 2011;13:149-59.

84. Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B. Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise. *Free Radic Biol Med.* 2010;48:342-7.

85. Bailey SJ, Fulford J, Vanhatalo A, et al. Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *J Appl Physiol.* 2010;109:135-48.

86. Bailey SJ, Winyard P, Vanhatalo A, et al. Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *J Appl Physiol.* 2009:107:1144-55.

87. Lansley KE, Winyard PG, Bailey SJ, et al. Acute dietary nitrate supplementation improves cycling time trial performance. *Med Sci Sports Exerc.* 2011;43:1125-31.

88. Lansley KE, Winyard PG, Fulford J, et al. Dietary nitrate supplementation reduces the O2 cost of walking and running: a placebo-controlled study. *J Appl Physiol.* 2011;110:591-600.

89. Vanhatalo A, Bailey SJ, Blackwell JR, et al. Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. *Am J Physiol Regul Integr Comp Physiol.* 2010;299:1121-31.

90. Bescós R, Rodriguez FA, Iglesias X, Ferrer MD, Iborra E, Pons A. Acute administration of inorganic nitrate reduces VO_{2peak} in endurance athletes. *Med Sci Sports Exerc.* 2011; 43:1979-1986.

91. Petersson J, Carlström M, Schreiber O, et al. Gastroprotective and blood pressure lowering effects of dietary nitrate are abolished by an antiseptic mouthwash. *Free Rad Biol Med.* 2009;46:1068-75.

92. Govoni M, Jansson EA, Weitzberg E, Lundberg JO. The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide.* 2008;19:333-7.

93. Sunderland KL, Greer F, Morales J. VO2max and ventilatory threshold of trained cyclists are not affected by 28-day l-arginine supplementation. *J Strength Cond Res.* 2011;25:833-7.

94. Camic CL, Housh TJ, Mielke M, et al. The effects of 4 weeks of an arginine-based supplement on the gas exchange threshold and peak oxygen uptake. *Appl Physiol Nutr Metab.* 2010;35:286-93.

95. Tsai PH, Tang TK, Juang CL, Chen KW, Chi CA, Hsu MC. Effects of arginine supplementation on post-exercise metabolic responses. *Chin J Physiol.* 2009;52:136-42.

96. Bescós R, Gonzalez Haro C, Pujol P, et al. Effects of dietary L-Arginine intake on cardiorespiratory and metabolic adaptation in athletes. *Int J Sport Nutr Exerc Metab.* 2009;19:355-65.

97. Chin-Dusting JP, Alexander CT, Arnold PJ, Hodgson WC, Lux AS, Jennings GL. Effects of in vivo and in vitro L-arginine supplementation on healthy human vessels. *J Cardiovasc Pharmacol.* 1996;28:158-66.

98. Adams MR, Forsyth CJ, Jessup W, Robinson J, Celermajer DS. Oral L-arginine inhibits platelet aggregation but does not enhance endothelium-dependent dilation in healthy young men. *J Am Coll Cardiol.* 1995;26:1054-61.

99. Hartman WJ, Torre PM, Prior RL. Dietary citrulline but not ornithine counteracts dietary arginine deficiency in rats by increasing splanchnic release of citrulline. *J Nutr.* 1994;124:1950-60.

100. Hickner RC, Tanner CJ, Evans CA, et al. L-citrulline reduces time to exhaustion and insulin response to a graded exercise test. *Med Sci Sports Exerc.* 2006;38:660-6.

101. Maeda S, Miyauchi T, Iemitsu M, Sugawara J, Nagata Y, Goto K. Resistance exercise training reduces plasma endothelin-1 concentration in healthy young humans. *J Cardiovasc Pharmacol.* 2004;44:443-6.

102. Thijssen DH, Rongen GA, Smits P, Hopman MT. Physical (in)activity and endothelium-derived constricting factors: overlooked adaptations. *J Physiol.* 2008;586:319-24.

103. Maeda S, Miyauchi T, Goto K, Matsuda M. Alteration of plasma endothelin-1 by exercise at intensities lower and higher than ventilatory threshold. *J Appl Physiol.* 1994;77:1399-402.

104. Maeda S, Miyauchi T, Kobayashi T, Goto K, Matsuda M. Exercise causes tissue-specific enhancement of endothelin-1 mRNA expression in internal organs. *J Appl Physiol.* 1998;85:425-31.

105. Maeda S, Miyauchi T, Sakane M, et al. Does endothelin-1 participate in the exercise-induced changes of blood flow distribution of muscles in humans? *J Appl Physiol.* 1997;82:1107-11.

106. Seals DR, Jablonski KL, Donato AJ. Aging and vascular endothelial function in humans. *Clin Sci.* 2011;120:357-75.

107. Donato AJ, Gano LB, Eskurza I, et al. Vascular endothelial dysfunction with aging: endothelin-1 and endothelial nitric oxide synthase. *Am J Physiol Heart Circ Physiol.* 2009;297:425-32.

108. Blair ML. Sex-based differences in physiology: what should we teach in the medical curriculum? *Adv Physiol Educ.* 2007;31:23-5.

109. De Souza MJ, Toombs RJ, Scheid JL, O'Donnell E, West SL, Williams NI. High prevalence of subtle and severe menstrual disturbances in exercising women: confirmation using daily hormone measures. *Hum Reprod.* 2010;25:491-503.

110. Hoch A, Dempsey RL, Carrera GF, et al. Is there an association between athletic amenorrhea and endothelial cell dysfunction? *Med Sci Sports Exerc.* 2003;35(3):377-83.

111. Lundberg JO, Weitzberg E. NO Generation from nitrite and its role in vascular control. *Arterioscler Thromb Vasc Biol*. 2005;25:915-22.

112. Curis E, Nicolis I, Moinard C, et al. Almost all about citrulline in mammals. *Amino Acids.* 2005;29:177-205.

113. Doutreleau S, Mettauer B, Piquard F, et al. Chronic but not acute oral L-arginine supplementation delays the ventilatory threshold during exercise in heart failure patients. *Can J Appl Physiol.* 2005;30:419-32.

114. Doutreleau S, Rouyer O, Di Marco P, Lonsdorfer E, Richard R, Piquard F, et al. L-arginine supplementation improves exercise capacity after a heart transplant. *Am J Clin Nutr.* 2010;91:1261-7.

115. Rogers MA, Vaughan TL, Davis S, Thomas DB. Consumption of nitrate, nitrite, and nitrosodimethylamine and the risk of upper aerodigestive tract cancer. *Cancer Epidemiol Biomarkers Prev.* 1995;4:29-36.

116. Lundberg JO, Carlstrom M, Larsen FJ, Weitzberg E. Roles of dietary inorganic nitrate in cardiovascular health and disease. *Cardiovasc Res.* 2010;89:525-32.

117. Alvares TS, Meirelles CM, Bhambhani YN, Paschoalin VM, Gomes PS. L-arginine as a potential ergogenic aid in healthy subjects. *Sports Med.* 2011;41:233-48.

118. Schwedhelm E, Maas R, Freese R, et al. Pharmacokinetic and pharmacodynamic properties of oral L-citrulline and L-arginine: impact on nitric oxide metabolism. *Br J Clin Pharmacol.* 2008;65:51-9.

119. Rassaf T, Lauer T, Heiss C, et al. Nitric oxide synthase-derived plasma nitrite predicts exercise capacity. *Br J Sports Med.* 2007;41:669-73.

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Effects of Dietary L-Arginine Intake on Cardiorespiratory and Metabolic Adaptation in Athletes

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To assess the effect of diet enrichment with L-arginine or supplementation at high doses on physiological adaptation during exercise, 9 athletes followed 3 different diets, each over 3 consecutive days, with a wash-out period of 4 d between training sessions: control diet (CD), 5.5 ± 0.3 g/d of L-arginine; Diet 1 (rich in L-arginine food), 9.0 ± 1.1 g/d of L-arginine; and Diet 2 (the same as CD but including an oral supplement of 15 g/d), 20.5 ± 0.3 g/d of L-arginine. Plasma nitrate levels of each participant were determined on the day after each treatment. Participants performed a submaximal treadmill test (initial speed 10–11 km/hr, work increments 1 km/hr every 4 min until 85–90% VO_{2max}, and passive recovery periods of 2 min). Oxygen uptake and heart rate were monitored throughout the test. Blood lactate concentration ($[La^-]_b$) was determined at the end of each stage. Repeated-measures ANOVA and paired Student's *t* tests were used to compare the various physiological parameters between diets. The level of significance was set at $p < .05$. $[La^-]_b$ showed a significant effect at the 5-min time point between CD and Diet 2 (CD 3.0 ± 0.5 mM, Diet 2 2.5 ± 0.5 mM, $p = .03$), but this tendency was not found at higher exercise intensities. No significant differences were observed in any of the cardiorespiratory or plasma nitrate levels. In conclusion, dietary L-arginine intake on the days preceding the test does not improve physiological parameters during exercise.

Keywords: nitric oxide, oxygen uptake, blood lactate concentration, heart rate, amino acid

L-arginine is the main physiological precursor of nitric oxide (NO). This molecule is an endogenous gas released from the vascular endothelium in response to a variety of stimuli such as acetylcholine, catecholamine, fluid shear stress, and

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local hypoxia (Vallance, Leone, Calver, Collier, & Moncada, 1992). In physiological adaptation to exercise, NO plays an important regulatory role by increasing blood flow to the muscles and modulating muscle contraction and glucose uptake (McConell & Kingwell, 2006). It is also involved in controlling cellular respiration through interaction with enzymes of the mitochondrial respiratory chain (Moncada & Erusalimsky, 2002), and it may act as an antioxidant in certain situations (Brune et al., 1997).

During exercise, oxygen consumption (VO_2; i.e., total energetic cost of the physical work) is an important parameter that reflects both mechanical efficiency and running performance until fatigue. The inhibition of NO synthesis by endogenous inhibitors such as N^G-nitro-L-arginine methyl ester and N^G-monomethyl-L-arginine has been associated with increased submaximal VO_2 levels in animals during exercise independent of the reduction in blood flow (Shen et al., 1995). This finding has also been reported in studies both in vitro (Brown, 1995) and in humans (Schrage, Joyner, & Dinenno, 2004). The increase in VO_2 during NOS blockage has been attributed to the fact that NO affects tissue respiration by reversible inhibition of the respiratory enzyme cytochrome c oxidase (Brown & Cooper, 1994). Other authors have related the increased VO_2 during NOS blockage to an inhibiting effect of NO on proton leakage over the mitochondrial membrane (Bohuslavs'kyi, Dmytriieva, & Sahach, 2005). Increases in VO_2 have been associated with decreased mechanical and energetic efficiency during physical effort (Jones, Wilkerson, & Campbell, 2004). Nevertheless, few attempts have been made to study the effect of exogenous NO delivery on physiological efficiency (VO_2) during exercise. Studies with pharmacological NO donors such as nitroprusside and nitroglycerine have shown divergent results: decreases in VO_2 (Loke et al., 1999), no effect (Nuñez et al., 2005), or increases (De Backer et al., 1996).

The effect of L-arginine on exercise capacity and NO metabolism in healthy participants and highly trained athletes is still not clear (Abel et al., 2005). The first investigations on oral supplementation with arginine aspartate or arginine glutamate suggested that they could reduce blood lactate concentration ($[La^-]_b$) and accumulation of ammonia after high-intensity exercise. These metabolites have been shown to be involved in the development of muscle fatigue caused by increased muscle acidity (Denis et al., 1991). However, the role of arginine in these studies may have been masked by the other components that might also contribute to delaying fatigue. In another study, oral arginine aspartate failed to produce metabolic benefit in endurance runners during 14 days of supplementation (Colombani et al., 1999).

To our knowledge, the dietary intervention of L-arginine intake in athletes has not been studied to date. We hypothesized that a diet rich in L-arginine and/or higher doses of this amino acid supplement might improve exercise economy in highly trained athletes. The aim of the current study was to determine whether increases in L-arginine intake increase NO delivery and optimize performance (i.e., reduce VO_2 and/or $[La^-]_b$ values) during treadmill exercise in athletes.

Methods

Participants

Nine highly trained national-level male tennis players (age 18.2 ± 3.7 years, VO_{2peak} 57.4 ± 3.9 ml · kg^{-1} · min^{-1}, body weight 67.7 ± 8.7 kg, body-mass index 22.0 ± 1.9 kg/m^2, sum of six skinfolds [triceps, subscapular, supraspinal, abdominal, medial calf, and front thigh] 65.5 ± 12.1 mm) participated in this study. The protocol was approved by the ethics committee of the Sant Cugat High Performance Center (CAR) in Barcelona, and all participants gave their written consent before participation. Exclusion criteria were the presence of cardiovascular disease or any medical treatment. The study was carried out during the early preseason training period. All the tests and analyses were performed in the Exercise Physiology and Nutrition Laboratories of the CAR.

Dietary Intervention

Three diets were designed: control diet (CD), a balanced composition of macronutrients (5.5 ± 0.3 g L-arginine; Maughan, Burke, & Coyle, 2004); Diet 1, the same as CD but enriched as much as possible without unbalancing the composition of macronutrients with foods high in L-arginine (9.1 ± 1.1 g L-arginine; Table 1); and Diet 2, the same as CD but further including an oral supplement of 5 g of L-arginine (Arginaid, Novartis, ref. 40285) three times per day (20.5 ± 0.3 g L-arginine). Sodium intake was set at a constant level (about 160 mol/day). The energy contents of the macronutrients and L-arginine (Table 1) in each diet were calculated using the software Fuel 3.0 (Human Kinetics, Champaign, IL, USA).

Experimental Intervention

The experiment was conducted over a period of 18 days, during which the participants were administered the three different randomized treatments (CD, Diet 1, and Diet 2). Participants had four meals every day (breakfast, lunch, afternoon snack, and dinner). Each treatment involved 3 days of controlled diet consumption, followed by a day of passive rest when individuals underwent blood sampling and cardiopulmonary exercise testing in the laboratory. A 4-day wash-out period was scheduled before the following stage of the experiment while participants continued with their normal training. This time has been shown to be sufficient for metabolized oral L-arginine intake (Bode-Böger, Boger, Galland, Tsikas, & Frolich, 1998). Training followed a set pattern throughout testing (i.e., morning, 1.5 hr of court-based technical training; midday, 1 hr of physical training in the gym; and afternoon, 2 hr of court-based technical training).

Blood Collection

Blood samples were obtained at baseline on the fourth morning of each treatment before breakfast. From the antecubital vein 2 ml of blood were collected in Vacutainer tubes with heparin (BD Vacutainer, USA) and centrifuged immediately at

Table 1 Main Food Included in Arginine-Enriched Diet (Diet 1)

	Arginine (g/100 g)
Soya flour	2,903
Soy seeds[a]	2,400
Sunflower seeds[a]	1,995
Sesame seeds	2,200
Almonds	1,995
Walnuts	1,580
Brazil nuts	1,834
Beef	1,831
Pork	1,684
Halibut	1,510
Chicken	1,410
Hazelnuts	1,310
Salmon	1,284
Parmesan cheese[a]	1,300
Chickpeas	708
Egg	749
Lentils	659
Spinach	324
White bread	308
Rice	174

Source: De Lorgeril 1998.

[a]Souci, Fachman, & Kraut, 1994.

3,000 *g* for 10 min (Heraeus Labofuge, USA); the plasma was stored at –20 °C until analysis.

Measurement of Nitrates

Plasma nitrate concentrations were determined by colorimetric fixing of nitrite with the Griess reagent as described by Moshage, Kok, Huizenga, and Cansen (1995). Nitrate was reduced to nitrite by incubating 50 µl of plasma in a final volume of 0.5-ml reaction mixture containing 50 mU nitrate reductase, 50 µM NADPH, 5 µM FAD, and 50 mM potassium phosphate buffer pH 7.5 for 20 min at 37 °C. After this, 30 µl of lactate dehydrogenase (0.2 mg/ml in potassium phosphate buffer 0.15 M pH 7.5) and 30 µl of sodium pyruvate 0.2 M were added, and incubation was continued for 5 additional min at 37 °C to oxidize the remaining NADPH. Finally, the samples were deproteinized by adding 30 µl of 300 g/L zinc sulfate, stored at 4 °C for 15 min, and centrifuged at 15,000 *g* for 5 min at 4 °C. Then, 500 µl of the supernatants were mixed in Eppendorf tubes with 500 µl of the Griess reagent (1 g/L sulphanilamide and 0.1 g/L N-[1 naphthyl] ethylenediamine in 25 g/L phosphoric acid), and, after incubation for 15 min at room temperature, absorbance at 540 nm was measured in a Shimadzu UV-160 (Kyoto, Japan) spec-

trophotometer. Results were compared with a standard curve with known concentrations of nitrite.

Measurement of Malondialdehyde

Malondialdehyde (MDA) level, an indicator of free-radical generation that increases at the end of lipid peroxidation, was estimated using the method of Santos, Valles, Aznar, and Vilches (1980). The principle of the method is the spectrophotometric measurement of the color generated by reaction of thiobarbituric acid (TBA) with MDA. For this purpose, 50 µl of plasma samples were mixed with 250 µl of physiological serum, 500 µl of trichloroacetic acid (100% p/v; in HCl 6 N), and 100 µl of TBA 0.12 M (in Tris-HCl 0.26 M, pH 7.0). After incubation for 30 min at 100 °C, 1.1 ml of distilled water was added and centrifugation was performed for 5 min at 3,000 *g* and 4 °C. Absorbance of the supernatants was measured at 532 nm using a spectrophotometer (Shimadzu UV-160, Kyoto, Japan). The concentration of MDA was calculated by the absorbance coefficient of the MDA–TBA complex.

Treadmill Test

All participants were tested in the afternoon. Incremental treadmill tests (Laufergotest LE/6, Jaeger, Germany) were performed at Stage 4 and were maintained for 4 min at an initial speed of 10 km/hr for 3 athletes and 11 km/hr for the others. Each level was followed by a 2-min rest period, after which the speed was increased by 1 km/hr until all participants reached an intensity corresponding to 85% or 90% of VO_{2max} or a respiratory quotient of 1. During the last 15 s of Minutes 5, 11, 17, and 23 of effort, $[La^{-}]_b$ was measured from the earlobe via the micro method (Lactate Pro LT 1710, Germany). Ventilatory frequency, tidal volume, fraction of O_2 exhaled, fraction of CO_2 exhaled, ventilatory volume, rate of ventilatory exchange, and VO_2 were measured in real time using a breath-by-breath cardiorespiratory analyzer (Jaeger Oxycon Champion, Germany), and heart rate (HR) was registered every 5 s (XtrainerPlus, Polar, Finland).

Statistical Analyses

Results were expressed as $M \pm SD$. To compare cardiovascular, metabolic, and biochemical variables between the diets at different task intensities, a two-way repeated-measures analysis (ANOVA) was carried out. Paired Student's t tests were used when only two measurements were involved. The level of significance was set as $p < .05$.

Results

The energy and macronutrient composition of the three diets are shown in Table 2. No significant differences were found in participants' body mass over the duration of the study (CD, 67.7 ± 8.7 kg; Diet 1, 67.8 ± 9.1 kg; and Diet 2, 67.4 ± 9.2 kg). No change was observed in MDA concentrations between treatments (Diet 1, 37.7 ± 3.3 µmol/L, and Diet 2, 42.6 ± 3.7 µmol/L, vs. CD, 41.3 ± 2.6 µmol/L). In

addition, no significant difference was found in plasma levels of nitrate (Diet 1, 31.9 ± 7.2 μmol/L, and Diet 2, 31.3 ± 7.9 μmol/L, vs. CD, 30.4 ± 5.8 μmol/L).

All results for cardiorespiratory (VO_2, HR) and metabolic ($[La^-]_b$) variables are displayed in Table 3. There was no significant difference in VO_2 or HR at any of the time points in the three trials. Blood lactate concentration showed a significant effect at 5 min between CD and Diet 2 (CD, 3.0 ± 0.5 mM, Diet 2, 2.5 ± 0.5 mM; $p = .03$), but this tendency was not found at higher exercise intensities.

Table 2 Daily Energy Uptake in the Diets

	Control diet	Diet 1	Diet 2
Energy intake (kcal/day)	3,024 ± 57.5	3,108 ± 61.5	3,044 ± 57.5
Protein (g/day)	128 ± 5.8	148 ± 11.1	133 ± 5.8
Carbohydrate (g/day)	385 ± 8.2	377 ± 8.2	385 ± 8.2
Fat (g/day)	108 ± 6.5	112 ± 6.5	108 ± 3.1
Caloric distribution ($g \cdot kg^{-1} \cdot day^{-1}$)			
protein	1.9 ± 0.3	1.9 ± 0.3	2.0 ± 0.3
carbohydrate	5.8 ± 0.9	5.8 ± 0.9	5.7 ± 0.8
fat	1.6 ± 0.2	1.6 ± 0.2	1.6 ± 0.2

Note. Diet 1 = enriched food L-arginine diet; Diet 2 = the same as control but further including oral supplement of L-arginine (5 g 3 times per day).

Table 3 Cardiorespiratory and Metabolic Results of the Laboratory Test

	Control diet	Diet 1	Diet 2
Oxygen uptake (L/min)			
5 min	3,047 ± 313	3,001 ± 419	3,096 ± 352
11 min	3,293 ± 340	3,258 ± 426	3,315 ± 373
17 min	3,514 ± 341	3,475 ± 420	3,563 ± 392
23 min	3,689 ± 363	3,671 ± 386	3,764 ± 459
Heart rate (beats/min)			
5 min	158 ± 12	157 ± 12	156 ± 11
11 min	171 ± 12	170 ± 12	169 ± 12
17 min	179 ± 12	178 ± 12	177 ± 12
23 min	185 ± 12	184 ± 11	184 ± 12
Blood lactate accumulation (mM)			
5 min	3.0 ± 0.5	2.9 ± 0.5	2.5 ± 0.5*
11 min	3.2 ± 0.8	3.0 ± 0.7	3.2 ± 1.0
17 min	4.2 ± 0.9	4.1 ± 0.8	4.3 ± 0.7
23 min	6.0 ± 1.4	5.7 ± 1.3	5.7 ± 0.8

*$p < .05$ between control diet and Diet 2.

Discussion

Our results suggest that a diet enriched with L-arginine or supplementation at high doses does not improve cardiorespiratory or metabolic parameters in highly trained athletes during an endurance task. Statistically significant differences were found in blood lactate accumulation between CD and Diet 1 at low exercise intensities. This unexpected effect occurred without any changes in the cardiorespiratory and plasma nitrate values, and it disappeared at higher effort intensities.

The effects of L-arginine supplementation in $[La^-]_b$ are controversial. Some studies have found significant decreases in $[La^-]_b$ and ammonia concentrations using oral combinations of L-arginine and L-aspartate (with the aim of increasing L-arginine absorption) at 3-g/day doses (Burtscher, Brunner, Faulhaber, Hotter, & Likar, 2005). On the other hand, a more recent analysis of arginine supplementation (6 g/day) in athletes showed no effect on $[La^-]_b$ during and after intermittent exercise in highly trained judo athletes or any increase in measured metabolites of NO (nitrates and citrulline; Liu et al., in press), nor did the study by Abel et al. (2005), which used arginine aspartate supplementation, find a positive effect on exercise economy in athletes.

The bioavailability of L-arginine in the human body depends on the administration route used. Oral L-arginine is absorbed in the small intestine and transported to the liver, where the greater part is taken up to be used in the hepatic urea cycle; however, a small part of dietary L-arginine may pass through the liver and be used for substrate NO production, as animal and human studies using ^{15}N-labeled L-arginine as a precursor have shown (Böger et al., 2004). One limiting factor of oral supplements is that the high hepatic activity of the enzyme arginase, which breaks down arginine to urea, is thought to prohibit the release of enterally administered L-arginine to the systemic circulation (Van de Poll, Soeters, Deutz, Fearon, & Dejong, 2004). Approximately 68% of total dietary L-arginine reaches the blood (Bode-Böger et al., 1998). The quantity of L-arginine in our control diet was adjusted to the average values of the intake of this amino acid in the general population (Venho et al., 2002). The L-arginine content of Diet 2 was 9.0 ± 1.1 g/day, similar to that used by Siani et al. (2000). Diet 3 provided 5.5 ± 0.3 g/day in the diet and 15 g/day in supplement form (approximate total = 20.5 g/day) of L-arginine, which was higher than those reported by Colombani et al. (1999; 15 g/day), Abel et al. (2005; 5.7 g/day), and Burtscher et al. (2005; 3 g/day).

Several studies indicated that dietary supplementation of L-arginine may be effective when endothelial L-arginine-NO metabolism presents an impairment that L-arginine can reverse (Coman, Yaplito-Lee, & Boneh, 2008). However, in agreement with the current study, certain investigations in sports sciences have reported poor results with L-arginine supplementation in NO metabolism during exercise in athletes (Liu et al., 2008). Nevertheless, when intravenous infusion of L-arginine was administered (acute dose of 3 g) with the aim of avoiding L-arginine losses during digestion and hepatic catabolism, decreases were found in $[La^-]_b$ and ammonia associated with endothelial-dependent vasodilation, although no positive effects were found on VO_2 or HR values during endurance exercise at submaximal intensities (Schaefer et al., 2002).

In our study the homogeneity of nitrate plasmatic levels could be associated with failure to improve exercise-economy variables (HR, VO_2, and $[La^-]_b$) at

submaximal running speeds. The rapid metabolism and short half-life of NO pose a considerable obstacle to its analysis in humans. Some authors have used nitrate and nitrite plasma levels to assess NO bioavailability in vivo, because a relationship has been observed between them (Wu & Morris, 1998). In the current study the nitrate values in plasma were higher than those reported by other authors in sedentary persons (Brown et al., 2000), suggesting that highly trained athletes have higher baseline concentrations of NO than the general population, but no increases were achieved with a diet enriched with L-arginine or supplementation of amino acid at high doses compared with the CD.

A fundamentally different way of generating NO in NOS-independent pathways was recently used to investigate NO metabolism in athletes. Larsen, Weitzberg, Lundberg, and Ekblom (2007) reported lower oxygen cost during submaximal work and reduced blood nitrite (NO_2^-) levels without an accompanying increase in plasma lactate, indicating that the energy production had become more efficient because of the intake of sodium nitrate supplementation in amounts achievable through a vegetable-rich diet. These findings open up a new line of research into action mechanisms. The main targets need to be clarified, but the process is likely to involve in vivo reduction of nitrate (NO_3^-) into bioactive nitrogen oxides including NO_2^- and NO.

On the other hand, some researchers have observed that L-arginine supplementation has an antioxidant effect in animals (Lin, Yang, Tsai, Huang, & Lee, 2006). This might be related to NO's positive effect on free-radical-scavenging activity in specific conditions (Fukai et al., 2000). However, our results did not show significant differences in MDA plasma values between the experimental diets and the CD, probably because the NO end-product concentrations did not differ between the experimental and control conditions.

Finally, none of the participants suffered any digestive disorder because of the high intake of L-arginine in Diet 3. Large quantities (up to 25 g/day) have been associated with gastrointestinal dysfunction (Barbul, Lazarou, Efron, Wasserkrug, & Efron, 1990). Nor did we observe changes in body mass, indicating that the energy intake over the trial period was enough to maintain energy balance (Rankin, 2002).

In summary, the findings show that a short period of diet enrichment with L-arginine or supplementation at high doses had no effect on either plasma nitrate concentration or on exercise-economy-related parameters in highly trained athletes. Significant differences were found in $[La^-]_b$ at the 5-min time point between CD and Diet 2, but not at high exercise intensities. Further investigations with intravenous L-arginine infusion or intake of inorganic nitrate-rich foods are required to investigate the effects on NO and exercise economy.

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References

Abel, T., Knechtle, B., Perret, C., Eser, P., von Arx, P., & Knecht, H. (2005). Influence of chronic supplementation of arginine aspartate in endurance athletes on performance and substrate metabolism—A randomized, double-blind, placebo-controlled study. *International Journal of Sports Medicine, 26,* 344–349.

Barbul, A., Lazarou, S.A., Efron, D.T., Wasserkrug, H.L., & Efron, G. (1990). Arginine enhances wound healing and lymphocyte immune response in humans. *Surgery, 108,* 331–336.

Bode-Böger, S.M., Boger, R.H., Galland, A., Tsikas, D., & Frolich, J.C. (1998). L-arginine-induced vasodilation in healthy humans: Pharmacokinetics-pharmacodynamic relationship. *British Journal of Clinical Pharmacology, 46,* 489–497.

Böger, R.H., Tsikas, D., Bode-Böger, S.M., Phivthong-Ngam, L., Schwedhelm, E., & Frölich, J.C. (2004). Hypercholesterolemia impairs basal nitric oxide synthase turnover rate: A study investigating the conversion of L-[guanidine-15N2]-arginine to 15N-labeled nitrate by gas chromatography-mass spectrometry. *Nitric Oxide, 11,* 1–8.

Bohuslavs'kyi, A., Dmytriieva, A.V., & Sahach, V.F. (2005). Effect of nitric oxide on the efficiency of oxygen consumption by the working skeletal muscle in fatigue. *Fiziologicheskii Zhurnal, 51,* 33–42.

Brown, G.C. (1995). Hypothesis: Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. *FEBBS Letters, 369,* 136–139.

Brown, G.C., & Cooper, C.E. (1994). Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBBS Letters, 356,* 295–298.

Brown, M.D., Srinivasan, M., Hogikyan, R.V., Dengel, D.R., Glickman, S.G., Galecki, A., et al. (2000). Nitric oxide biomarkers increase during exercise-induced vasodilation in the forearm. *International Journal of Sports Medicine, 21,* 83–89.

Brune, B., Gotz, C., Messmer, U.K., Sandau, K., Hirvonen, M.R., & Lapetina, E.G. (1997). Superoxide formation and macrophage resistance to nitric oxide-mediated apoptosis. *The Journal of Biological Chemistry, 272,* 7253–7258.

Burtscher, M., Brunner, F., Faulhaber, M., Hotter, B., & Likar, R. (2005). The prolonged intake of L-arginine-L-aspartate reduces blood lactate accumulation and oxygen consumption during submaximal exercise. *Journal of Sports Sciences, 4,* 314–322.

Colombani, P.C., Bitzi, R., Frey-Rindova, P., Frey, W., Arnold, M., & Langhans, W. (1999). Chronic arginine aspartate supplementation in runners reduces total plasma amino acid level at rest and during a marathon run. *European Journal of Nutrition, 38,* 263–270.

Coman, D., Yaplito-Lee, J., & Boneh, A. (in press).New indications and controversies in arginine therapy. *Clinical Nutrition (Edinburgh, Lothian),* .

De Backer, D., Berre, J., Moraine, J.J., Melot, C., Vanfraechem, J., & Vincent, J.L. (1996). Effects of dobutamine on the relationship between oxygen consumption and delivery in healthy volunteers: Comparison with sodium nitroprusside. *Clinical Science, 90,* 105–111.

De Lorgeril, M. (1998). Dietary arginine and the prevention of cardiovascular diseases. *Cardiovascular Research, 37,* 560–563.

Denis, C., Dormois, D., Linossier, M.T., Eychenne, J.L., Hauseux, P., & Lacour, J.R. (1991). Effect of arginine aspartate on the exercise-induced hyperammoniemia in humans: A two periods cross-over trial. *Archives Internationales de Physiologie et de Biochimie, 99,* 123–127.

Fukai, T., Siegfried, M.R., Ushio-Fukai, M., Cheng, Y., Kojda, G., & Harrison, D.G. (2000). Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. *The Journal of Clinical Investigation, 105,* 1631–1639.

Jones, A.M., Wilkerson, D.P., & Campbell, I.T. (2004). Nitric oxide synthase inhibition with L-NAME reduces maximal uptake but not gas exchange threshold during incremental cycle exercise in man. *The Journal of Physiology, 560,* 329–338.

Larsen, F.J., Weitzberg, E., Lundberg, J.O., & Ekblom, B. (2007). Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiologica, 191,* 59–66.

Lin, W.T., Yang, S.C., Tsai, S.C., Huang, C.C., & Lee, N.Y. (2006). L-arginine attenuates xanthine oxidase and myeloperoxidase activities in hearts of rats during exhaustive exercise. *The British Journal of Nutrition, 95,* 67–75.

Liu, T.H., Wu, C.L., Chiang, C.W., Lo, Y.W., Tseng, H.F., & Chang, C.K. (in press).No effect of short-term arginine supplementation on nitric oxide production, metabolism and performance in intermittent exercise in athletes. *The Journal of Nutritional Biochemistry, .*

Loke, K.E., Laycock, S.K., Mital, S., Wolin, M.S., Bernstein, S., Oz, M., et al. (1999). Nitric oxide modulates mitochondrial respiration in failing human heart. *Circulation, 100,* 1291–1297.

Maughan, R.J., Burke, L.M., & Coyle, E.F. (2004). *Food, nutrition and sports performance II*. New York: Routledge.

McConell, G.K., & Kingwell, B.A. (2006). Does nitric oxide regulate skeletal muscle glucose uptake during exercise? *Exercise and Sport Sciences Reviews, 34,* 36–41.

Moncada, S., & Erusalimsky, J.D. (2002). Does nitric oxide modulate mitochondrial energy respiration and apoptosis? *Nature Reviews. Molecular Cell Biology, 3,* 214–220.

Moshage, H., Kok, B., Huizenga, J.R., & Cansen, P.L.M. (1995). Nitrite and nitrate determinations in plasma: A critical evaluation. *Clinical Chemistry, 41,* 892–896.

Nuñez, C., Victor, V.M., Tur, R., Alvarez-Barrientos, A., Moncada, S., Esplugues, J.V., et al. (2005). Discrepancies between nitroglycerin and NO-releasing drugs on mitochondrial oxygen consumption, vasoactivity, and the release of NO. *Circulation Research, 97,* 1063–1069.

Rankin, J.W. (2002). Weight loss and gain in athletes. *Current Sports Medicine Reports, 1,* 208–213.

Santos, M.T., Valles, J., Aznar, J., & Vilches, J. (1980). Determination of plasma malondialdehyde-like material and its clinical application in stroke patients. *Journal of Clinical Pathology, 33,* 973–976.

Schaefer, A., Piquard, F., Geny, B., Doutreleau, S., Lampert, E., Mettauer, B., et al. (2002). L-arginine reduces exercise induced increase in plasma lactate and ammonia. *International Journal of Sports Medicine, 23,* 403–407.

Schrage, W.G., Joyner, M.J., & Dinenno, F.A. (2004). Local inhibition of nitric oxide and prostaglandins independently reduces forearm exercise hyperaemia in humans. *The Journal of Physiology, 557,* 599–611.

Shen, W., Zhang, X., Zhao, G., Wolin, M.S., Sessa, W., & Hintze, T.H. (1995). Nitric oxide production and NO synthase gene expression contribute to vascular regulation during exercise. *Medicine and Science in Sports and Exercise, 27,* 1125–1134.

Siani, A., Pagano, E., Iacone, R., Iacoviello, L., Scopacasa, F., & Strazzullo, P. (2000). Blood pressure and metabolic changes during dietary L-arginine supplementation in humans. *American Journal of Hypertension, 13,* 547–551.

Souci, S.W., Fachman, W., & Kraut, W. (1994). *Food composition and nutrition table*. Stuttgart, Germany: Medpharm Scientific Publications, CRC Press.

Vallance, P., Leone, A., Calver, A., Collier, J., & Moncada, S. (1992). Accumulation of an endogenous inhibitor of NO synthesis in chronic renal failure. *Lancet, 339,* 572–575.

van de Poll, M.C., Soeters, P.B., Deutz, N.E., Fearon, K.C., & Dejong, C.H. (2004). Renal metabolism of amino acids: Its role in interorgan amino acid exchange. *The American Journal of Clinical Nutrition, 79,* 185–197.

Venho, B., Voutilainen, S., Valkonen, V.P., Virtanen, J., Lakka, T.A., Rissanen, T.H., et al. (2002). Arginine intake, blood pressure, and the incidence of acute coronary events in men: the Kuopio Ischaemic Heart Disease Risk Factor Study. *The American Journal of Clinical Nutrition, 76,* 359–364.

Wu, G., & Morris, S.M. (1998). Arginine metabolism: Nitric oxide and beyond. *The Biochemical Journal, 336,* 1–17.

MANUSCRIPT II

Acute Administration of Inorganic Nitrate Reduces $\dot{V}O_{2peak}$ in Endurance Athletes

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ABSTRACT

BESCÓS, R., F. A. RODRÍGUEZ, X. IGLESIAS, M. D. FERRER, E. IBORRA, and A. PONS. Acute Administration of Inorganic Nitrate Reduces $\dot{V}O_{2peak}$ in Endurance Athletes. *Med. Sci. Sports Exerc.*, Vol. 43, No. 10, pp. 1979–1986, 2011. **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 yr, $\dot{V}O_{2peak}$ = 65.1 ± 6.2 mL·kg^{-1}·min^{-1}) participated in this randomized, double-blind, crossover study. Subjects received dietary supplementation with nitrate ($NaNO_3$ 10 mg·kg^{-1} of body mass) or a placebo (NaCl) 3 h before exercise. They then performed a cycle ergometer test that consisted of four 6-min submaximal workloads, corresponding to 2.0, 2.5, 3.0, and 3.5 W·kg^{-1} of body mass, interspersed with 3 min of passive recovery. After a 5-min recovery period, subjects performed one incremental exercise test until exhaustion. **Results**: Plasma nitrate and nitrite were significantly higher ($P < 0.05$) 3 h after supplementation (nitrate = 250 ± 80 μM, nitrite = 2313 ± 157 nM) than after the placebo (nitrate = 29 ± 8 μM, nitrite = 1998 ± 206 nM) at resting conditions. Nitrate supplementation significantly reduced $\dot{V}O_{2peak}$ (nitrate = 4.64 ± 0.35 L·min^{-1}, placebo = 4.82 ± 0.33 L·min^{-1}, $P = 0.010$) and the ratio between $\dot{V}O_2$ and power at maximal intensity (nitrate = 11.2 ± 1.1 mL·min^{-1}·W^{-1}, placebo = 11.8 ± 1.1 mL·min^{-1}·W^{-1}, $P = 0.031$). This reduction of $\dot{V}O_2$ occurred without changes in the time to exhaustion (nitrate = 416 ± 32 s, placebo = 409 ± 27 s) or in the maximal power (nitrate = 416 ± 29 W, placebo = 410 ± 28 W). **Conclusions**: A single oral dose of inorganic nitrate acutely reduces $\dot{V}O_{2peak}$ without compromising the maximal exercise performance. **Key Words:** NITRIC OXIDE, NITRATE, NITRITE, EXERCISE PERFORMANCE, EXERCISE ECONOMY, OXYGEN UPTAKE

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 during 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%. Most 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 nitrate to 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 classic 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 the 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 because diet constitutes the main source of nitrate in humans, with vegetables accounting for 60%–80% of our daily intake.

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 ($\dot{V}O_2$) at low and moderate intensities of exercise in healthy subjects (1,2,15). In addition,

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two recent studies showed that $\dot{V}O_2$ 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 degradation, which diminishes the ATP cost of muscle force production.

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 classic precursor of NO (L-arginine) (4,19). However, in elderly populations with endothelial dysfunction, L-arginine supplements effectively enhance exercise capacity (9). In addition, a recent study by Koppo et al. (13) reported that L-arginine supplementation speeds $\dot{V}O_2$ 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 d) 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 because it lowers blood pressure 3 h after ingestion in healthy subjects (36). One very recent study assessed the effect of acute ingestion of nitrate on physically active people, but these subjects were not highly trained (34).

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 nitrate and nitrite 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. Eleven male cyclists and triathletes (age = 34.3 ± 4.8 yr, body weight = 73.3 ± 5.6 kg, body mass index = 23.7 ± 1.5 $kg \cdot m^{-2}$, $\dot{V}O_{2peak}$ = 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 used 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. Subjects were randomly assigned in a double-blind crossover design to receive a single dose of either sodium nitrate (10 $mg \cdot kg^{-1} \cdot kg^{-1}$ of body mass; code 18211 [Acofarma, Madrid, 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 because this period 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 3 d before the tests. During this time, athletes received nutritional guidelines and were encouraged to follow a high-CHO diet to optimize glycogen deposition. In addition, they were told to avoid alcohol, caffeine products, and dietary supplements 48 h before the exercise test. A 7-d washout separated the supplementation periods.

Ergometry test. 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 nitrate and 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 the study. All tests were performed at the same time of day (±1 h) on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands) under controlled conditions (22°C ± 1°C, 40%–60% relative humidity, P_b = 760–770 mm Hg). 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 and 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 3 min of passive recovery. Five minutes after completion of the submaximal workloads, subjects performed

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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 the formula:

$$W_{max} = W_E + (W_I / t)\, t_E \quad [1]$$

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. During all the tests, oxygen uptake ($\dot{V}O_2$), minute ventilation ($\dot{V}_E$), carbon dioxide production ($\dot{V}CO_2$), and the RER were measured breath-by-breath by a computerized gas analyzer (Cosmed Quark PFT-Ergo, Rome, 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, Plumsteadville, PA) and a 3-L calibration syringe (Hans Rudolph, Shawnee, KS), respectively, following the manufacturer's instructions.

Data analysis procedures. Breath-by-breath $\dot{V}O_2$ data from submaximal bouts of exercise were initially examined to exclude errant breaths caused by coughing, swallowing, sighing, and others. Values greater than 4 SD from the local mean were removed. The first 20 s of data after the onset of exercise (i.e., the phase 1, cardiodynamic component) was deleted, and a nonlinear least squares algorithm was used to fit the data thereafter (SigmaPlot 8.0; SPSS, Inc., Chicago, IL). A single-exponential model was used to analyze the oxygen uptake kinetics of four submaximal rates of exercise, as described in the following equation:

$$\dot{V}O_2(t) = \dot{V}O_{2baseline} + A_P\left[1 - e^{-(t - TD_P/\tau_P)}\right] \quad [2]$$

where $\dot{V}O_2(t)$ represents the absolute $\dot{V}O_2$ at a given time; $\dot{V}O_{2baseline}$ represents the mean $\dot{V}O_2$ in the baseline period; A_P, TD_P and τ_P represent the amplitude, time delay, and time constant, respectively, describing the phase 2 (i.e., primary component) increase in $\dot{V}O_2$ above baseline. $\dot{V}O_{2baseline}$ and end-exercise $\dot{V}O_2$ were defined as the mean $\dot{V}O_2$ measured during the final 30 s before starting each submaximal workload and during 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:

$$GE(\%) = \text{work rate (W)} / \text{energy expended } (J \cdot s^{-1}) \times 100 \quad [3]$$

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

$$\text{energy expenditure } (J \cdot s^{-1}) = [(3.869\,\dot{V}O_2) + (1.195\,\dot{V}CO_2)] \times (4.186/60) \times 1000 \quad [4]$$

The $\dot{V}O_{2peak}$ during the incremental test was determined as the mean $\dot{V}O_2$ measured over the final 60 s of exercise. To determine the ventilatory threshold (VT) and the respiratory compensation point (RCP), data were averaged at 30-s intervals and analyzed by two independent reviewers, according to methods described by Wasserman et al. (35). HR was continuously recorded during the test with a portable HR monitor and HR_{max} was defined as the HR at the point of exhaustion (RS800 SD; Polar, Kempele, Finland).

Blood sampling. A small catheter was inserted into an antecubital vein for venous blood sampling. Four blood samples were collected to analyze nitrate and nitrite: 1) during resting conditions, 2) 3 h after supplement or placebo ingestion, 3) in the first minute after the fourth submaximal load, and 4) in the first minute after the maximal test. Venous blood was drawn with a 5-mL syringe EDTA and was immediately centrifuged at 1000*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, Billerica, MA) 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, Boulder, CO).

Nitrate levels were determined following an adaptation of the method described by Braman and Hendrix (6). Briefly, the purge vessel was loaded with a saturated VCl_3 solution in 1 M HCl and heated to 90°C with a current of hot water. To prevent damage to the NOA from the hydrochloric acid vapor, a gas bubbler filled with 1 M 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 was 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).

Nitrite levels were determined following an adaptation of the method described by Castegnaro et al. (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).

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, Berlin, Germany): 1) during resting conditions, 2) in the first minute after each submaximal load, and 3) at 3 and 5 min after the maximal test.

Statistics. Results are expressed as means ± SEM. A paired *t*-test was used to evaluate the differences between the placebo and the nitrate groups, where appropriate. To investigate the influence of time and treatment, the data were treated with two-way ANOVA with repeated measures on both time and treatment. The data were assessed to determine the normal distribution, and *post hoc* analyses

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were performed via Tukey HSD. The significance level was set at $P < 0.05$, whereas a trend was noted when $P < 0.10$.

RESULTS

Plasma nitrate and nitrite kinetics. The concentrations of nitrate were similar (nitrate = 30 ± 12 μM, placebo = 28 ± 10 μM) before intake. Three hours after ingestion, the plasma levels of nitrate had increased significantly in the nitrate group (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 (Fig. 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 after supplementation (250 ± 80 μM) (Fig. 1).

There were no differences between treatments in the levels of nitrite under fasting conditions (nitrate = 2005 ± 158, placebo = 2053 ± 278 nM). Conversion of nitrate to nitrite was evident from the increased plasma nitrite levels 3 h after nitrate supplementation (2313 ± 157 nM, $P = 0.017$) compared with the placebo (1998 ± 206 nM). During nitrate treatment, nitrite levels were significantly lower after maximal exercise (2126 ± 251 nM, $P = 0.044$) than the peak value reached 3 h after supplementation (2313 ± 157 nM) (Fig. 1). Nitrite also tended to be lower after the placebo treatment and maximal exercise (1916 ± 168 nM) than under fasting conditions (2053 ± 278 nM, $P = 0.056$) (Fig. 1).

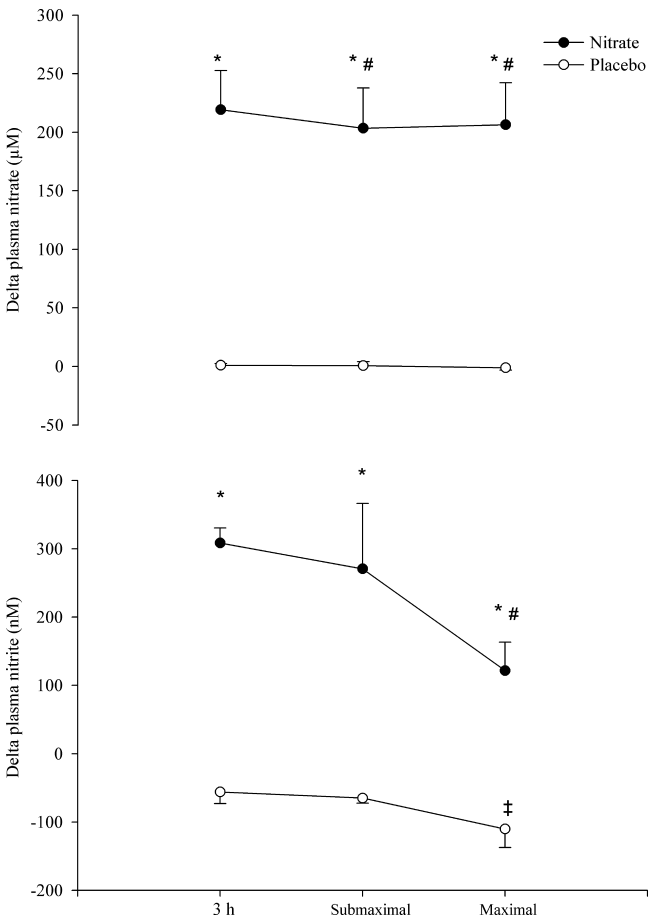

FIGURE 1—Plasma nitrate and nitrite change (Δ) relative to pre-supplementation baseline in plasma ($n = 11$). *Statistical significance between nitrate and placebo ($P < 0.05$). #Statistical significance in nitrate levels between 3 h before supplementation and after submaximal and maximal workloads of exercise ($P < 0.05$). ‡Statistical tendency in placebo condition between nitrite levels at 3 h and after maximal test of exercise ($P < 0.10$).

Submaximal work parameters. 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 $\dot{V}O_2$, $\dot{V}CO_2$, $\dot{V}_E$, RER, HR, and GE. In addition, we did not find changes in the time constant and primary amplitude of $\dot{V}O_2$ at any submaximal load (Table 1). The mean work rate was 147 ± 11 W at 2 W·kg^{-1}, 183 ± 14 W at 2.5 W·kg^{-1}, 220 ± 17 W at 3 W·kg^{-1}, and 257 ± 20 W at 3.5 W·kg^{-1}. The chosen cadence was 87 ± 8 rpm on the two occasions (nitrate and placebo).

Maximal work parameters. After nitrate supplementation, $\dot{V}O_{2peak}$ dropped from 4.82 ± 0.33 to 4.64 ± 0.35 L·min^{-1} ($P = 0.010$) (Table 2). In addition, $\dot{V}O_2$ tended to be lower at the respiratory compensation point after nitrate supplementation (4.31 ± 0.28 L·min^{-1}) than after the placebo (4.44 ± 0.23 L·min^{-1}, $P = 0.068$) (Table 2). The ratio between oxygen consumption and power was significantly decreased at the $\dot{V}O_{2peak}$ level after nitrate ingestion ($P = 0.031$) (Fig. 2). Other cardiorespiratory parameters such as HR, 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 $\dot{V}O_{2peak}$ was 416 ± 29 W for nitrate and 410 ± 28 W for the placebo ($P = 0.318$).

Blood lactate concentration. No differences were found in blood lactate accumulation between conditions at any point of submaximal or maximal exercise intensity (Fig. 3).

DISCUSSION

In agreement with our first hypothesis, this research showed that cardiorespiratory adaptation at low to moderate intensities of exercise was not modified by a single administration of nitrate (10 mg·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 $\dot{V}O_{2peak}$ 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.

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TABLE 1. Cardiorespiratory dynamics during low- to 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**
$\dot{V}O_2$								
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
$\dot{V}CO_2$								
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
$\dot{V}_E$								
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.

RER, respiratory exchange ratio; HR, heart rate; $\dot{V}CO_2$, expired carbon dioxide; $\dot{V}_E$, minute ventilation; $\dot{V}O_2$, oxygen uptake.

Effects of an acute dose of nitrate on blood levels of nitrate and nitrite. The levels of plasma nitrate had increased by 86.9% ± 8.4% ($P < 0.05$) 3 h after supplementation compared with the placebo, which is consistent with previous studies (1,2,15,18,36). In addition, 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 after supplementation (250 ± 80 μM). This fact is difficult to attribute to the effect of exercise alone because the level of nitrate was no different after the incremental than after submaximal exercise. Previous research showed that nitrate remained stable after exercise (15). One likely explanation for this finding is related to the pharmacokinetics of nitrate after dietary ingestion. There is evidence that the plasma levels of nitrate increased rapidly within 30 min 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 because athletes completed submaximal and maximal workloads at 3 h 45 min (±10 min) and 4 h 5 min (±14 min), respectively.

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

Treatment	Placebo	Nitrate
$\dot{V}O_{2peak}$ (L·min^{-1})	4.82 ± 0.33	4.64 ± 0.35*
$\dot{V}O_2$ at RCP (L·min^{-1})	4.44 ± 0.23	4.31 ± 0.28**
$\dot{V}O_2$ at VT (L·min^{-1})	3.52 ± 0.32	3.45 ± 0.23
$\dot{V}CO_{2peak}$ (L·min^{-1})	5.25 ± 0.45	5.18 ± 0.50
$\dot{V}_{Emax}$ (L·min^{-1})	156.4 ± 16.5	151.0 ± 22.6
RER	1.09 ± 0.08	1.12 ± 0.07
HR_{max} (beats·min^{-1})	182 ± 14	182 ± 14

Values are means ± SD.

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

** Statistical tendency between nitrate and placebo ($P < 0.10$).

$\dot{V}CO_{2peak}$, peak of expired carbon dioxide; $\dot{V}_{Emax}$, maximum minute ventilation; $\dot{V}O_2$ at RCP, oxygen consumption at respiratory compensation point; $\dot{V}O_2$ at VT, oxygen consumption at ventilatory threshold; $\dot{V}O_{2peak}$, peak of oxygen uptake.

Additional studies are needed to pinpoint the exact mechanisms behind this finding.

Nitrite takes longer to appear in the circulation than nitrate, peaking between 2.5 and 3 h (36). This delay is due to the enterosalivary circulation of these compounds. Most of the 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). Because 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).

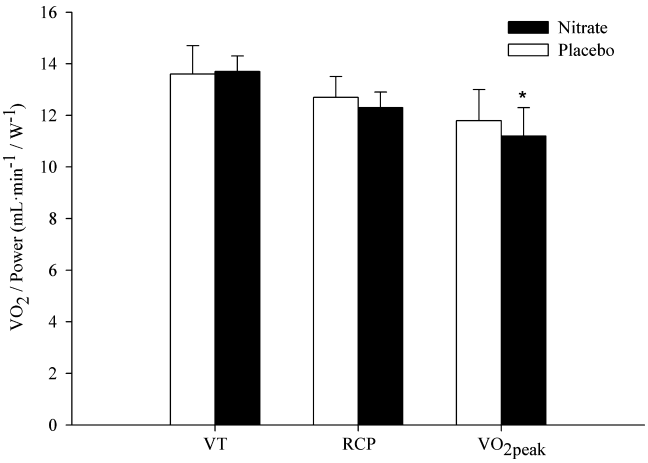

FIGURE 2—Rate between oxygen consumption and power at ventilatory threshold (VT), at respiratory compensation point (RCP), and at peak of oxygen consumption ($\dot{V}O_{2peak}$) (n = 11). *Statistical significance between nitrate and placebo ($P < 0.05$).

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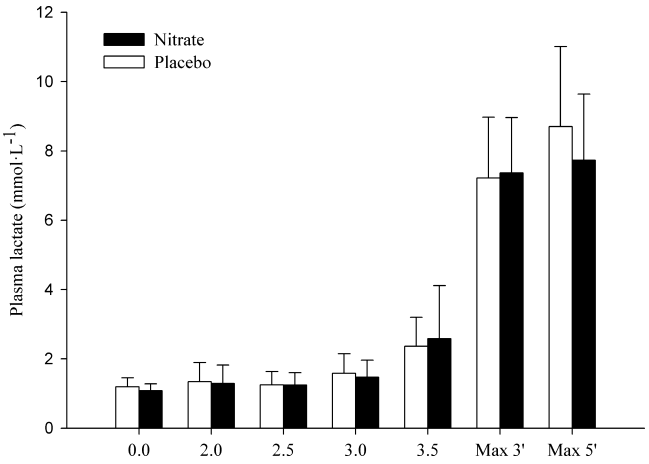

FIGURE 3—Plasma lactate concentration at rest conditions, after every submaximal workloads equivalents to 2.0, 2.5, 3.0, and 3.5 W·kg^{-1}, and at 3 and 5 min after maximal exercise in both conditions (nitrate and placebo).

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 because 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 nitrite in highly trained athletes.

Effects of an acute dose of nitrate on the physiological response to low to moderate exercise. We found no statistical differences in cardiorespiratory adaptation to exercise at low to moderate intensities between the nitrate and placebo groups (Table 1). In contrast, previous studies showed significant improvements in exercise efficiency at low to moderate intensities after dietary nitrate supplementation (1,2,15). The present study design differed from these studies in two main aspects. The first is the duration of the treatment. Although previous studies followed several days (between 3 and 6 d) of nitrate supplementation, we assessed the effect of only one dose before exercise. Interestingly, we showed that an acute dose of sodium nitrate equivalent to 10 mg·kg^{-1} produced a similar increase in plasma nitrate (218 ± 68 μM) as a 3-d supplement of sodium nitrate (212 ± 28 μM), in which 8.5 mg·kg^{-1}·d^{-1} were ingested (18). Thus, the difference in $\dot{V}O_2$ 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 $\dot{V}O_{2peak}$ between 45 and 58 mL·kg^{-1}·min^{-1}(1,2,14,15, 18,34). In our study, all subjects were endurance athletes with high $\dot{V}O_{2peak}$ (65.1 ± 6.2 mL·kg^{-1}·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 seems 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 because 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 exercise might 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. Nitrate supplementation showed a trend toward 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 ($\dot{V}O_{2peak}$). In addition, we found that the mean ratio between $\dot{V}O_{2peak}$ 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 d with inorganic nitrate and nitrate-rich beetroot juice, respectively (14,18). However, these surprising reductions in $\dot{V}O_{2peak}$ and in the ratio of $\dot{V}O_{2peak}$ 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, $\dot{V}_E$, $\dot{V}CO_2$ and RER), as well as lactate concentrations, which suggests that the reduction in $\dot{V}O_{2peak}$ could not be originated from alterations in the energetic cost of cardiorespiratory support processes.

However, the mechanistic bases for the reduced $\dot{V}O_{2peak}$ after 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 before exercise, it was found that plasma nitrite levels decreased significantly just after finishing maximal workload, suggesting activation of nitrate–nitrite–NO pathway (Fig. 1). There is evidence that NO donors, which evoke a 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

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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 improvement in 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 $\dot{V}O_{2peak}$ 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 $\dot{V}O_{2peak}$ reduction in well-trained athletes after dietary nitrate consumption.

In conclusion, acute dietary nitrate administration 3 h before an exercise test increases plasma levels of nitrate and nitrite. In contrast with previous studies carried out in moderately 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 $\dot{V}O_{2peak}$ 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 role in oxygen consumption at maximal intensity of exercise in well-trained athletes.

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The results of the present study do not constitute endorsement by the American College of Sports Medicine.

REFERENCES

1. Bailey SJ, Fulford J, Vanhatalo A, et al. Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *J Appl Physiol*. 2010;109:135–48.
2. Bailey SJ, Winyard P, Vanhatalo A, et al. Dietary nitrate supplementation reduces the O_2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *J Appl Physiol*. 2009;107:1144–55.
3. Bergstrom M, Hultman E. Energy cost and fatigue during intermittent electrical stimulation of human skeletal muscle. *J Appl Physiol*. 1988;65:1500–5.
4. Bescós R, Gonzalez-Haro C, Pujol P, et al. Effects of dietary L-arginine intake on cardiorespiratory and metabolic adaptation in athletes. *Int J Sport Nutr Exerc Metab*. 2009;19:355–65.
5. Bohuslavs'kyi A, Dmytriieva AV, Sahach VF. [Effect of nitric oxide on the efficiency of oxygen consumption by the working skeletal muscle in fatigue]. *Fiziol Zh*. 2005;51:33–42.
6. Braman RS, Hendrix SA. Nanogram nitrite and nitrate determination in environmental and biological materials by vanadium (III) reduction with chemiluminescence detection. *Anal Chem*. 1989;61:2715–8.
7. Brouwer E. On simple formulae for calculating the heat expenditure and the quantities of carbohydrate and fat oxidized in metabolism of men and animals, from gaseous exchange (oxygen intake and carbonic acid output) and urine-N. *Acta Physiol Pharmacol Neerl*. 1957;6:795–802.
8. Castegnaro M, Massey RC, Walters CL. The collaborative evaluation of a procedure for the determination of N-nitroso compounds as a group. *Food Addit Contam*. 1987;4:37–43.
9. Chen S, Kim W, Henning SM, Carpenter CL, Li Z. Arginine and antioxidant supplement on performance in elderly male cyclists: a randomized controlled trial. *J Int Soc Sports Nutr*. 2010;23:7–13.
10. Duncan C, Dougall H, Johnston P, et al. Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nat Med*. 1995;1:546–51.
11. Erzurum SC, Ghosh S, Janocha AJ, et al. Higher blood flow and circulating NO products offset high-altitude hypoxia among Tibetans. *Proc Natl Acad Sci U S A*. 2007;104:17593–8.
12. Hopker J, Passfield L, Coleman D, et al. The effects of training on gross efficiency in cycling: a review. *Int J Sports Med*. 2009;30:845–50.
13. Koppo K, Taes YE, Pottier A, Boone J, Bouckaert J, Derave W. Dietary arginine supplementation speeds pulmonary $\dot{V}O_2$ kinetics during cycle exercise. *Med Sci Sports Exerc*. 2009;41(8):1626–32.
14. Lansley KE, Winyard PG, Fulford J, et al. Dietary nitrate supplementation reduces the O_2 cost of walking and running: a placebo-controlled study. *J Appl Physiol*. 2011;110:591–600.
15. Larsen F, Weitzberg E, Lundberg JO, Ekblom B. Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol*. 2007;191:55–66.
16. Larsen FJ, Ekblom B, Sahlin K, Lundberg JO, Weitzberg E. Effects of dietary nitrate on blood pressure in healthy volunteers. *N Engl J Med*. 2006;355:2792–3.
17. Larsen FJ, Schiffer TA, Borniquel S, et al. Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell Metab*. 2011;12(2):149–59.
18. Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B. Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise. *Free Radic Biol Med*. 2010;48:342–7.
19. Liu TH, Wu CL, Chiang CW, Lo YW, Tseng HF, Chang CK. No effect of short-term arginine supplementation on nitric oxide production, metabolism and performance in intermittent exercise in athletes. *J Nutr Biochem*. 2008;20:462–8.

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20. Lundberg JO, Giovoni M. Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic Biol Med*. 2004;37: 395–400.
21. Lundberg JO, Weitzberg E, Gladwin M. The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov*. 2008;7:156–67.
22. Lundberg JO, Weitzberg E, Lundberg JM, Alving K. Intragastric nitric oxide production in humans: measurements in expelled air. *Gut*. 1994;35:1543–6.
23. Maiorana A, O'Driscoll G, Taylor R, Green D. Exercise and the nitric oxide vasodilator system. *Sports Med*. 2003;33:1013–35.
24. McKnight GM, Smith LM, Drummond RS, Duncan CW, Golden M, Benjamin N. Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. *Gut*. 1997;40:211–4.
25. Rassaf T, Lauer T, Heiss C, et al. Nitric oxide synthase–derived plasma nitrite predicts exercise capacity. *Br J Sports Med*. 2007;41: 669–73.
26. Reid MB. Role of nitric oxide in skeletal muscle: synthesis, distribution and functional importance. *Acta Physiol Scand*. 1998;162: 401–9.
27. Shen W, Xu X, Ochoa M, Zhao G, Wolin MS, Hintze TH. Role of nitric oxide in the regulation of oxygen consumption in conscious dogs. *Circ Res*. 1994;75:1086–95.
28. Shiva S, Sack MN, Greer JJ, et al. Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. *J Exp Med*. 2007;204:2089–102.
29. Sureda A, Cordova A, Ferrer MD, Perez G, Tur JA, Pons A. L-Citrulline–malate influence over branched chain amino acid utilization during exercise. *Eur J Appl Physiol*. 2010;110:341–51.
30. Sureda A, Cordova A, Ferrer MD, et al. Effects of L-citrulline oral supplementation on polymorphonuclear neutrophils oxidative burst and nitric oxide production after exercise. *Free Radic Res*. 2009;43:828–35.
31. Sureda A, Tauler P, Aguiló A, et al. Blood cell NO synthesis in response to exercise. *Nitric Oxide*. 2006;15:5–12.
32. Tannenbaum SR, Weisman M, Fett D. The effect of nitrate intake on nitrite formation in human saliva. *Food Cosmet Toxicol*. 1976;14:549–52.
33. Tonkonogi M, Sahlin K. Physical exercise and mitochondrial function in human skeletal muscle. *Exerc Sport Sci Rev*. 2002;30(3): 129–37.
34. Vanhatalo A, Bailey SJ, Blackwell JR, et al. Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. *Am J Physiol Regul Integr Comp Physiol*. 2010;299: 1121–31.
35. Wasserman K, Hansen JE, Sue DY, Stringer WW, Whipp BJ. *Principles of Exercise Testing and Interpretation*. 4th ed. Philadelphia (PA): Lippincott Williams & Wilkins; 2004. p. 32–48.
36. Webb AJ, Loukogeorgakis S, Okorie M, et al. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension*. 2008;51:784–90.

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MANUSCRIPT III

Dietary nitrate supplementation does not enhance performance of endurance athletes during a 40-minute cycle time trial

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Abstract

Purpose: Inorganic nitrate supplementation has been suggested to be an ergogenic aid for athletes. However, few data are available on the effect of inorganic nitrate ingestion on competitive athletes. Here we tested the hypothesis that a 3-day supplementation with inorganic nitrate enhances the performance of endurance athletes. **Methods:** Thirteen endurance athletes (age 32.6 ± 5.6 yrs; body mass index 23.4 ± 2.0 $kg \cdot m^{-2}$) 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) for 3 days. They then performed a cycle ergometer 40-minute time-trial test. Cardio-respiratory parameters, as well as distance, power output and cadence were measured during the test. **Results:** Plasma nitrate increased significantly ($P < 0.05$) after supplementation (256 ± 53 μM) than after placebo (44 ± 9 μM; $P < 0.001$). In contrast, plasma nitrite did not increase (nitrate: 4.5 ± 0.5 μM; placebo: 4.2 ± 0.4 μM; $P = 0.124$). The mean results of distance (nitrate: 26.328 ± 1.091 km; placebo: 26.320 ± 1.212 km; $P = 0.61$) and power output (nitrate: 258 ± 28 W; placebo: 257 ± 28 W; $P = 0.89$) did not differ between treatments. Neither were differences ($P > 0.05$) found in the oxygen consumption of athletes (nitrate: 3.63 ± 0.33 $L \cdot min^{-1}$; placebo: 3.63 ± 0.33 $L \cdot min^{-1}$). Plasma endothelin-1 increased just after exercise in placebo (2.3 ± 1.5 $pmol \cdot L^{-1}$) and nitrate (4.1 ± 2.9 $pmol \cdot L^{-1}$; $P < 0.05$) groups compared with resting values (placebo: 0.9 ± 0.9 $pmol \cdot L^{-1}$; nitrate: 1.2 ± 0.9 $pmol \cdot L^{-1}$). In addition, this effect was ($P = 0.010$) greater in the group receiving nitrate than in the placebo group. **Conclusion:** A 3-day supplementation with inorganic nitrate does not improve the performance of endurance-trained subjects. In addition, the significant increase in plasma endothelin-1 may implicate ingested inorganic nitrate in the regulation of blood flow during exercise, independently of NO synthesis.

Keywords: nitric oxide, nitrate, nitrite, exercise performance, endothelin-1, oxygen uptake.

Introduction

Inorganic nitrate has been indicated to be an important donor of nitric oxide (NO) and other bioactive nitrogen oxides that complement the classical L-Arginine NO synthase pathway. The bioactivation of dietary nitrate requires its initial reduction to nitrite. This conversion is performed mainly by commensal bacteria in the gastrointestinal tract (1). Once nitrite is formed, a large amount of this anion enters the systematic circulation where it is reduced to NO and other bioactive nitrogen oxides by hemoglobin (2) under hypoxic conditions. Furthermore, several other body tissues can also reduce nitrite to NO (3-6). This alternative NO pathway makes a critical contribution to the regulation of blood pressure and blood flow (7), gastric integrity (8), and tissue protection against ischemic injury (9). Thus interest in inorganic dietary nitrate has increased substantially during the last decade (10-11).

Such interest has not gone unnoticed in exercise physiology, and inorganic nitrate supplementation has been suggested to be a potential ergogenic aid for athletes (12). The first research group to assess the effect of inorganic nitrate on exercise performance demonstrated a significant decrease in oxygen demand at low-to-moderate as well as at maximal exercise intensity (VO_{2peak}) after nitrate ingestion (13-14). They attributed this reduction to improved mitochondrial efficiency (15). However, despite these findings, these authors did not observe that nitrate supplementation improved performance measured as time-to-exhaustion during a cycling exercise test (14). In agreement with these data, in a recent study we found that pharmacological sodium nitrate supplementation significantly decreased VO_{2peak}, but did not improve exercise performance measured as time-to-exhaustion during an incremental cycle ergometer test in endurance athletes (16). In contrast, another research group has recently showed that inorganic nitrate supplementation in the form of beetroot juice (500 ml) significantly improved exercise performance measured as time-to-exhaustion during a fixed workload and during an incremental exercise test in healthy humans (17-19). Furthermore, the same group found that acute beetroot juice ingestion (500 ml) increased power output (5%) and reduced completion time (2.8%) during 4 and 16.1 km cycle ergometer tests. These results have been attributed to an attenuation of the VO_2 slow component

and an associated blunting of changes in the muscle metabolic milieu (reduction of phosphocreatine degradation and decrease in the cost of ATP production) (20).

There thus remains some controversy about the ergogenic effect of inorganic nitrate supplementation. One explanation for the diverging results is the training status of subjects (21). In well-trained individuals, NO donors have a lower effect on increasing NO markers and on endurance performance (21). The benefits of chronic exercise appear to outweigh those derived from dietary intervention with NO donors (22-23). From this viewpoint, authors examining the effect of nitrate supplementation in subjects with high fitness level, measured as VO_{2peak}, did not report an increase in exercise performance (14, 16). However, these studies analyzed performance throughout time-to-exhaustion tests, which do not efficiently measure endurance capacity (24). Time-trial protocols are a better way to measure changes in exercise performance (24).

Apart from the effect of dietary inorganic nitrate on plasma NO markers, its action on other plasma markers related with vasodilator and/or vasoconstrictor mechanisms such as endothelin-1 (ET-1) has not been addressed. ET-1 is a potent vasoconstrictor peptide produced by vascular endothelial cells that contributes to the regulation of vascular tone in humans (25). During exercise, NO and prostanoids (PGI_2) reduce the production and release of ET-1. In addition, NO can also nitrosylate endothelin receptors and reduce the affinity for ET-1 (26). Thus, in addition to its direct vasodilator effects, NO induces vasodilation indirectly by limiting the production of and/or vasoconstriction triggered by ET-1.

Accordingly, here we assessed the ergogenic effect of dietary inorganic nitrate supplementation in endurance-trained subjects using a 40-minute time-trial test. In addition, we evaluated plasma ET-1 before and after exercise in two experimental groups, one receiving inorganic nitrate supplementation and the other a placebo. We hypothesized that in well endurance-trained subjects dietary nitrate supplementation has a little enhancing effect on performance. In addition, we also studied whether inorganic nitrate supplementation increases NO synthesis, as this effect may also lead to a decrease in plasma ET-1 in resting subjects and/or after an exercise test.

Methods

Subjects

Thirteen non-professional male cyclists and triathletes (age 32.6 ± 5.6 yrs; body weight 72.4 ± 9.7 kg^{-1}; body mass index 23.4 ± 2.0 $kg \cdot m^{-2}$; body fat: 9.6 ± 3.3 %) 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. They had 8 ± 5 years of experience in endurance events, and their average weekly training volume was 15.7 ± 5.0 hours per week. All of them competed from 15 to 40 cycling and triathlon events per year at national level. 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

Subjects were randomly assigned in a double-blind, crossover design to follow 3-days of supplementation of either sodium nitrate (10 $mg \cdot kg^{-1}$ of body mass; Acofarma, code 18211, Spain) or the placebo (sodium chloride) dissolved in water. Supplementation was ingested each morning before to the breakfast. On the last day, subjects ingested the supplement or placebo 3 hours before to exercise test. A diet with low levels of moderate or high nitrate content foods (green vegetables, beetroot, strawberries, grapes and tea) was followed two 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 24 h prior to the exercise test. A 4-day washout separated the supplementation periods.

Ergometry test

The subjects were required to report to the laboratory on four occasions separated each by one week. The first week subjects performed an anthropometric evaluation and an incremental exercise test under laboratory controlled conditions to determine maximal oxygen uptake (VO_{2max}), maximal power output (W_{max}), ventilatory threshold (VT) and respiratory compensation point (RCP). The exercise protocol started at 50 W and increased 25 W every minute until voluntary exhaustion. An electronically braked cycle ergometer (Schoberer Rad Messtechnik, SRM, Germany) was used for the all tests. The configuration of the ergometer as the crank length, pedals, saddle and handlebar position was adapted to the measures of own road bicycles of subjects. Before each test cycle ergometer was calibrated following the manufacturer's instructions. The pedaling cadence was individually chosen within the range of 70 – 100 rpm. In the next three weeks subjects performed three time trials in the laboratory with controlled environmental conditions (23.8 ± 1.0 ºC). The first of them was carried out to familiarize subjects with the bicycle ergometer, gas analyzer and the testing procedure. The following two time trials were carried out in both conditions (placebo and nitrate) at the same time of day (± 1 h). They were asked to perform the maximum distance as much as possible during 40 min. We choose this duration because was related with distance (22 km) and time (~ 35 – 40 min) of the regional and national time trial championships. Some athletes of the current study were training for these championships and they accepted to participate in this study as part of their training for these events. Before to the test, athletes performed 15 min of warm up at 60% of VO_{2max} which was followed by 5-10 min of passive recovery before to start time trial. The ergometer (Schoberer Rad Messtechnik, SRM, Germany) was programmed in the mode "open end test". The subjects started the test in "gear 9" and were allowed to change gear. In this mode, the power output is changed if either pedal rate or the gear is changed at a constant pedal rate. For each time trial, time, distance, power and torque was recorded every second by SRM software. To avoid any reference, the only feedback available to cyclists during the time-trial was time elapsed. In addition, they were strongly verbally encouraged during both time trials. During the test food and fluid ingestion was forbidden.

Gas analysis

During the incremental exercise test, oxygen uptake (VO_2), minute ventilation (V_E), carbon dioxide production (VCO_2) and the respiratory exchange ratio (RER) were measured continuously breath-by-breath by a computerized gas analyzer (Jaeger Oxycon Mobile, Germany). The VO_{2max} was determined as the mean VO_2 measured over the final 60 s of exercise. W_{max} and HR_{max} were defined as the HR and W at the point of exhaustion during the test. 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 (27).

During time trials respiratory response was not measured continuously. Three samples of respiratory gas exchange were taken during the test: 1) between 12 and 15 min; 2) between 22 and 25 min, and 3) between 32 and 35 min. Data of VO_2, V_E, VCO_2 and RER were recorded breath-by-breath and values of the last minute were averaged and used to assess the respiratory response during exercise. In addition, HR was continuously recorded (beat-by-beat) with a portable heart rate monitor (Polar RS800 SD, Finland).

Blood sampling

Two blood samples were collected from the antecubital vein to analyze nitrate and nitrite: 1) after three days of nitrate supplementation or placebo in resting conditions before exercise test; 2) during the first three minutes after time trials (placebo and nitrate). 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) as it has been described previously (16).

Endothelin-1 levels in plasma were measured using commercially available immunoassay kits (Assay Designs, Inc., MI, USA) following the manufacturer's instructions. Assays were performed in duplicate and optical density was determined using a microplate reader set to 450 nm.

In addition, during time trials four samples of capillary blood (10 μL) were collected from the ear lobe to analyze lactate ([Hla]) using a Lange Miniphotometer LP2 (Germany): 1-3) in the minute 10, 20 and 30 of the test; and 4) at three minute after the maximal test.

Urine sampling

To analyze nitrate – nitrite urine levels three samples were collected at the same points of plasma samples: 1) during resting conditions (previous to VO_{2max} test and without nitrate restricted diet); 2) after three days of nitrate supplemention or placebo ingestion and before to start exercise test; 3) the first urine during the first hour after time trials. The same method used for blood samples was applied to analyze nitrate and nitrite concentration in urine.

Statistics

Results are expressed as means ± standard deviation of the mean. The coefficient of variation (CV) for distance and power output among the third and fourth time trial was calculated by dividing each subject standard deviation (SD) by his mean. A spreadsheet that analyzes validity by linear regression proposed by Hopkins was used for calculations. (HopkinsWG. Analysis of validity by linear regression (Excel spreadsheet). *In: A new view of statistics. sportsci.org: Internet Society for Sport Science, sportsci.org/resource/stats/xvalid.xls. 2000)*. In addition, an intraclass correlation coefficient (ICC) for the same variables was also computed. To investigate the influence of treatment (*S*) and time (*T*), and interaction between these both variables (*S***T*) the data were treated with two-way analysis of variance (ANOVA) with repeated measures. The sets of data in which

there was significant *S*T* interaction were tested by ANOVA one-way test. When significant effects of *S* or *T* were found, a Student's *t*-test for paired data was used to determine the differences between the groups (nitrate and placebo) involved. 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

Performance during the VO_{2max} test

Mean results of VO_{2max}, HR_{max} and W_{max} during the incremental exercise test were 4.3 ± 0.3 $L \cdot min^{-1}$ (59.7 ± 7.0 $mL \cdot kg^{-1} \cdot min^{-1}$), 180 ± 11 $beats \cdot min^{-1}$ and 378 ± 30 W (5.3 ± 0.8 $W \cdot kg^{-1}$), respectively. The ventilatory threshold (VT) was determined at mean intensity of 215 ± 38 W, 144 ± 12 $beats \cdot min^{-1}$ (80 ± 4 % of HR_{max}) and 38.6 ± 7.7 $mL \cdot kg^{-1} \cdot min^{-1}$ of VO_2 (64.3 ± 8.3 % of VO_{2max}), whereas respiratory compensation point (RCP) was estimated at average intensity of 301 ± 37 W, 166 ± 12 $beats \cdot min^{-1}$ (92 ± 3 % of HR_{max}) and 50.8 ± 7.8 $mL \cdot kg^{-1} \cdot min^{-1}$ of VO_2 (85.1 ± 8.1 % of VO_{2max}).

Plasma and urinary levels of nitrate and nitrite

The concentration of plasma nitrate increased significantly following nitrate supplementation (256 ± 53 μM) compared with placebo (44 ± 9 μM; $P < 0.001$) (**Figure 1**). Plasma nitrite showed a slight increase, but not statistically significant, after nitrate supplementation in resting conditions (nitrate: 4.5 ± 0.5 μM; placebo: 4.2 ± 0.4 μM; $P = 0.124$) (**Figure 1**). Otherwise, physical exercise did not alter significantly plasma nitrate and nitrite levels just after 40-min time trial in both groups (placebo and nitrate) (**Figure 1**). Plasma nitrate remained significantly increased compared with placebo after exercise (nitrate: 272 ± 60 μM; placebo: 52 ± 7 μM $P < 0.001$).

In urine, nitrate excretion increased significantly when subjects ingested dietary inorganic nitrate compared with placebo in resting conditions and after exercise (nitrate: 7,624 ± 468 μM; placebo: 1,299 ± 90 μM; $P < 0.001$). After nitrate treatment, the losses of nitrate in the urine corresponded to 65 ± 30 % (473 ± 189 mg) of the total amount of nitrate supplemented (724 ± 97 mg). Additionally, 40-min time trial did not alter urinary excretion levels of nitrate and nitrite after exercise (**Figure 1**). The nitrate plasma/urinary ratio in resting conditions was 0.07 ± 0.05 and 0.05 ± 0.03 after placebo and nitrate treatment, respectively. After exercise, this ratio did not show significant changes (placebo: 0.09 ± 0.05; nitrate: 0.06 ± 0.04; $P = 0.08$). For nitrite, plasma/urinary ratio in resting conditions was 1.32 ± 0.41 and 1.41 ± 0.53 for placebo and nitrate condition, respectively. This ratio was not significant modified after exercise (placebo: 1.49 ± 0.57; nitrate: 1.31 ± 0.40; $P = 0.28$).

Plasma levels of endothelin-1 (ET-1)

In resting conditions, plasma ET-1 levels did not differ between nitrate and placebo conditions (**Figure 2**). However, a significant increase was showed just after exercise in placebo ($P = 0.030$) and nitrate ($P < 0.001$) groups compared with resting values. In addition, this effect was significantly ($P = 0.010$) greater in the nitrate group compared with placebo (**Figure 2**).

Performance during 40-min time trial

The mean coefficient of variation (CV %) for distance and power output between the third and fourth time trial independent of treatment was 1.1% (95% CI = 0.9 – 1.8) and 2.2% (95% CI = 1.7 – 3-5), respectively. These differences were not statistically significant ($P > 0.05$). Additionally, the intraclass correlation coefficient ICC for distance was 0.98 (95% CI = 0.95 – 1.00) and 0.99 (95% CI = 0.96 – 1.00) for power output.

Average distance and power output profile are shown in **Figure 3**. There were no significant differences between the nitrate and placebo groups in the overall distance (nitrate: 26.328 ± 1.091 km; placebo: 26.320 ± 1.212 km; $P = 0.61$) and power output (nitrate: 258 ± 28 W and 3.6 ± 0.6 $W \cdot kg^{-1}$; placebo: 257 ± 28 W and 3.6 ± 0.6 $W \cdot kg^{-1}$; $P = 0.89$) performed during 40-min time trial. The mean cadence output during time trials was 93 ± 7 and 93 ± 6 revolutions per minute (rpm) for placebo and nitrate, respectively.

Cardiorespiratory and metabolic response during 40 min time trial

Main respiratory variables (VO_2, VCO_2, VE and RER) were unaffected after nitrate supplementation compared with placebo (**Table 1**). The HR increased significantly during the test in both treatments. It was found significant differences between average HR at 15 min and 25 min in placebo ($P < 0.001$) and nitrate ($P < 0.001$) conditions, as well as between average HR at 15 min and 35 min (placebo and nitrate = $P < 0.001$) (**Table 1**). In addition, in the nitrate group the average HR data at 15 min and 35 min differed significantly ($P = 0.018$). However, there were not differences in the HR response between nitrate and placebo conditions ($P > 0.05$) (**Table 1**). Blood lactate accumulation increased significantly at three minutes after exercise in respect values at 30 min in placebo group ($P < 0.017$) and at 10 min in nitrate group ($P < 0.024$) (**Figure 4**). However, there were not differences in average blood lactate concentration at any point of the test between placebo and nitrate groups. Average blood lactate accumulation throughout the test was 7.4 ± 3.7 and 7.5 ± 3.3 $mmol \cdot L^{-1}$ in placebo and nitrate conditions respectively.

Discussion

The main finding of this study is that supplementation with inorganic nitrate did not enhance the performance of endurance-trained athletes during a 40-minute time-trial. These results confirm our first hypothesis, as well as findings from a previous study (16) suggesting that the effect of this

supplementation in well trained athletes is limited compared with moderately trained ones (28). In addition, plasma ET-1 in arms increased significantly after nitrate supplementation compared with the placebo group. Although this finding contradicts the second hypothesis, it is consistent with data from other studies assessing plasma levels of ET-1 in working and non-working muscles during exercise (29-30). These findings suggest that, in addition to NO, dietary inorganic nitrate may stimulate the release of other molecules that also regulate blood flow during exercise.

Effects of dietary inorganic nitrate ingestion on blood and urine levels of nitrate and nitrite

Plasma nitrate in resting conditions increased an average of 212 ± 54 μM ($P < 0.05$) after 3 days of supplementation compared to the placebo group, a finding that is consistent with results from previous studies (13-14, 16, 31). Furthermore, urinary nitrate excretion also increased after supplementation with nitrate. Physical exercise did not alter plasma or urinary nitrate levels, which remained significantly higher than the placebo group after nitrate treatment. These results corroborate our previous hypothesis (16). We found that plasma nitrate was significantly reduced after a submaximal and maximal cycle ergometer test compared with values in resting conditions. This finding contrasts with those of another study indicating that plasma nitrate is not altered by exercise (13). We hypothesized that the differences we detected in plasma nitrate were related to the pharmacokinetics of nitrate after dietary ingestion since we assessed the effect of only one dose of inorganic nitrate 3 hours before an exercise test. The half-life of plasma nitrate in humans is approximately 5 hours and there is a substantial decrease 4 hours after ingestion (31). In a previous study, athletes completed submaximal and maximal workloads at 3 hours and 45 minutes (± 10 min) and 4 hours and 5 minutes (± 14 min) after nitrate ingestion (16), times at the borderline nitrate half-life. Thus, our results confirm that without a pharmacokinetic effect, the levels of plasma nitrate are not modified by physical exercise.

Plasma and urinary nitrite levels were not significantly altered after nitrate supplementation in resting conditions compared with the placebo group. This effect contrasts with

that found in other studies (13-14, 16-20, 28), which reported a substantial increase in plasma nitrite after ingestion of this compound. We estimated that around 65% of supplemented nitrate was excreted in the urine while the remaining 35% remained in the body. These results are in agreement with previous data (32). However, this is only estimation since urinary nitrate could also be increased by the activity of NO synthase (NOS). Plasma nitrate that remains in the body is actively taken up by salivary glands and excreted in saliva (32). In the mouth, facultative anaerobic bacteria on the surface of the tongue reduce nitrate to nitrite (33). Salivary nitrite is then further converted to NO in the stomach (34), but a substantial part of swallowed nitrite is absorbed intact and increases circulating plasma nitrite (1). However, this enterosalivary circulation of nitrate and reduction to nitrite can be disrupted by factors that alter oral flora. For instance, studies by Petersson et al. (35) and Govoni et al. (36) showed that the increase in plasma nitrite after nitrate supplementation is markedly attenuated by daily use of a commercial antibacterial mouthwash for 1 week as a result of the suppression of oral microflora. We asked athletes about their use of antibacterial mouthwashes; none reported use of these products. Therefore discarding this possibility, it is difficult to explain the lower response of some athletes to increased plasma nitrite after dietary ingestion of this compound. In addition, like in our previous study (16), here we found higher plasma nitrite compared with other reports (13-14, 17-20, 28). We previously proposed that these levels were related to methodological issues or training level of the subjects involved since there is evidence that plasma nitrite increases with exercise capacity (16). However, further studies are required to analyze the lower response of some endurance athletes to increased plasma nitrite.

Plasma nitrite levels remained unchanged after a 40-minute time-trial. Given that the athletes performed exercise at a mean intensity equivalent to the respiratory compensation point (RCP) (~ 85% of VO_{2max} and ~ 91% of HR_{max}), these results confirm our previous findings showing that plasma nitrite levels are not altered when exercise is performed at intensities close to or below the RCP (16). Thus, since nitrite is the main precursor of NO and other bioactive nitrogen oxides, the absence of an increase in plasma nitrite and/or decrease in this parameter after exercise may explain the limited effect of the dietary nitrate supplementation on exercise performance.

Effects of dietary inorganic nitrate ingestion on blood levels of endothelin-1 (ET-1)

Plasma ET-1 increased significantly after exercise in both treatments, but this increase was higher in the group supplemented with nitrate than in the placebo group. An increase in basal plasma ET-1 has traditionally been associated with a cardiovascular impairment function related to aging (37). However, there is also evidence that acute exercise causes a tissue-specific change in the production of ET-1 and that these alterations participate in the integrated physiological response during exercise (38). Studies by Maeda et al. (29-30) in humans exercising one leg showed that the concentration of ET-1 increased in the venous blood of the non-exercising leg, whereas it remaining unchanged in the exercising one. The mechanism behind the difference in ET-1 production between working and non-working muscles remains to be elucidated. Two endogenous mechanisms have been put forward to explain this response. The first is associated with the stimulus of shear stress induced by physical exercise. The release of ET-1 in cultured vascular endothelial cells is linked to low levels of shear stress while higher levels of stress depress the release of this molecule (30, 39). The second mechanism is related to neurohumoral factors, such as NO, prostacyclin, and arginine vasopressin, which may be released during exercise. For instance, a low dose of ET-1 potentiates vascular contractions to norepinephrine (40), thus there may be interactions between the blood flow, the sympathetic nervous system, and the release of various endothelium-derived vasoconstricting and vasodilating factors in the regulation of blood flow in exercising and non-exercising muscles. Thus, it has been hypothesized that neuronal-endothelial interactions in working and non-working muscles affect the release of ET-1 (30).

In the present study athletes performed a cycle ergometer test exercising basically muscle legs; however, blood samples were taken from the antecubital vein. Therefore, on the basis of the above data, an increase in plasma ET-1 would be expected in forearms just after exercise. We did not measure plasma ET-1 in the main exercising muscles (femoral venous) and therefore we cannot corroborate that this parameter changed or remained stable in these tissues. To the best of our knowledge, this is the first study to address the effect of dietary inorganic nitrate ingestion

on plasma ET-1 after exercise. However, there is evidence that acute ingestion of beetroot juice rich in nitrate increases blood flow before and after an ischemic stimulus in the brachial artery of the arm (31). These results were attributed to an increase in NO release; however, other molecules associated with vasodilator response were not examined. Although further research is required to corroborate that dietary nitrate ingestion alters plasma ET-1 after exercise, we propose that nitrate is involved in the release of other molecules, in addition to NO, and that these molecules contribute to the regulation of blood flow during exercise. On the basis of studies by Maeda et al. (29-30, 38), we hypothesize that an increase in plasma ET-1 in non-active tissues causes enhanced vascular tone and the consequent decrease in blood flow in these tissues, which could contribute to increasing the blood flow in exercising muscles or in the lungs.

Effects of dietary inorganic nitrate ingestion on the cardiorespiratory and metabolic response to exercise

The respiratory response was not assessed continuously in this study because athletes felt uncomfortable with the face mask during an exercise test lasting 40-minutes at maximal intensity. Thus we measured the respiratory response at three points during the test (3 minutes each). The data collected at these points showed that the respiratory gas exchange was constant (**Table 1**). No modifications of VO_2 at any point in either condition (nitrate and placebo) were detected. This finding is consistent with our previous study (16). However, in contrast to these results, other authors have reported a reduction in oxygen demands at submaximal exercise intensity after dietary nitrate supplementation in the form of sodium nitrate (13) as well as beetroot juice (17, 19-20). These findings have been related to a reduction of the ATP cost of muscle force production (20) and an improvement in mitochondrial efficiency (15). Like the results on performance, it is difficult to explain the contrasting results on the effects of nitrate supplementation on oxygen demands during exercise. Again, we propose that differences could be attributed to the training status of subjects. In addition to the benefits induced by chronic training on the cardiovascular and NO

system, there is also evidence showing that training (especially at high intensities) improves energy efficiency (41). The mechanisms responsible for training-related increases in efficiency may include muscle fiber type transformation (42), aerobic enzyme capacity within the muscle (43) and the expression of proteins such as PGC1α, ANT and UCP3 (41). Although the athletes included in our study were not professional, they trained an average of 15.7 ± 5.0 hours per week and competed frequently (15-40 days of competition per year) in cycling and triathlon events. In addition, they had 8 ± 5 years of experience in endurance events. At this level of fitness, the effect of exercise training may induce physiological and metabolic adaptations that overcome dietary intervention of NO donors (21).

In conclusion, dietary inorganic nitrate supplementation (10 $mg \cdot kg^{-1}$ of body mass) for 3 days did not enhance the performance of endurance-trained subjects in a 40-minute time-trial. In addition, although plasma nitrate increased significantly, plasma nitrite remained unchanged after nitrate ingestion. This effect was unexpected since other studies have shown an increase in plasma nitrite after dietary nitrate ingestion. Further research is required to analyze whether some endurance-trained subjects have a lower capacity to increase plasma nitrite from dietary nitrate ingestion. Furthermore, we found that nitrate supplementation induced a significant increase in ET-1 in forearms just after exercise. This is the first study to show this intriguing response. On the basis of this observation, we propose that dietary nitrate ingestion plays a crucial role in the release of vasoconstrictor molecules during exercise.

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References

1. Lundberg JO, Giovoni M. Inorganic Nitrate is a possible source for systemic generation of nitric oxide. *Free Rad Bio Med*. 2004;37:395-400.
2. Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, et al. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med*. 2003;9:1498-505.
3. Shiva S, Huang Z, Grubina R, Sun J, Ringwood LA, MacArthur PH, et al. Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration. *Circ Res*. 2007;100:654-61.
4. Millar TM, Stevens CR, Benjamin N, Eisenthal R, Harrison R, Blake DR. Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. *FEBS Lett.* 1998;427:225-8.
5. Carlsson S, Wiklund NP, Engstrand L, Weitzberg E, Lundberg JO. Effects of pH, nitrite, and ascorbic acid on nonenzymatic nitric oxide generation and bacterial growth in urine. *Nitric Oxide*. 2001;5(6):580-6.
6. Peri L, Pietraforte D, Scorza G, Napolitano A, Fogliano V, Minetti M. Apples increase nitric oxide production by human saliva at the acidic pH of the stomach: a new biological function for polyphenols with a catechol group? *Free Radic Biol Med.* 2005;39(5):668-81.
7. Larsen FJ, Ekblom B, Sahlin K, Lundberg JO, Weitzberg E. Effects of dietary nitrate on blood pressure in healthy volunteers. *N Engl J Med.* 2006;355:2792-3.
8. Lundberg JO, Weitzberg E, Lundberg JM, Alving K. Intragastric nitric oxide production in humans: measurements in expelled air. *Gut.* 1994;35:1543-6.
9. Shiva S, Sack MN, Greer JJ, et al. Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. *J Exp Med.* 2007;204:2089-102.
10. Lundberg JO, Weitzberg E, Gladwin M. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov.* 2008;7:156-67.

11. Gilchrist M, Winyard PG, Benjamin N. Dietary nitrate--good or bad? *Nitric Oxide.* 2010;22:104-9.
12. Lundberg JO, Larsen FJ, Weitzberg E. Supplementation with nitrate and nitrite salts in exercise: a word of caution. *J Appl Physiol.* 2011;111:616-7.
13. Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B. Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol.* 2007;191:55-66.
14. Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B. Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise. *Free Radic Biol Med.* 2010;48:342-7.
15. Larsen FJ, Schiffer TA, Borniquel S, et al. Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell Metab.* 2011;13:149-59.
16. Bescos R, Rodriguez FA, Iglesias X, Ferrer MD, Iborra E, Pons A. Acute administration of inorganic nitrate reduces VO_{2peak} in endurance athletes. *Med Sci Sports Exerc.* 2011;43:1979-86.
17. Bailey SJ, Winyard P, Vanhatalo A, et al. Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *J Appl Physiol.* 2009; 104:1144-55.
18. Vanhatalo A, Bailey SJ, Blackwell JR, et al. Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. *Am J Physiol Regul Integr Comp Physiol.* 2010;299:1121-31.
19. Lansley KE, Winyard PG, Fulford J, et al. Dietary nitrate supplementation reduces the O2 cost of walking and running: a placebo-controlled study. *J Appl Physiol.* 2010;110:591-600.
20. Bailey SJ, Fulford J, Vanhatalo A, et al. Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *J Appl Physiol.* 2010;109:135-48.
21. Bescos R, Sureda A, Tur JA, Pons A. The effect of nitric oxide related supplements on human performance. *Sports Med.* (In press).

22. Maiorana A, O'Driscoll G, Taylor R, Green D. Exercise and the nitric oxide vasodilator system. *Sports Med.* 2003;33:1013-35.

23. Sureda A, Tauler P, Aguiló A, et al. Blood cell NO synthesis in response to exercise. *Nitric Oxide.* 2006;15:5-12.

24. Currell K, Jeukendrup AE. Validity, reliability and sensitivity of measures of sporting performance. *Sports Med.* 2008;38:297-316.

25. Maeda S, Miyauchi T, Iemitsu M, Sugawara J, Nagata Y, Goto K. Resistance exercise training reduces plasma endothelin-1 concentration in healthy young humans. *J Cardiovasc Pharmacol.* 2004;44:S443-6.

26. de Beer VJ, Bender SB, Taverne YJ, et al. Exercise limits the production of endothelin in the coronary vasculature. *Am J Physiol Heart Circ Physiol.* 2011;300:1950-9.

27. Wasserman K, Hansen JE, Sue DY, Stringer WW, Whipp BJ. Principles of exercise testing and interpretation. Fourth ed. Philadelphia (USA): Lippincott Williams & Wilkins; 2004.

28. Lansley KE, Winyard PG, Bailey SJ, et al. Acute Dietary Nitrate Supplementation Improves Cycling Time Trial Performance. *Med Sci Sports Exerc.* 2011;43:1125-31.

29. Maeda S, Miyauchi T, Goto K, Matsuda M. Alteration of plasma endothelin-1 by exercise at intensities lower and higher than ventilatory threshold. *J Appl Physiol.* 1994;77:1399-402.

30. Maeda S, Miyauchi T, Sakane M, et al. Does endothelin-1 participate in the exercise-induced changes of blood flow distribution of muscles in humans? *J Appl Physiol.* 1997;82:1107-11.

31. Webb AJ, Loukogeorgakis S, Okorie M, et al. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension.* 2008;51:784-90.

32. Tannenbaum SR, Weisman M, Fett D. The effect of nitrate intake on nitrite formation in human saliva. *Food Cosmet Toxicol.* 1976;14:549-52.

33. Duncan C, Dougall H, Johnston P, et al. Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nat Med.* 1995;1:546-51.

34. McKnight GM, Smith LM, Drummond RS, Duncan CW, Golden M, Benjamin N. Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. *Gut.* 1997;40:211-4.

35. Petersson J, Carlström M, Schreiber O, et al. Gastroprotective and blood pressure lowering effects of dietary nitrate are abolished by an antiseptic mouthwash. *Free Rad Biol Med.* 2009;46:1068-75.

36. Govoni M, Jansson EA, Weitzberg E, Lundberg JO. The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide.* 2008;19:333-7.

37. Donato AJ, Gano LB, Eskurza I, et al. Vascular endothelial dysfunction with aging: endothelin-1 and endothelial nitric oxide synthase. *Am J Physiol Heart Circ Physiol.* 2009;297:425-32.

38. Maeda S, Miyauchi T, Kobayashi T, Goto K, Matsuda M. Exercise causes tissue-specific enhancement of endothelin-1 mRNA expression in internal organs. *J Appl Physiol.* 1998;85:425-31.

39. Kuchan MJ, Frangos JA. Shear stress regulates endothelin-1 release via protein kinase C and cGMP in cultured endothelial cells. *Am J Physiol.* 1993;264:150-6.

40. Yang ZH, Richard V, von Segesser L, et al. Threshold concentrations of endothelin-1 potentiate contractions to norepinephrine and serotonin in human arteries. A new mechanism of vasospasm? *Circulation.* 1990;82:188-95.

41. Hopker J, Passfield L, Coleman D, Jobson S, Edwards L, Carter H. The effects of training on gross efficiency in cycling: a review. *Int J Sports Med.* 2009;30:845-50.

42. Jansson E, Kaijser L. Muscle adaptation to extreme endurance training in man. *Acta Physiol Scand.* 1977;100:315-24.

43. Burgomaster KA, Hughes SC, Heigenhauser GJ, Bradwell SN, Gibala MJ. Six sessions of sprint interval training increases muscle oxidative potential and cycle endurance capacity in humans. *J Appl Physiol.* 2005;98:1985-90.

Table 1. Cardiorespiratory response during 40-minutes time trials after after dietary inorganic nitrate and placebo supplementation (n= 13).

Time	15 min		25 min		35 min		Average				
Treatment	Placebo	Nitrate	Placebo	Nitrate	Placebo	Nitrate	Placebo	Nitrate	*S*	*T*	*S*T*
VO_2											
$L \cdot min^{-1}$	3.64 ± 0.31	3.70 ± 0.32	3.64 ± 0.28	3.57 ± 0.32	3.62 ± 0.31	3.63 ± 0.42	3.63 ± 0.26	3.63 ± 0.33			
$mL \cdot min^{-1} \cdot kg^{-1}$	51.1 ± 7.6	51.9 ± 7.9	50.9 ± 6.6	50.2 ± 7.9	50.6 ± 6.4	50.9 ± 8.7	50.9 ± 6.6	51.0 ± 7.9			
VCO_2 ($L \cdot min^{-1}$)	3.52 ± 0.30	3.53 ± 0.29	3.50 ± 0.36	3.41 ± 0.33	3.44 ± 0.30	3.48 ± 0.45	3.49 ± 0.27	3.47 ± 0.32			
VE ($L \cdot min^{-1}$)	110 ± 13	112 ± 13	109 ± 12	110 ± 13	110 ± 13	116 ± 18	110 ± 11	113 ± 14			
RER	0.97 ± 0.04	0.96 ± 0.05	0.96 ± 0.05	0.96 ± 0.05	0.95 ± 0.04	0.96 ± 0.05	0.96 ± 0.04	0.96 ± 0.04			
HR ($beats \cdot min^{-1}$)	160 ± 11	160 ± 11	166 ± 12*	165 ± 11*	167 ± 10*	167 ± 12*#	164 ± 11	165 ± 11		X	

Values are means ± SD. VO_2: oxygen uptake; VCO_2: expired carbon dioxide; VE: minute ventilation; RER: respiratory exchange ratio; HR: heart rate.
X indicates significant effects (*S* or *T*) or significant interaction (*S*T*) of two-way ANOVA of repeated measures ($P < 0.05$).
* significant difference with respect to 15 min values
# significant difference with respect to 25 min values

Figure 1. Plasma and urinary levels of nitrate (A, C) and nitrite (B, D) in rest conditions and just after 40-minutes time trial after 3-days of supplementation with inorganic dietary nitrate or placebo (n= 13).
* Statistical significance between nitrate and placebo ($P < 0.05$).

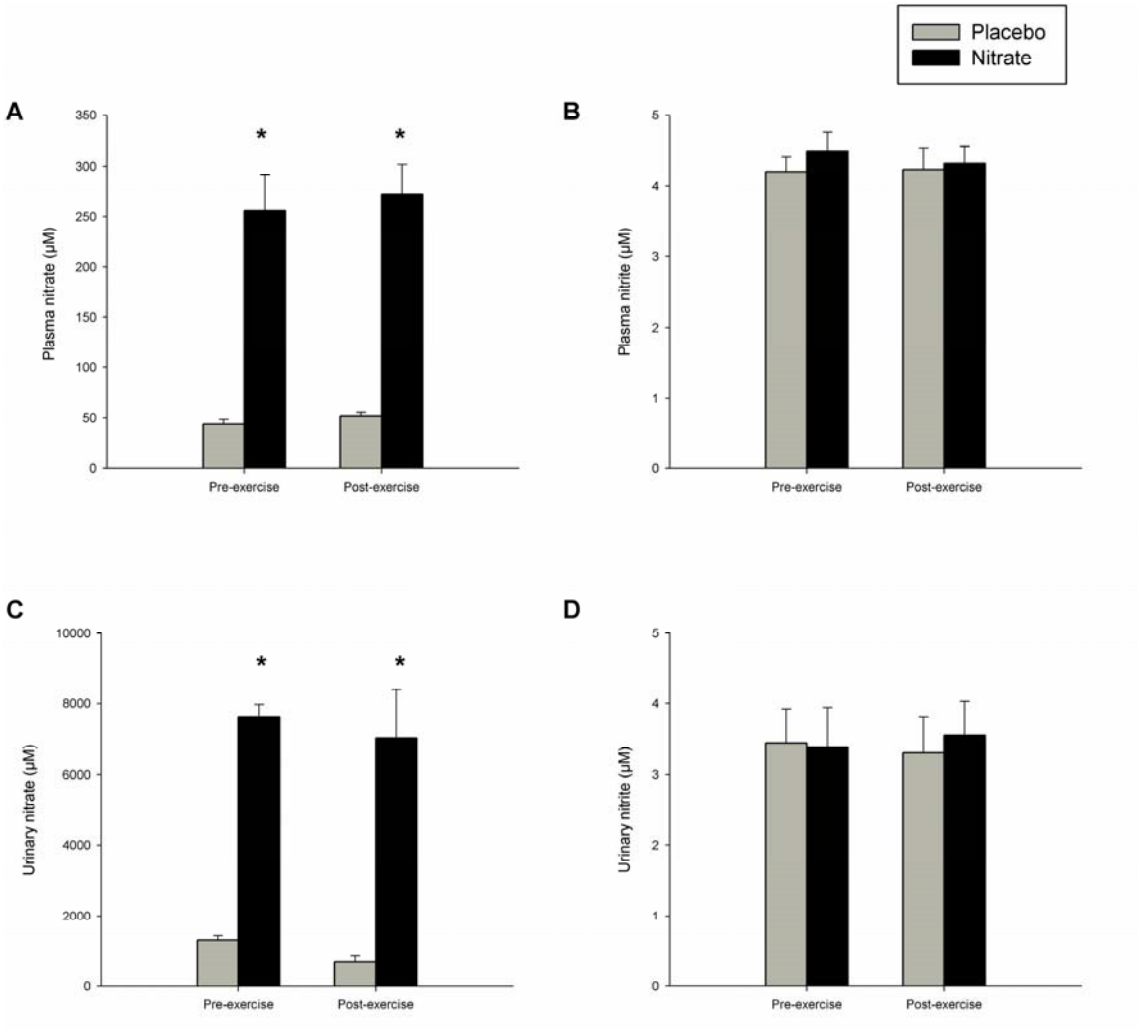


Figure 2. Plasma levels of endothelin-1 (ET-1) before and after 40-minutes time in both conditions (nitrate and placebo) (n= 13).

* Statistical significance between nitrate and placebo ($P < 0.05$).
# Statistical significance between pre-exercise and post-exercise values ($P < 0.05$).

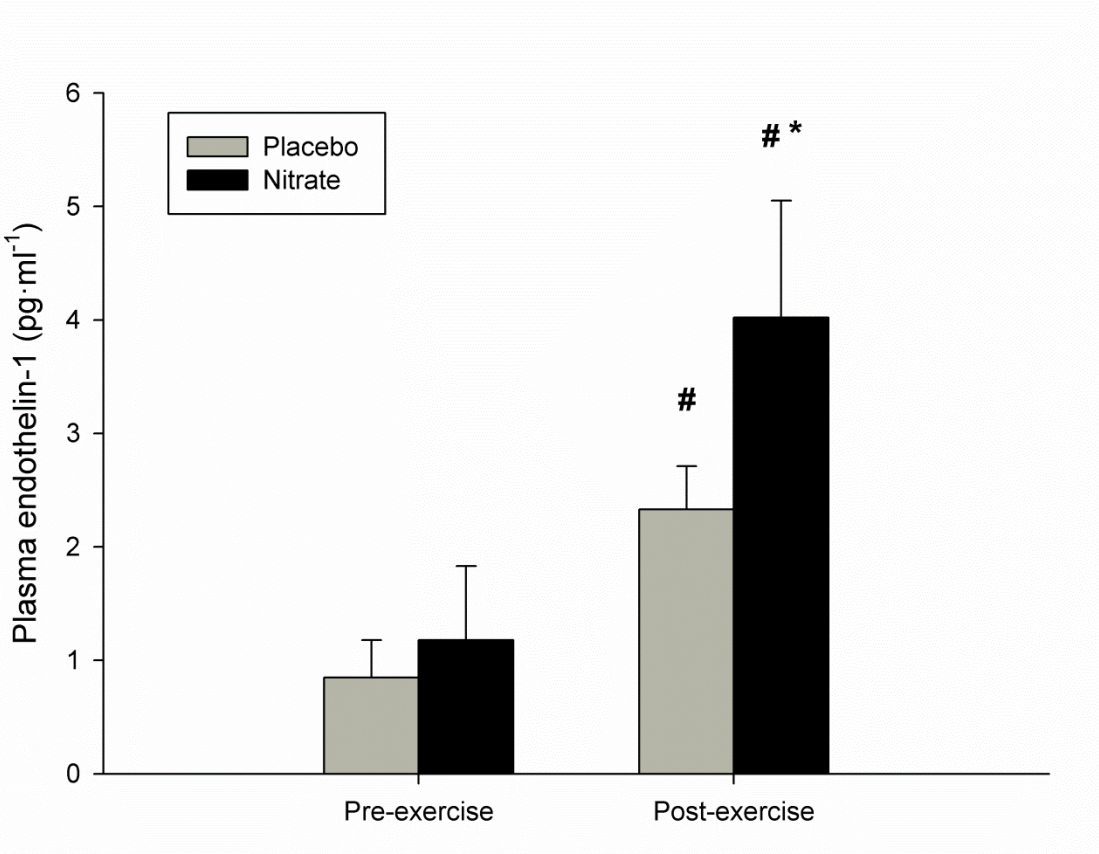


Figure 3. Profile of distance (A) and power output (B) performed by subjects during the 40-minutes time trials after dietary inorganic nitrate and placebo supplementation (n= 13).

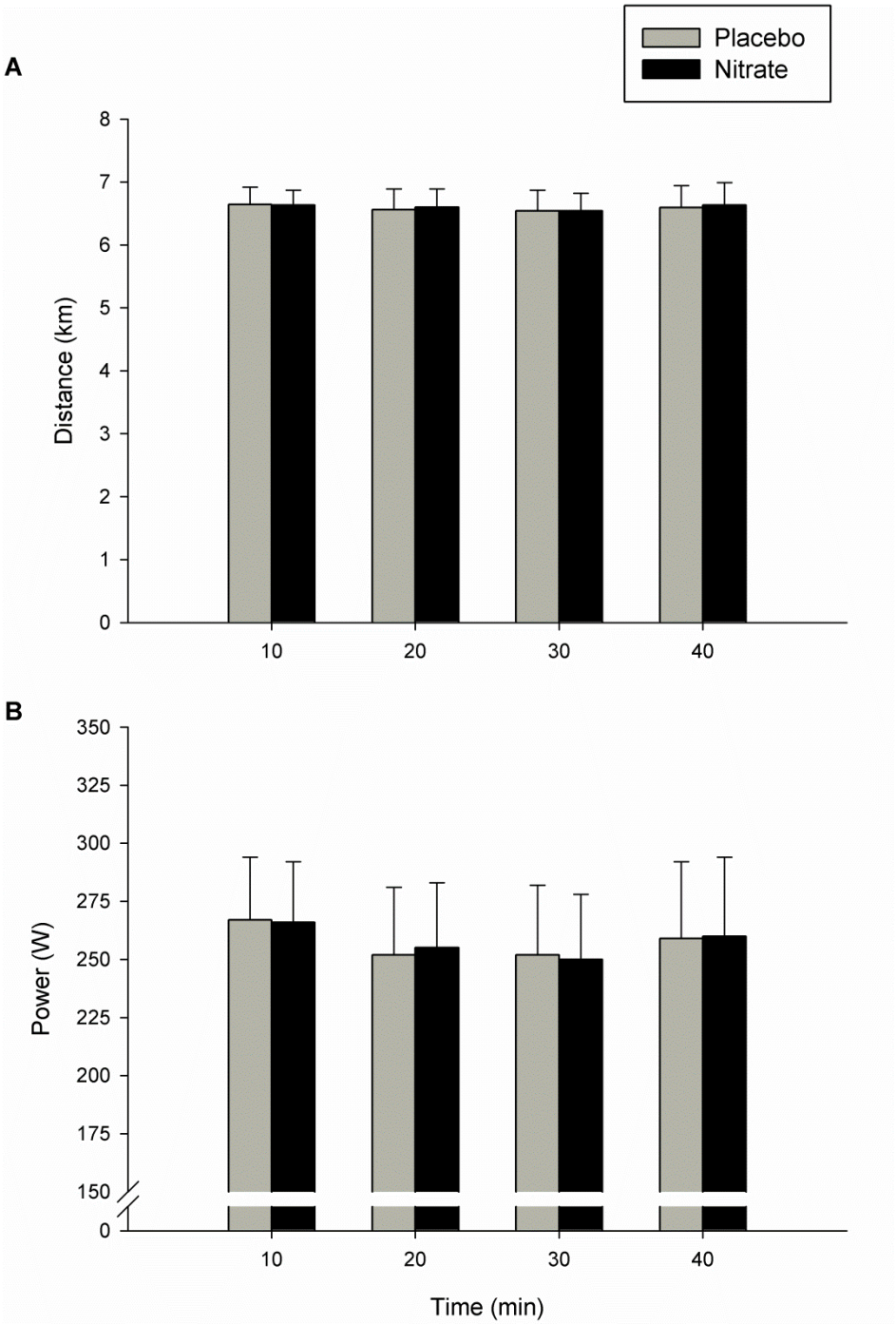
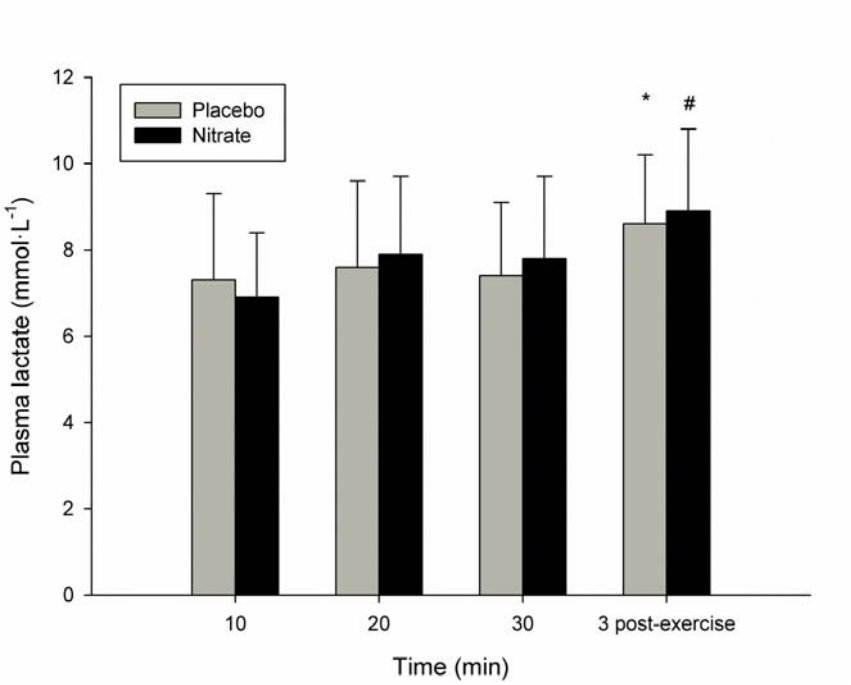


Figure 4. Plasma lactate concentration during 40-minutes time trial and just after 3 minutes post-exercise in both conditions (nitrate and plecebo) (n= 13).



* Statistical significance between mean values at 30 minutes and 3 minutes post-exercise in placebo group. Two-way ANOVA of repeated measures ($P < 0.05$).
# Statistical significance between mean values at 10 minutes and 3 minutes post-exercise in nitrate group. Two-way ANOVA of repeated measures ($P < 0.05$).

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The Effect of Nitric-Oxide-Related Supplements on Human Performance

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Contents

Abstract

Nitric oxide (NO) has led a revolution in physiology and pharmacology research during the last two decades. This labile molecule plays an important role in many functions in the body regulating vasodilatation, blood flow, mitochondrial respiration and platelet function. Currently, it is known that NO synthesis occurs via at least two physiological pathways: NO synthase (NOS) dependent and NOS independent. In the former, L-arginine is the main precursor. It is widely recognized that this amino acid is oxidized to NO by the action of the NOS enzymes. Additionally, L-citrulline has been indicated to be a secondary NO donor in the NOS-dependent pathway, since it can be converted to L-arginine. Nitrate and nitrite are the main substrates to produce NO via the NOS-independent pathway. These anions can be reduced *in vivo* to NO and other bioactive nitrogen oxides. Other molecules, such as

the dietary supplement glycine propionyl-L-carnitine (GPLC), have also been suggested to increase levels of NO, although the physiological mechanisms remain to be elucidated.

The interest in all these molecules has increased in many fields of research. In relation with exercise physiology, it has been suggested that an increase in NO production may enhance oxygen and nutrient delivery to active muscles, thus improving tolerance to physical exercise and recovery mechanisms. Several studies using NO donors have assessed this hypothesis in a healthy, trained population. However, the conclusions from these studies showed several discrepancies. While some reported that dietary supplementation with NO donors induced benefits in exercise performance, others did not find any positive effect. In this regard, training status of the subjects seems to be an important factor linked to the ergogenic effect of NO supplementation. Studies involving untrained or moderately trained healthy subjects showed that NO donors could improve tolerance to aerobic and anaerobic exercise. However, when highly trained subjects were supplemented, no positive effect on performance was indicated. In addition, all this evidence is mainly based on a young male population. Further research in elderly and female subjects is needed to determine whether NO supplements can induce benefit in exercise capacity when the NO metabolism is impaired by age and/or estrogen status.

1. Introduction

Nitric oxide (NO) is a labile lipid soluble gas synthesized at several locations in the body. The endogenous formation and biological significance of NO were revealed in a series of studies in the 1980s and for these seminal discoveries, three American researchers were subsequently awarded the Nobel Prize in Physiology or Medicine in 1998. Soon after the identification of NO as a signalling molecule in mammals, it was reported that specific nitric oxide synthase (NOS) enzymes catalyze a complex enzymatic reaction leading to NO formation from the substrates L-arginine and molecular oxygen.[1] Later, an alternative NOS-independent pathway of NO synthesis was discovered, based on the simple reduction of nitrate and nitrite,[2,3] the main oxidation products of NO. During this period, interest in the biological role of NO has led a revolution in pharmacological and physiological research. Currently, NO is known to regulate important functions as a mediator in noradrenergic and non-cholinergic neurotransmission in learning and memory, synaptic plasticity and neuroprotection.[4]

In exercise physiology, NO has also received much interest, and supplements of NO are thought to be an ergogenic aid.[5] This fact is based on the evidence that NO is an important modulator of blood flow and mitochondrial respiration during physical exercise.[6] In addition, it is suggested that the increase of blood flow derived from NO synthesis may improve recovery processes of the activated tissues.[7] These supposed benefits are claimed in most sport supplements, which are currently sold in the market and linked with stimulation of NO production. However, a careful examination of the composition of NO-stimulating supplements shows that, in many cases, they are 'cocktails' of a great variety of ingredients such as creatine, carbohydrates, amino acids, vitamins, minerals, etc. It is known that some of these components (creatine, carbohydrates and amino acids) may have an ergogenic effect in themselves.[8-10] In addition, the scientific evidence behind these 'cocktails' of supplements related with NO stimulation is very scarce. Only one study has evaluated the effect of some of these products, indicating that their effectiveness at increasing NO and/or improving

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performance is very limited.[11] In comparison with data reported in scientific studies, it has been suggested that the amounts of NO ingredients (mainly L-arginine and L-citrulline) that contain commercial NO-stimulating supplements are extremely low and ineffective to induce changes in NO.[11]

For this reason, most studies involving NO donors have used pharmaceutical products to assess the effect on human performance.[12-18] Furthermore, there are some recent studies that have also assessed the effect of natural foods rich in NO donors, such as beetroot juice.[19-23] Results from these studies show great controversy. Some of them showed that dietary NO supplements may enhance human performance in healthy subjects,[19,24,25] but others did not find any positive effect.[16,18,26] One reason to explain this fact could be the large methodological differences between studies: duration of treatment, exercise protocol and training status differ significantly between studies, making a comparison between them difficult. Additionally, many studies have used NO donors in combination with other components such as malate, glutamate, aspartate, etc., in an attempt to increase the bioavailability of NO donors. This fact adds more difficulty because some of these additional products may participate in the independent NO-synthesis pathways in the body.

Accordingly, this review focuses on pathways and donors of NO synthesis and elucidates the effect of NO supplements on human performance. Scientific articles were retrieved based on an extensive search in MEDLINE (1980–2011) and Google Scholar (1990–2011) databases. Computer search engines used the following combined keywords: 'L-arginine', 'L-citrulline', 'nitrate', 'glycine-propionyl-L-carnitine', 'supplementation', 'nitric oxide', 'exercise' and 'performance'. After using these initial keywords, the search engines were limited to human studies excluding research with animals, as well as in humans in pathological states. As a result, 42 articles related to the effects of dietary ingredients linked with NO and performance in response to exercise were considered. References cited in the retrieved articles were also considered in this review.

2. Synthesis of Nitric Oxide (NO) from the NO Synthase (NOS)-Dependent Pathway

L-arginine amino acid participates in the NOS-dependent pathway in a reaction catalyzed by specific NOS enzymes[4] (figure 1). Additionally, it has been suggested that L-citrulline could be an alternative donor of NO, due to the fact that it can increase the levels of L-arginine.

2.1 L-Arginine: Sources and Metabolism

L-arginine is considered a conditional essential proteinogenic amino acid that is a natural constituent of dietary proteins. L-arginine is relatively high in seafood, watermelon juice, nuts, seeds, algae, meat, rice protein concentrate and soy protein isolate.[27] The typical dietary intake of L-arginine is approximately 4–5 g per day. Furthermore, L-arginine could be endogenously synthesized, mainly in the kidney, where L-arginine is formed from L-citrulline.[28] The liver is also able to synthesize considerable amounts of L-arginine, although this is completely reutilized in the urea cycle.[28] Normal plasma L-arginine concentrations depend upon the age of the individual and homeostasis is primarily achieved via its catabolism.[29] The usual mean ± standard deviation range of plasma L-arginine in humans has been determined between 70 and 115 $\mu mol \bullet L^{-1}$[30] Extracellular L-arginine can be quickly taken up by endothelial cells; in the presence of molecular oxygen and nicotinamide adenosine dinucleotide phosphate, L-arginine is subsequently oxidized to NO.[1,4] This is a complex reaction, which is catalyzed by NOS enzymes that contain a binding site for L-arginine. There are three isoforms of NOS that have been recognized: type I (neuronal NOS; nNOS), type II (inducible NOS; iNOS) and type III (endothelial NOS; eNOS). eNOS and iNOS are constitutive enzymes that are controlled by intracellular Ca^{2+}/calmodulin. nNOS is inducible at the level of gene transcription, Ca^{2+} independent and expressed by muscle activity[31] the aging process,[32] as well as by macrophages and other tissues in response to inflammatory mediators.[33]

L-arginine participates in other metabolic pathways independent of NO synthesis. For in-

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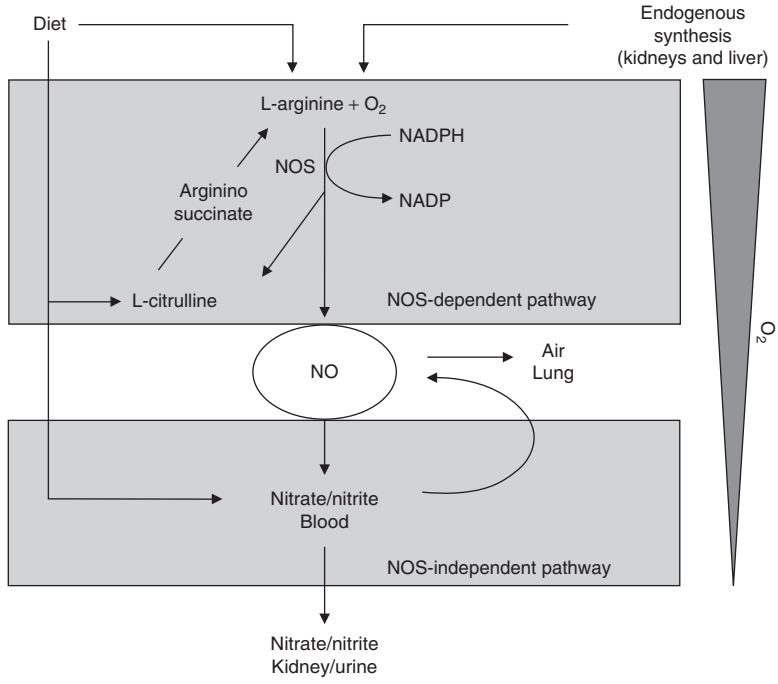

Fig. 1. Metabolic pathways of NO synthesis in humans. **NADP**/**NADPH** = nicotinamide adenine dinucleotide phosphate-oxidase; **NO** = nitric oxide; **NOS** = NO synthases.

stance, L-arginine is essential for the normal function of the urea cycle, in which ammonia is detoxified through its metabolism into urea.[34] L-arginine is also a potent hormone secretagogue. L-arginine infusion at rest increases plasma insulin, glucagon, growth hormone (GH), prolactin and catecholamines concentrations.[35] Such hormonal changes affect the metabolism. For instance, insulin and GH are important anabolic hormones with a remarkable degree of synergy in regulating glucose and fat metabolism. While insulin facilitates glucose entry into cells and an increase in glycogen stores, GH stimulates lipolysis and reduces glucose oxidation to maintain blood glucose levels.[36] Thus, it has been suggested that GH and insulin release may enhance exercise performance by increasing fatty acid oxidation and sparing glycogen stores.[36] In addition, GH also causes the release of insulin-like growth factor (IGF)-1 that increases amino acid uptake and protein synthesis.[37] These effects could also improve performance through increased muscle mass and strength.[37]

2.1.1 Ergogenic Effect of L-Arginine Supplements Alone

Seven studies have analysed the effect of L-arginine supplementation alone.[12-18] Two of these studies were carried out in healthy, but not well trained, males[12,14] and one in healthy, postmenopausal women.[13] In this study, females were supplemented with high doses of L-arginine ($14.2\,g \bullet day^{-1}$) for 6 months. After this period, a significant increase in the maximal power in relation with body mass ($power \bullet kg^{-1}$) measured as peak jump power (counter-movement jump) was found.[13] In male studies, it has been indicated that L-arginine supplementation could enhance the respiratory response. Koppo et al.[12] showed a significant increase in speed in phase II of pulmonary oxygen consumption ($\dot{V}O_2$) at the onset of moderate intensity endurance cycle exercise after 14 days'

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L-arginine supplementation ($6\,g \bullet day^{-1}$). Faster $\dot{V}O_2$ kinetics of phase II reduces the O_2 deficit that follows the onset of exercise and can reduce intracellular perturbation (e.g. increased lactic acid, decreased phosphocreatine).[38] This fact could be interesting in order to enhance tolerance to endurance exercise, mainly in subjects with slow $\dot{V}O_2$ kinetics (time it takes to reach 63% of steady state [tau] >30 seconds). However, all these findings were not linked with NO synthesis since the above studies did not report data related to NO markers, such as plasma ratio of L-arginine : L-citrulline and/or plasma levels of nitrate and nitrite. On the other hand, Olek et al.[14] assessed the effect of an acute dose of L-arginine in a low dose (2 g) 60 minutes before exercise. They showed that this amount of L-arginine did not induce any increase in the total work performed or mean power output during Wingate cycle tests (30 seconds), or $\dot{V}O_2$ either.[14] Additionally, plasma levels of nitrate/nitrite were unchanged after L-arginine supplementation compared with placebo.

The remaining studies were performed in well trained athletes using different types of athletic populations such as judo athletes,[17,18] tennis players[16] and cyclists.[15] Despite analysing supplements during different durations (between 1 and 28 days) and doses (between 6 g and 12 g), no benefit was indicated in parameters linked with performance, such as power in a cycle ergometer test[18] or $\dot{V}O_2$ during a treadmill test.[15,16] Additionally, the levels of some exercise metabolites (lactate and ammonia) were unchanged after L-arginine supplementation compared with placebo.[17,18] Moreover, three of these studies analysed the level of plasma nitrate/nitrite as NO markers showing that they did not increase after dietary L-arginine ingestion.[16-18] The other study did not include data regarding NO metabolites.[15]

Apart from dietary supplementation, other studies have analysed the effect of intravenous infusion of L-arginine in an attempt to increase its bioavailability,[39,40] since dietary L-arginine bioavailability is only about 60%. This fact is due to the high activity of arginases in the liver.[41] Arginases are enzymes that participate in the fifth and final step of the urea cycle, competing with NOS for L-arginine.[42] In athletes, there is evidence that exhaustive exercise increases arginase activity in lymphocytes nearly 6-fold, limiting L-arginine availability for lymphocyte iNOS activity.[43] However, despite the fact that the bioavailability of intravenous infusion of L-arginine could be high compared with dietary consumption, no positive effect in parameters of performance, such as maximal workload during an incremental cycle ergometer test or the amount of work completed in a 15-minute test after intravenous L-arginine infusion, has been reported.[39,40]

2.1.2 Ergogenic Effect of L-Arginine Supplements in Combination with Other Components

There are several studies that have shown an increase in exercise performance after L-arginine supplementation in combination with other components in untrained or moderately trained subjects. For instance, a recent study of Bailey et al.[24] showed that L-arginine ($6\,g \times 3$ days) in combination with other amino acids and vitamins induced a decrease in $\dot{V}O_2$ (L-arginine: $1.48 \pm 0.12\,L \bullet min^{-1}$; placebo: $1.59 \pm 0.14\,L \bullet min^{-1}$; $p < 0.05$) in a low to moderate bout of exercise (6 minutes at 82 ± 14 W); and an increase in time to exhaustion (L-arginine: 707 ± 232 seconds; placebo: 562 ± 145 seconds; $p < 0.05$) during an incremental cycling test.[21] In another recent study, Camic et al.[25] found an increase in power output (5.4%) during an incremental test to exhaustion (cycle ergometer) when L-arginine (3 g) in combination with grape seed extract was administered for 28 days. Similarly, in elderly males, Chen et al.[44] found that supplementation of L-arginine ($5.2\,g \bullet day^{-1} \times 21$ days) with L-citrulline and antioxidants increased power output (~21%) during an incremental cycle ergometer test until exhaustion. These surprising findings have been related to an increase in gas exchange threshold after L-arginine supplementation.[44,45] It has been suggested that the attenuation of metabolic products such as potassium, ammonia and lactate, may be the result of increased clearance from the circulation related to NO synthesis and increased blood flow.[45] However, it is only speculation, since there is evidence indicating that higher doses of dietary L-arginine (>10 g) are ineffective to increase blood flow in healthy humans.[46,47]

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Other studies have also reported benefits of a mixture of L-arginine supplements in strength and power performance in moderately trained subjects. Campbell et al.[48] indicated a significant increase of one-maximum repetition (1-RM) of bench press, as well as peak power during a 30-second Wingate test after L-arginine supplementation ($6\,g \bullet day^{-1} \times 56$ days) in combination with α-ketoglutarate. Futhermore, Buford and Koch,[49] and Stevens et al.[50] showed that an acute dose of L-arginine (6 g of L-arginine) in the form of α-ketoisocaproic increased the mean power performed during Wingate tests (10 seconds) and work sustained during continuous isokinetic concentric/eccentric knee extension repetitions, respectively.

In well trained athletes, two studies have assessed dietary L-arginine supplementation in combination with aspartate. In the first, Colombani et al.[51] supplemented ($15\,g \bullet day^{-1} \times 14$ days) endurance-trained runners. They showed that the plasma level of somatotropic hormone (STH), glucagon, urea and arginine were significantly increased, and the level of plasma amino acids was significantly reduced after a marathon run following L-arginine supplementation. The conclusion of this study was that there was no metabolic or performance benefit derived from L-arginine. More recently, similar findings were reported by Abel et al.[52] They supplemented endurance-trained cyclists with L-arginine and aspartate at high (5.7 g of L-arginine; 8.7 g of aspartate) and low (2.8 g of L-arginine; 2.2 g of aspartate) doses for 28 days. After an incremental endurance exercise test (cycle ergometer) in laboratory conditions, no modification was found in endurance performance ($\dot{V}O_2$ peak [$\dot{V}O_{2peak}$], time to exhaustion), or in endocrine (concentration of growth hormone, glucagon, cortisol and testosterone) or in metabolic (concentration of lactate, ferritine and urea) parameters.[52]

Therefore, including all studies with L-arginine supplementation alone and with other components (tables I and II), no study in well trained athletes reported benefits in human performance.[15-18,51,52] One important factor that may explain the reduced effect of L-arginine in well trained athletes, could be explained by the physiological and metabolic adaptation derived from chronic physical training. The effect of exercise training on the enhancement of endothelial function has been well established.[69] Repetitive exercise over weeks results in an upregulation of endothelial NO activity. This is not a localized but rather a systemic response in endothelial function when large muscle mass is regularly activated, as in aerobic exercise.[70] Perhaps benefits in pulmonary, cardiovascular and neuromuscular systems induced by long-term training may overcome any potential effects of dietary L-arginine supplementation in well trained athletes. However, there are other factors that may also reduce the effect of dietary L-arginine, such as the L-arginine : lysine ratio. The amino acid lysine competes with L-arginine for entry into cells and also inhibits arginase activity.[71] Under normal feeding conditions, the total amount of L-arginine in the diet should not be more than 150% greater than that of lysine (namely, L-arginine : lysine <2.5).[72]

In addition, in most of the above mentioned studies, there is a lack of data concerning NO metabolites. Only Bailey et al.[24] analysed the plasma levels of nitrite, reporting a significant increase after L-arginine supplementation. However, only one study[24] states that there is too little scientific evidence to corroborate that dietary L-arginine supplementation increases NO synthesis in healthy humans. Some of the benefits shown in the previous studies could be related to other metabolic pathways independent of NO synthesis, as well as to the other ingredients included in L-arginine supplements. For example, there is evidence that L-arginine supplementation in combination with glutamate and aspartate is effective at reducing blood levels of ammonia,[55,56] as well as blood lactate,[40,57] during exercise. This response could explain the results reported by the aforementioned studies of Camic et al.[45] and Stevens et al.[50] Moreover, L-arginine is known to actively participate in the synthesis of creatine.[73] Diets supplemented with L-arginine increase intramuscular creatine phosphate concentrations between 1% and 2% in laboratory animals; thus, this may enhance the response to anaerobic exercise.[74] This finding may be a suitable response to the study of Buford and Koch[49] who indicated that a supplement of

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Table I. Studies with nitric oxide supplements that reported an increase in performance

Substance	Dose per day	Duration (days)	Other components	Design	Sample size	Training status	Effects	References
L-arg	14.2 g	≈180		DB, R	23	U	↑ Maximal power	13
L-arg	6.0 g	3	Vitamins and amino acids	DB, CO	9	M	↑ Efficiency and time to exhaustion	24
L-arg	1.5 g	28	Grape seed extract	DB, R	50	U	↑ Work capacity	25
L-arg	1.5 g	28	Grape seed extract	DB, R	41	U	↑ Increase of gas exchange threshold and power output	45
L-arg	5.2 g	21	L-citrulline and antioxidants	DB, R	16	M	↑ Power output	44
L-arg	6.0 g	56	α-ketoglutarate	DB, R	35	M	↑ Increase of 1-RM	48
L-arg	6.0 g	1	Glycine α-ketoisocaproic	DB, R	19	M	↑ Power performance	49
L-arg	6.0 g	1	Glycine α-ketoisocaproic	DB, R, CO	13	U	↑ Work sustained during anaerobic exercise	50
L-citr	8.0 g	1	Malate	DB, R, CO	41	M	↑ Work capacity	53
Nitrate	5.5 mmol	6	Beetroot juice	DB, R, CO	8	M	↑ Efficiency and time to exhaustion	19
Nitrate	5.1 mmol	6	Beetroot juice	DB, R, CO	7	M	↑ Increase time-to-task failure	20
Nitrate	6.2 mmol	6	Beetroot juice	DB, R, CO	9	M	↑ Efficiency and time to exhaustion	22
Nitrate	6.2 mmol	1	Beetroot juice	DB, R, CO	9	M	↑ Power output	23
Nitrate	5.2 mmol	15	Beetroot juice	DB, R, CO	8	U	↑ Increase efficiency and peak power	21
GPLC	4.5 g	1		DB, R, CO	24	M	↑ Peak power and reduced power decrement	54

1-RM = one-repetition maximum; **CO** = crossover; **DB** = double blind; **GPLC** = glycine propionyl-L-carnitine; **L-arg** = L-arginine; **L-citr** = L-citrulline; **M** = moderately-trained subjects; **R** = randomized; **U** = untrained subjects; ↑ indicates improvement in performance.

glycine-arginine-α-ketoisocaproic acid (GAKIC) enhances performance during repeated bouts of anaerobic cycling performance.

In summary, current evidence of L-arginine supplementation in sports performance suggests that (i) L-arginine, mainly in combination with other components, could induce some benefit in untrained or moderately trained subjects, improving tolerance to aerobic and anaerobic physical exercise. However, as the studies do not show a well defined relationship between dietary L-arginine supplementation and NO synthesis, the benefit in exercise performance shown in some studies could be derived from other ingredients of supplements, as well as other metabolic pathways independent of NO synthesis; and (ii) in well trained athletes, there is a lack of data indicating that L-arginine supplementation induces benefits in performance. A recent review analysing the potential ergonegic effects of acute and chronic L-arginine supplementation did not reach a clear conclusion as to the benefits in exercise performance either.[75]

2.2 L-Citrulline: Sources and Metabolism

The organic compound L-citrulline, is a non-essential α-amino acid. Its name is derived from *Citrullus*, the Latin word for watermelon from which it was first isolated in 1930, and which is

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Table II. Studies with NO supplements that reported no or negative effects on performance

Substance	Dose per day	Duration (days)	Other components	Design	Sample size	Training status	Performance	Other findings	References
L-arg	6.0 g	14		DB, CO	7	M	NM	Increase of phase II pulmonary $\dot{V}O_2$	12
L-arg	2.0 g	1		DB, R, CO	6	M	None		14
L-arg	12.0 g	28		DB, R	18	H	None		15
L-arg	20.5 g	3		DB, R	9	H	NM		16
L-arg	≈7.6 g	1		R	15	H	NM	Increase of glucose and insulin	17
L-arg	6.0 g	3		DB, R	10	H	None		18
L-arg	30.0 g[a]	1		DB, R	9	H	None	Increase of glucose	39
L-arg	3.0 g[a]	1		DB, R	8	M	None	Lower blood lactate and ammonia	40
L-arg	5.7 g	28	L-asp	R	30	H	None		52
L-arg	15.0 g	14	L-asp	DB, CO	20	H	NM	Increase of somatotropic hormone, glucagon and urea	51
L-arg	20.0 g	1	L-glut	DB, CO	3	U	NM	Lower ammonia	55
L-arg	5.0 g	10	L-asp	DB, R	15	U	NM	Lower ammonia	56
L-arg	3.0 g	21	L-asp	DB	16	M	NM	Lower blood lactate and $\dot{V}O_2$	57
L-citr	6.0 g	1	Malate	DB, R	17	H	NM	Increased levels of NO metabolites	58
L-citr	9.0 g	1		DB	17	M	↓	Decrease of time to exhaustion	59
L-citr	6.0 g	15	Malate	?	18	U	NM	Increase ATP production	60
L-citr	6.0 g	1	Malate	DB, R	17	H	NM	Increase of plasma nitrite	61
Nitrate	10.0 mg • kg^{-1}	1	SN	DB, R, CO	11	H	None	Reduce $\dot{V}O_{2peak}$	26
Nitrate	0.1 mmol • kg^{-1}	3	SN	DB, R, CO	9	M	None	Increase efficiency	62
Nitrate	0.1 mmol • kg^{-1}	2	SN	DB, R, CO	9	M	None	Reduce $\dot{V}O_{2peak}$	63
Nitrate	0.1 mmol • kg^{-1}	3	SN	DB, R, CO	14	M	NM	Increase mitochondrial efficiency	64
GPLC	4.5 g	1		DB, R, CO	19	M	None	Decrease of malondialdehyde	11
GPLC	3.0 g	28		DB, R, CO	15	M	NM	Increased levels of NO metabolites	65
GPLC	1.5–4.5 g[b]	56		DB, R	30	U	NM	Increased levels of NO metabolites	66
GPLC	1.5–4.5 g[b]	56		DB, R, CO	32	U	None		67
2-ethyl	–	1		R, CO	10	M	NM	No changes in plasma nitrate/nitrite	68

a Intravenous supplementation.

b Data is presented in ranges.

2-ethyl = 2-(nitrooxy) ethyl 2-amino-3-methylbutanote; **ATP** = adenosine triphosphate; **CO** = crossover; **DB** = double blind; **GPLC** = glycine propionyl-L-carnitine; **H** = highly trained subjects; **L-arg** = L-arginine; **L-asp** = L-aspartate; **L-citr** = L-citrulline; **L-glut** = L-glutamate; **M** = moderately trained subjects; **NM** = none measured; **R** = randomized; **SN** = sodium nitrate; **U** = untrained subjects; **$\dot{V}O_2$** = oxygen consumption; **$\dot{V}O_{2peak}$** = peak $\dot{V}O_2$; ↓ indicates decrease in performance; – indicates no supplement composition shown therefore amount not shown; ? indicates design not stated.

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the main dietary source of this amino acid.[76] L-citrulline is also produced endogenously via the following two main pathways: (i) it is synthesized from glutamine in enterocytes by condensation of ornithine and carbamyl phosphate in a reaction catalyzed by ornithine carbamyl-transferase;[77,78] and (ii) L-citrulline is produced via the conversion of L-arginine to NO in a reaction catalyzed by NOS enzymes (figure 1). The normal value of L-citrulline reported in healthy populations is approximately 25 μmol • L^{-1},[79] although lower values have recently been found (10–15 μmol • L^{-1}) in professional cyclists.[58]

The dietary interest for this amino acid has substantially increased in the last decade as a result of the importance of L-citrulline as a precursor of L-arginine.[80,81] It is interesting, because, unlike L-arginine, it bypasses the hepatic metabolism and is not a substrate of arginase enzymes. For this reason, it has been indicated that systemic administration of L-citrulline could be a more efficient way to elevate extracellular levels of L-arginine by itself.[82] Dietary L-citrulline is taken up and released by enterocytes in the portal circulation, bypasses metabolism by periportal hepatocytes and is transported to the kidneys where around 80% is catabolized to L-arginine by cells of the proximal tubules.[83] Apart from the function as a precursor of L-arginine, it is known that L-citrulline is an essential component participating in the urea cycle in the liver.[77]

2.2.1 Ergogenic Effect of L-Citrulline Supplements Alone

Only one study has been carried out involving L-citrulline supplementation without the addition of other products. In this study, Hickner et al.[59] assessed the effect of one dose of L-citrulline administered 3 hours (3 g) or 24 hours (9 g) before an incremental treadmill test until exhaustion in young healthy subjects. Contrary to the hypothesis of the authors, the results showed that L-citrulline supplementation impaired exercise performance measured as time to exhaustion compared with placebo. To explain this surprising response, it was indicated that L-citrulline ingestion might reduce nitric-oxide-mediated pancreatic insulin secretion or increase insulin clearance. This hypothesis was based on the lower plasma insulin levels found after L-citrulline ingestion.[59] Additionally, lower levels of plasma NO markers (nitrates/nitrites) were also indicated following L-citrulline supplementation compared with placebo.

2.2.2 Ergogenic Effect of L-Citrulline Supplements with Malate

The other studies that have analysed the effect of L-citrulline combined this amino acid with malate, which is an intermediate component of the tricarboxylic acid cycle (TCA). The first of these studies examined the rate of adenosine triphosphate (ATP) production during an exercise of finger flexions using ^{31}P-magnetic resonance spectroscopy (^{31}P-MRS).[60] This study concluded that 6 g • day^{-1} of L-citrulline with malate for 16 days resulted in a significant increase (34%) in the rate of oxidative ATP production during exercise, and a 20% increase in the rate of phosphocreatine recovery after exercise.[60] However, there is some criticism around this research, because it used a very simple design without a placebo group or a blind condition. More recently, two studies conducted by the same research group showed an increase in plasma NO metabolites in well trained endurance athletes after a cycling competition; these athletes were supplemented with only one dose of L-citrulline with malate (6 g) 2 hours before exercise.[58,61] In addition, an increase in plasma arginine availability was linked with substrate for NO synthesis, as well as polymorphonuclear neutrophils (PMNs).[58] PMNs play an important role in the defense against infections, the inflammatory response, and muscle repair and regeneration.[84,85] Unfortunately, these findings were unable to be associated with variables of exercise performance because of the characteristics of the study design. Many factors, such as strategy, environmental conditions, nutrition, drafting and breakdown of material, can affect the results during field sport events, limiting the use of these data to assess the association between dietary supplement and performance. Another recent study by Pérez-Guisado and Jakeman[53] showed that a single dose of L-citrulline with malate (8 g) increased work capacity by an average of 19%, measured as the number of repetitions

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performed until exhaustion during a flat barbell bench-press test at 80% of 1-RM. However, this finding cannot be related to NO delivery because plasma NO markers were not determined in this study.[53]

Taking all this overview together, it is evident that there is a lack of data linking an increase in exercise performance to an increase in NO production derived from L-citrulline supplementation (tables I and II). Performance enhancement reported by L-citrulline in combination with malate could be explained by the interaction of these molecules in other metabolic pathways independent of NO production. For example, L-citrulline increases levels of plasma L-arginine indirectly; it could also enhance the synthesis of creatine, since it has been reported that L-arginine supplementation stimulates an increase in intramuscular creatine concentration.[74] Therefore, this mechanism may improve the response to anaerobic exercise. In addition, malate may be involved in the beneficial effects on energy production because it is an intermediate of TCA.[59,86] It has been suggested that hyperactivation of aerobic ATP production coupled to a reduction in anaerobic energy supply, may contribute to the reduction in fatigue sensation reported by the subjects.[87]

In short, the conclusions that we can extract from the studies using L-citrulline as a dietary supplementation in sport are as follows:

- Dietary supplementation with L-citrulline alone does not improve exercise performance.
- Addition of malate to dietary L-citrulline supplements may increase levels of NO metabolites.
- However, this response has not been related to an improvement in athletic performance.

3. Synthesis of NO from the NOS-Independent Pathway

The NOS-independent pathway is a novel pathway that was discovered by two independent research groups during the 1990s.[2,3] Nitrate and nitrite are the main precursors for NO synthesis in this alternative system. Interestingly, the NOS-dependent pathway is O_2 dependent; whereas the nitrate/nitrite-NO pathway is gradually activated as O_2 tension falls[88] (figure 1).

3.1 Nitrate and Nitrite: Sources and Metabolism

The main providers of nitrate in the diet of humans are vegetables such as lettuce, spinach or beetroot.[89] Drinking water can also contain considerable amounts of nitrate. It has been estimated that nitrate consumption derived from food and beverages is on average 100–150 $mg \bullet day^{-1}$ in adults.[90] However, the amount of nitrate in food has been regulated for a long time and there is currently an acceptable daily intake (ADI) for humans of 5 mg sodium nitrate or 3.7 mg nitrate $\bullet\ kg^{-1}$ of body weight, which equals 222 mg for a 60 kg adult. This is due to the fact that nitrate has been considered a carcinogenic substance and a toxic residue in our food and water. The supposed carcinogenic mechanism is the nitrite–dependent formation of nitrosating agents, which can react with dietary amines, forming nitrosamines, substances with known carcinogenic properties.[91] However, despite extensive research, no causal link between dietary nitrate intake and gastric cancer in humans has been found.[92]

Apart from the diet, nitrate and nitrite is generated endogenously in our bodies. The NO generated by L-arginine and NOS enzymes is oxidized in the blood and tissues to form nitrate and nitrite.[4] Thus, the NOS-dependent pathway significantly contributes to the overall nitrate and nitrite production, which indicates an active recycling pathway for generating NO in the human body. The normal plasma level of nitrate is within the 20–40 μM range, while the nitrite level is substantially lower (50–1000 nM), although many factors such as training and diet can modify these levels.[93]

Nitrate circulating in plasma distributes to the tissues and has a half life of approximately 5 hours. By not yet fully defined mechanisms, circulating nitrate is actively taken up by the salivary glands and concentrated in the saliva (10- to 20-fold higher than in the blood).[94] In the oral cavity, facultative anaerobic bacteria on the surface of the tongue reduces nitrate to nitrite by the action of nitrate reductase enzymes.[95] In the absence of O_2, these bacteria use nitrate as an alternative electron acceptor to gain ATP. When

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swallowed, one part of nitrite in the saliva is metabolized to NO locally in the acidic environment of the stomach, but the other part of swallowed nitrite is absorbed intact to increase circulating plasma nitrite.[93] Such nitrite can be converted to NO and other bioactive nitrogen oxides in the blood and tissues under appropriate physiological conditions.[3] These findings demonstrate that a complete reverse pathway (nitrate–nitrite–NO) exists in mammals.

3.1.1 Ergogenic Effect of Sodium Nitrate Supplementation

This alternative pathway of NO generation has not gone unnoticed in exercise physiology. Currently, four studies have assessed the effect of dietary nitrate supplementation in the form of sodium nitrate.[26,62-64] The first was carried out by Larsen et al.,[62] which showed that the ingestion of sodium nitrate ($0.1\,mmol \bullet kg^{-1} \times 3$ days) reduced $\dot{V}O_2$ ($\sim 160\,mL \bullet min^{-1}$) during work rates at mean intensities of 40–80% of $\dot{V}O_{2peak}$ performed on a cycle ergometer. Gross efficiency, defined as the ratio of mechanical work output to the metabolic energy input, was also significantly improved (~0.4%). This highly surprising effect occurred without changes in other cardiorespiratory parameters (ventilation, carbon dioxide production, heart rate and respiratory exchange ratio) or lactate concentration, which suggests that energy production became more efficient after dietary nitrate consumption. Interestingly, in the second study by Larsen et al.,[63] it was reported that $\dot{V}O_2$ at maximal intensity of exercise ($\dot{V}O_{2peak}$) was also significantly reduced ($\sim 100\,mL \bullet min^{-1}$) after nitrate supplementation ($0.1\,mmol \bullet kg^{-1} \times 2$ days). Despite this decrease in $\dot{V}O_{2peak}$, exercise performance measured until time to exhaustion during an incremental exercise test did not decrease compared with placebo (nitrate: 564 ± 30 seconds; placebo: 524 ± 31 seconds; $p > 0.05$). To explain this physiological response during endurance exercise, it was suggested that nitrate and nitrite modulated mitochondrial respiration via NO synthesis, since both studies showed a significant increase in plasma NO metabolites (nitrate and nitrite) after nitrate treatment.[62,63] This hypothesis was investigated by the same research group in an interesting recent study.[64] They reported that human mitochondrial efficiency, measured *in vitro* as the amount of O_2 consumed per ATP produced, termed P/O ratio, was significantly improved after sodium nitrate ingestion, compared with placebo using a similar amount of supplementation as in previous studies ($0.1\,mmol \bullet kg^{-1} \times 3$ days).[64] Nevertheless, despite these interesting findings, these studies did not report an enhancement in specific parameters of sports performance such as power output, time to exhaustion or total work performed.

While all the above studies assessed moderately trained subjects, one recent study by another independent research group assessed the effect of nitrate supplementation in well trained endurance athletes.[26] Following acute supplementation of sodium nitrate ($10\,mg \bullet kg^{-1}$ of body mass) 3 hours before exercise, 11 trained cyclists and triathletes completed a cycle ergometer test performing four intermittent workloads at submaximal intensities (between 2 and $3.5\,W \bullet kg^{-1}$ of body mass) and one continuous incremental test until volitional exhaustion.[26] Interestingly, this study showed that plasma nitrate levels increased at the same level after only one dose of nitrate ingestion ($10\,mg \bullet kg^{-1}$ of body mass) compared with 2-days' supplementation ($8.5\,mg \bullet kg^{-1}$ of body mass $\bullet\, day^{-1}$).[63] Furthermore, results of exercise tests showed that, contrary to previous studies,[62,63] $\dot{V}O_2$ at low to moderate intensities and increase in gross efficiency were not improved. However, in agreement with Larsen et al.,[62,63] at maximal intensity of exercise, the $\dot{V}O_{2peak}$ was significantly reduced ($\sim 180\,mL \bullet min^{-1}$) without a decrease in time to exhaustion after nitrate supplementation.

3.1.2 Ergogenic Effect of Nitrate Supplementation in the Form of Beetroot Juice

Five studies by the same research group have used dietary nitrate supplementation in the form of beetroot juice to assess the effect on human performance.[19-23] Interestingly, to isolate the effects of dietary nitrate from the other potentially active ingredients found in beetroot juice (betaine, quercetin and resveratrol), a process was developed to selectively remove the nitrate from beetroot juice using a commercially available resin.[22] In the first of these studies, Bailey et al.[19] showed a significant en-

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hancement of $\dot{V}O_2$ kinetics after supplementation with 500 mL • day^{-1} of nitrate-rich beetroot juice (468 mg of sodium nitrate • day^{-1}) for 6 days. During low- to moderate-intensity exercise (cycle ergometer exercise), there was a 19% reduction in the amplitude of the pulmonary response. In addition, it was shown that the $\dot{V}O_2$-slow component was reduced (~23%) and the time to exhaustion during an incremental cycle ergometer test was extended (~14%) after beetroot juice supplementation compared with placebo.[19] In an attempt to extend these findings to other forms of exercise (walking and running on a treadmill), the same research group performed another study using the same protocol of beetroot juice ingestion (500 mL • day^{-1} equivalent to 527 mg of sodium nitrate × 6 days).[22] This study concluded that beetroot supplementation induced similar changes in respiratory response in a treadmill exercise compared with the previous data in a cycle ergometer test.[19]

However, the effects of beetroot juice derived from nitrate seem to be fast, and acute ingestion of food rich in nitrate can affect the cardiovascular response in a few hours.[96] This fact was analysed in the study of Vanhatalo et al.[21] In this research, subjects ingested only one dose of beetroot juice (500 mL equivalent to 434 mg of sodium nitrate) 2.5 hours before a cycle ergometer test that included two moderate workloads at 90% of the gas exchange threshold followed by a ramp test. Moreover, subjects performed the same test after 5 and 15 days of beetroot juice ingestion and placebo. The steady-state $\dot{V}O_2$ during moderate-intensity exercise was significantly reduced 2.5 hours after supplementation and remained low on day 5 and 15 compared with placebo. However, contrary to the previous studies, the gas exchange threshold, maximal $\dot{V}O_2$ ($\dot{V}O_{2max}$) and peak power output were not affected 2.5 hours post-ingestion or after 5 days of supplementation. Surprisingly, these parameters showed a significant increase (peak power: ~3%; $\dot{V}O_{2max}$: ~4%) after 15 days of beetroot juice ingestion. However, several factors, such as training or resting conditions, as well as diet (subjects did not follow a nitrate-restricted diet at any time during the study period) could be the reason for these changes after 15 days of beetroot juice ingestion. Furthermore, in another very recent study by Lansley et al.,[23] following the same protocol of supplementation (500 mL of beetroot juice equivalent to 527 mg of sodium nitrate 2.5 hours before exercise), a significant improvement of average power output (5%) and mean completion time (2.8%) was indicated during 4 and 16.1 km cycle ergometer time trials compared with placebo.

To explain all these findings derived from beetroot juice ingestion, Bailey et al.[20] suggested that the nitrate content of beetroot juice could play an important role in the reduction of ATP turnover in contracting myocytes. With the utilization of ^{31}P-MRS, these authors reported that the decrease of O_2 cost at moderate and high intensities after beetroot ingestion (500 mL • day^{-1} equivalent to 468 mg of sodium nitrate × 6 days) was accompanied by a reduction in muscle phosphocreatine of a similar magnitude.[20] From this viewpoint, one of the most costly energy processes during skeletal muscle contraction is sarcoplasmic reticulum calcium pumping, which may account for up to 50% of the total ATP turnover.[97] There is evidence that small elevations of NO improves muscle metabolism, preventing excess calcium release and subsequently modulates the ATP cost of force production.[98] Interestingly, in beetroot juice studies, plasma nitrite levels measured as NO markers showed a significant increase after beetroot juice ingestion. Therefore, this is another alternative metabolic pathway to mitochondrial respiration indicated by Larsen et al.,[64] which may explain the reduction of O_2 demands during exercise derived from ingestion of food rich in nitrate.

Nevertheless, in all studies involving beetroot juice supplementation, moderately trained but not well trained subjects participated. In only one study that evaluated nitrate supplementation (sodium nitrate) in well trained endurance athletes, no reduction of O_2 consumption was found, or gross efficiency at low to moderate intensities of exercise either.[26] This study concluded that at low to moderate intensities of exercise, dietary nitrate supplementation could have a low effect in well trained endurance athletes compared with moderately trained subjects. Further research is needed in

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highly trained athletes to assess the effect of sodium nitrate or beetroot juice supplementation on performance.

In conclusion, in the field of exercise physiology, studies indicate that nitrate supplementation could (i) be effective at enhancing exercise efficiency and tolerance to exercise in untrained or moderately-trained subjects; and (ii) have shown that there is a lack of data assessing the effect of nitrate supplementation in the form of sodium nitrate as well as beetroot juice in well trained athletes (tables I and II). Results derived from the only study that assessed well trained endurance athletes, concluded that sodium nitrate does not enhance efficiency at low to moderate intensities of exercise.

4. Other Components Related to NO Synthesis

4.1 Glycine Propionyl-L-Carnitine (GPLC)

Glycine propionyl-L-carnitine (GPLC) is a new United States Pharmacopeial Convention-grade dietary supplement that consists of a molecular bonded form of propionyl-L-carnitine and one of the carnitine precursor amino acids, glycine. This molecule has also been proposed to improve NO metabolism[65] via two mechanisms: first, it has been reported in animal studies that the protective action of GPLC is derived from its antioxidant action, which may prevent vessels from peroxidative damage.[99] In accordance with this fact, other authors have suggested that the lower release of reactive oxygen species could be linked with a decrease in NO breakdown.[100] Second, eNOS gene expression has been demonstrated to increase within cultured human endothelial cells following carnitine incubation.[101] Thus, it has also been hypothesized that GPLC could stimulate NO synthesis via eNOS expression.[102]

Separating the components of GPLC, glycine is considered a glucogenic amino acid, in that it helps to regulate blood glucose levels, and is also important in the formation of creatine. Interestingly, glycine has been shown to have its own independent vasodilatory effects in rats.[103] On the other, hand L-carnitine in combination with propionyl (propionyl-L-carnitine) is a pharmaceutical agent that has been examined primarily as a treatment in clinical populations with apparent muscle carnitine deficiencies.[104,105]

4.1.1 Ergogenic Effect of GPLC

Recent studies assessed the effect of GPLC as a NO donor in sport exercise with different conclusions. First, Bloomer et al.[65] showed an increase in plasma NO metabolites (nitrate/nitrite) in active males after GPLC supplementation ($4.5\,g \bullet day^{-1} \times 4$ weeks). These findings were confirmed in the second study by the same research group.[66] However, contrary results were found in the third study published by Bloomer et al.[11] They showed that an acute dose (4.5 g) of GPLC did not increase NO markers. This controversy was attributed to the fact that in the latter study,[11] a single dose of GPLC was provided prior to exercise, whereas in the first two studies, GPLC was administered for 4 and 8 weeks, respectively.[65,66] Nevertheless, an important limitation of the studies performed by Bloomer et al.[11,65,66] was the lack of evidence indicating some benefit of GPLC in exercise performance. Two recent studies assessed this issue showing different results. Smith et al.[67] showed that ingestion of $3\,g \bullet day^{-1}$ of GPLC for 8 weeks did not enhance peak power, mean power or total work during a 30-second Wingate test. In contrast, Jacobs et al.[54] indicated that only one dose of GPLC (4.5 g) 90 minutes before performing a test consisting of five 10-second Wingate cycle sprints separated by 1-minute of active recovery periods, significantly improved peak power (~5.2%) and reduced power decrement (~5.2%) through sprints, compared with placebo. In addition, lactate measures taken 14 minutes post-exercise were 16.2% ($p < 0.05$) lower with GPLC.[54] However, these findings were not linked to NO delivery.

In summary, current scientific evidence of GPLC supplementation indicates that (i) in healthy and moderately trained subjects, GPLC could induce a mild increase in plasma NO metabolites, although the mechanism behind this response has not been defined; and (ii) evidence seems to indicate that the ergogenic effect of GPLC could be very limited; only one study indicated

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benefits in exercise performance after GPLC supplementation.[54] However, this finding could not be related to NO production as a result of the absence of analysis of NO markers

4.2 2-(Nitrooxy) Ethyl 2-Amino-3-Methylbutanoate

Recently, a new molecule 2-(nitrooxy) ethyl 2-amino-3-methylbutanoate has been claimed to increase NO delivery in the body, with this being more efficient and effective than the traditional NO donors.[68] However, to the best of our knowledge, there is only one study that has assessed the acute effect of 2-(nitrooxy) ethyl 2-amino-3-methylbutanoate in plasma NO markers, measured as nitrate/nitrite in moderately trained resistance males.[68] This study concluded there was no effect on circulating nitrate and nitrite within 1 hour post-ingestion of two tablets (no data was given on dose).

5. Side Effects of NO Supplements

Dietary supplementation with L-arginine and L-citrulline is not lacking in side effects, with gastrointestinal disturbances such as nausea, vomiting or diarrhoea as the most common adverse effects.[106] However, there is great inter-individual variation in the tolerance of these amino acids; high doses (>9 g • day^{-1}) can increase the risk of gastrointestinal distress. It has been suggested that smaller, divided doses might lead to fewer side effects.[34] In addition, the pre-existing proabsorptive or prosecretory state of the intestine may be important. The combination of secretory state and the extra stimulus provided by an acute dose (>9 g • day^{-1}) of L-arginine and/or L-citrulline may overwhelm the reserve-absorption capacity of the colon.[106]

As has been indicated previously, the amount of inorganic nitrate in food and water has been strictly regulated because of their proposed role in the development of malignancies such as metahaemoglobinaemia and cancer;[107] however, this view is currently changing. It is now thought that the nitrate concentrations commonly encountered in food and water are unlikely to cause metahaemoglobinaemia.[108,109] Moreover, an effect of exogenous nitrite on cancer seems less likely, because large amounts of nitrite are formed endogenously. Fasting saliva contains ≈2 mg • L^{-1}, and after consumption of an amount of nitrate equivalent to 200 g of spinach, nitrite concentration in saliva may rise to as much as 72 mg • L^{-1}. This is much higher than the ADI of 4.2 mg of nitrite • day^{-1}. Interestingly, all the above studies assessing nitrate supplementation in sports performance used amounts that were possible to achieve with natural foods, such as beetroot juice or green leafy vegetables (lettuce, spinach).

Studies have not reported adverse effects related with GPLC and 2-(nitrooxy) ethyl 2-amino-3-methylbutanoate supplementation. Nevertheless, this lack of data does not mean that this supplement is completely safe. For instance, it is known that amounts larger than 2 g • day^{-1} of L-carnitine may induce slight gastrointestinal distress.[110,111]

6. Conclusion and Future Perspectives

The current available data indicate that L-arginine supplementation, mainly in combination with other components, may be effective in moderately trained or untrained subjects for enhancing cardiorespiratory adaptation and tolerance to endurance exercise,[24,25,45] although a relationship between these findings and NO synthesis has not been established. On the other hand, L-citrulline in combination with malate could be a more efficient way to elevate extracellular levels of L-arginine by itself and with plasma NO markers. However, despite these effects, there is a lack of data indicating an improvement in exercise performance after L-citrulline supplementation. Therefore, it seems that some of the benefits shown after L-arginine and L-citrulline supplementation could be derived from the other ingredients included in supplements, as well as other metabolic pathways, independently of NO synthesis, which these amino acids participate in. Alternatively, a NOS-independent pathway has been reported by nitrate and nitrite oxidation. There is evidence that nitrate supplementation reduces the O_2 cost of endurance exercise, increasing the efficiency of energy production. An explanation for this intriguing physiological response has been linked

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with an increase in mitochondrial efficiency,[64] as well as ATP turnover functions.[20]

While some of the benefits linked with NO donors have been shown in moderately trained subjects, in well trained athletes, scientific data show that the effect of these supplements is low. Thus, it seems that the training status is an important factor linked with the effectiveness of dietary NO donors. One reason to explain this fact may be attributable, in part, to the positive effect of exercise in the regulation of NO metabolism. While short-term training rapidly increases NO bioactivity, if training is maintained, the short-term functional adaptation is succeeded by NO-dependent structural changes, leading to arterial remodelling and structural normalization of shear.[112] This structural remodelling and consequent normalization of shear obviates the need for ongoing functional dilatation, including enhanced NO dilator system function. The conclusion that we can extract is that training performed by competitive athletes has a greater effect on improving the NO system compared with NO supplementation. This fact raises the intriguing possibility of a threshold effect – volume and intensity – for training and mechanisms associated with NO production. Further investigation is needed to elucidate where this limit of physical exercise lies.

Moreover, almost all studies analysing NO donors and exercise performance were carried out, mainly, in young male subjects. It is known that vascular function and NO availability is impaired with age; thus, other studies should be planned in order to assess the effect of NO supplements in healthy adults (>40 years). Additionally, almost all research has been focused on endurance performance and few data exist concerning the effect of NO supplements in the regulation of hypertrophy and stimulation of satellite cells. This point needs more attention, not only for sports performance, but also for muscle mass losses associated with age and convalescence periods after injuries. Finally, gender differences have not been analysed, although it is recognized that there are structural/morphological differences between adult males and females for many, if not all, organ systems, which may have a significant impact on physiological function.[113] The female reproductive system is highly sensitive to physiological stress, and reproductive abnormalities including delayed menarche, primary and secondary amenorrhoea and oligomenorrhoea occur in 6–79% of women engaged in athletic activity.[114] The prevalence of observed irregularities varies with athletic discipline and level of competition.[115] It has also been indicated that amenorrhoea is associated with altered endothelial function.[116] We consider this point needs further research to analyse the potential effects of NO supplementation and physical exercise, specifically in females.

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References

1. Schmidt HH, Nau H, Wittfoht W, et al. Arginine is a physiological precursor of endothelium-derived nitric oxide. Eur J Pharmacol 1988; 154 (2): 213-6
2. Benjamin N, O'Driscoll F, Dougall H, et al. Stomach NO synthesis [abstract]. Nature 1994; 368 (6471): 502
3. Lundberg JO, Weitzberg E, Lundberg JM, et al. Intragastric nitric oxide production in humans: measurements in expelled air. Gut 1994; 35 (11): 1543-6
4. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 1993; 329 (27): 2002-12
5. Petroczi A, Naughton DP. Potentially fatal new trend in performance enhancement: a cautionary note on nitrite. J Int Soc Sports Nutr 2010; 7 (1): 25
6. Shen W, Xu X, Ochoa M, et al. Role of nitric oxide in the regulation of oxygen consumption in conscious dogs. Circ Res 1994; 75 (6): 1086-95
7. Bloomer RJ. Nitric oxide supplements for sports. J Strength Cond Res 2010; 32 (2): 14-20
8. Schoch RD, Willoughby D, Greenwood M. The regulation and expression of the creatine transporter: a brief review of creatine supplementation in humans and animals. J Int Soc Sports Nutr 2006; 3: 60-6
9. Burke LM, Kiens B, Ivy JL. Carbohydrates and fat for training and recovery. J Sports Sci 2004; 22 (1): 15-30
10. Tipton KD, Ferrando AA. Improving muscle mass: response of muscle metabolism to exercise, nutrition and anabolic agents. Essays Biochem 2008; 44: 85-98
11. Bloomer RJ, Farney TM, Trepanowski JF, et al. Comparison of pre-workout nitric oxide stimulating dietary supplements on skeletal muscle oxygen saturation, blood nitrate/nitrite, lipid peroxidation, and upper body exercise

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performance in resistance trained men. J Int Soc Sports Nutr 2010; 7: 16

12. Koppo K, Taes YE, Pottier A, et al. Dietary arginine supplementation speeds pulmonary VO2 kinetics during cycle exercise. Med Sci Sports Exerc 2009; 41 (8): 1626-32
13. Fricke O, Baecker N, Heer M, et al. The effect of L-arginine administration on muscle force and power in postmenopausal women. Clin Physiol Funct Imaging 2008; 28 (5): 307-11
14. Olek RA, Ziemann E, Grzywacz T, et al. A single oral intake of arginine does not affect performance during repeated Wingate anaerobic test. J Sports Med Phys Fitness 2010; 50 (1): 52-6
15. Sunderland KL, Greer F, Morales J. VO_{2max} and ventilatory threshold of trained cyclists are not affected by 28-day l-arginine supplementation. J Strength Cond Res 2011; 25 (3): 833-7
16. Bescós R, Gonzalez Haro C, Pujol P, et al. Effects of dietary L-Arginine intake on cardiorespiratory and metabolic adaptation in athletes. Int J Sport Nutr Exerc Metab 2009; 19: 355-65
17. Tsai PH, Tang TK, Juang CL, et al. Effects of arginine supplementation on post-exercise metabolic responses. Chin J Physiol 2009; 52 (3): 136-42
18. Liu TH, Wu CL, Chiang CW, et al. No effect of short-term arginine supplementation on nitric oxide production, metabolism and performance in intermittent exercise in athletes. J Nutr Biochem 2008; 20 (6): 462-8
19. Bailey SJ, Winyard P, Vanhatalo A, et al. Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. J Appl Physiol 2010; 109 (5): 1394-403
20. Bailey SJ, Fulford J, Vanhatalo A, et al. Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. J Appl Physiol 2010; 109 (1): 135-48
21. Vanhatalo A, Bailey SJ, Blackwell JR, et al. Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. Am J Physiol Regul Integr Comp Physiol 2010; 299 (4): 1121-31
22. Lansley KE, Winyard PG, Fulford J, et al. Dietary nitrate supplementation reduces the O2 cost of walking and running: a placebo-controlled study. J Appl Physiol 2011; 110 (3): 591-600
23. Lansley KE, Winyard PG, Bailey SJ, et al. Acute dietary nitrate supplementation improves cycling time trial performance. Med Sci Sports Exerc 2011; 43 (6): 1125-31
24. Bailey SJ, Winyard PG, Vanhatalo A, et al. Acute L-arginine supplementation reduces the O2 cost of moderate-intensity exercise and enhances high-intensity exercise tolerance. J Appl Physiol 2010; 109 (5): 1394-403
25. Camic CL, Housh TJ, Zuniga JM, et al. Effects of arginine-based supplements on the physical working capacity at the fatigue threshold. J Strength Cond Res 2010; 24 (5): 1306-12
26. Bescos R, Rodriguez FA, Iglesias X, et al. Acute administration of inorganic nitrate reduces VO_{2peak} in endurance athletes. Med Sci Sports Exerc 2011; 43 (10): 1979-86
27. King DE, Mainous AG, Geesey ME. Variation in L-arginine intake follow demographics and lifestyle factors that may impact cardiovascular disease risk. Nutr Res 2008; 28 (1): 21-4
28. Boger RH, Bode-Boger SM. The clinical pharmacology of L-arginine. Annu Rev Pharmacol Toxicol 2001; 41: 79-99
29. Castillo L, Chapman TE, Sanchez M, et al. Plasma arginine and citrulline kinetics in adults given adequate and arginine-free diets. Proc Natl Acad Sci 1993; 90 (16): 7749-53
30. Boger RH. L-Arginine therapy in cardiovascular pathologies: beneficial or dangerous? Curr Opin Clin Nutr Metab Care 2008; 11 (1): 55-61
31. Balon TW, Nadler JL. Evidence that nitric oxide increases glucose transport in skeletal muscle. J Appl Physiol 1997; 82 (1): 359-63
32. Capanni C, Squarzoni S, Petrini S, et al. Increase of neuronal nitric oxide synthase in rat skeletal muscle during ageing. Biochem Biophys Res Commun 1998; 245 (1): 216-9
33. Shen W, Zhang X, Zhao G, et al. Nitric oxide production and NO synthase gene expression contribute to vascular regulation during exercise. Med Sci Sports Exerc 1995; 27 (8): 1125-34
34. Cynober L. Pharmacokinetics of arginine and related amino acids. J Nutr 2007; 137 (6): 1646S-9
35. McConell GK. Effects of L-arginine supplementation on exercise metabolism. Curr Opin Clin Nutr Metab Care 2007; 10 (1): 46-51
36. Sonksen PH. Insulin, growth hormone and sport. J Endocrinol 2001; 170 (1): 13-25
37. Gibney J, Healy ML, Sonksen PH. The growth hormone/insulin-like growth factor-I axis in exercise and sport. Endocr Rev 2007; 28 (6): 603-24
38. Jones AM, Poole DC. Oxygen uptake kinetics in Sport, Exercise and Medicine. New York: Routledge, 2005
39. McConell GK, Huynh NN, Lee-Young RS, et al. L-Arginine infusion increases glucose clearance during prolonged exercise in humans. Am J Physiol Endocrinol Metab 2006; 290 (1): E60-E6
40. Schaefer A, Piquard F, Geny B, et al. L-arginine reduces exercise-induced increase in plasma lactate and ammonia. Int J Sports Med 2002; 23 (6): 403-7
41. Bode-Boger SM, Boger RH, Galland A, et al. L-arginine-induced vasodilation in healthy humans: pharmacokinetic-pharmacodynamic relationship. Br J Clin Pharmacol 1998; 46 (5): 489-97
42. Mori M, Gotoh T. Regulation of nitric oxide production by arginine metabolic enzymes. Biochem Biophys Res Commun 2000; 275 (3): 715-9
43. Sureda A, Batle JM, Tauler P, et al. Vitamin C supplementation influences the antioxidant response and nitric oxide handling of erythrocytes and lymphocytes to diving apnea. Eur J Clin Nutr 2006; 60 (7): 838-46

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44. Chen S, Kim W, Henning SM, et al. Arginine and antioxidant supplement on performance in elderly male cyclists: a randomized controlled trial. J Int Soc Sports Nutr 2010; 7 (1): 13
45. Camic CL, Housh TJ, Mielke M, et al. The effects of 4 weeks of an arginine-based supplement on the gas exchange threshold and peak oxygen uptake. Appl Physiol Nutr Metab 2010; 35 (3): 286-93
46. Chin-Dusting JP, Alexander CT, Arnold PJ, et al. Effects of in vivo and in vitro L-arginine supplementation on healthy human vessels. J Cardiovasc Pharmacol 1996; 28 (1): 158-66
47. Adams MR, Forsyth CJ, Jessup W, et al. Oral L-arginine inhibits platelet aggregation but does not enhance endothelium-dependent dilation in healthy young men. J Am Coll Cardiol 1995; 26 (4): 1054-61
48. Campbell B, Roberts M, Kerksick C, et al. Pharmacokinetics, safety, and effects on exercise performance of L-arginine alpha-ketoglutarate in trained adult men. Nutrition 2006; 22 (9): 872-81
49. Buford BN, Koch AJ. Glycine-arginine-alpha-ketoisocaproic acid improves performance of repeated cycling sprints. Med Sci Sports Exerc 2004; 36 (4): 583-7
50. Stevens BR, Godfrey MD, Kaminski TW, et al. High-intensity dynamic human muscle performance enhanced by a metabolic intervention. Med Sci Sports Exerc 2000; 32 (12): 2102-8
51. Colombani PC, Bitzi R, Frey-Rindova P, et al. Chronic arginine aspartate supplementation in runners reduces total plasma amino acid level at rest and during a marathon run. Eur J Nutr 1999; 38 (6): 263-70
52. Abel T, Knechtle B, Perret C, et al. Influence of chronic supplementation of arginine aspartate in endurance athletes on performance and substrate metabolism - a randomized, double-blind, placebo-controlled study. Int J Sports Med 2005; 26 (5): 344-9
53. Perez-Guisado J, Jakeman PM. Citrulline malate enhances athletic anaerobic performance and relieves muscle soreness. J Strength Cond Res 2010; 24 (5): 1215-22
54. Jacobs PL, Goldstein ER, Blackburn W, et al. Glycine propionyl-L-carnitine produces enhanced anaerobic work capacity with reduced lactate accumulation in resistance trained males. J Int Soc Sports Nutr 2009; 6: 9
55. Eto B, Peres G, Le Moel G. Effects of an ingested glutamate arginine salt on ammonemia during and after long lasting cycling. Arch Int Physiol Biochim Biophys 1994; 102 (3): 161-2
56. Denis C, Dormois D, Linossier MT, et al. Effect of arginine aspartate on the exercise-induced hyperammoniemia in humans: a two periods cross-over trial. Arch Int Physiol Biochim Biophys 1991; 99 (1): 123-7
57. Burtscher M, Brunner F, Faulhaber M, et al. The prolonged intake of L-Arginine-L-Aspartate reduces blood lactate accumulation and oxygen consumption during submaximal exercise. J Sports Sci Med 2005; 4: 314-22
58. Sureda A, Cordova A, Ferrer MD, et al. Effects of L-citrulline oral supplementation on polymorphonuclear neutrophils oxidative burst and nitric oxide production after exercise. Free Radic Res 2009; 6: 1-8
59. Hickner RC, Tanner CJ, Evans CA, et al. L-citrulline reduces time to exhaustion and insulin response to a graded exercise test. Med Sci Sports Exerc 2006; 38 (4): 660-6
60. Bendahan D, Mattei JP, Ghattas B, et al. Citrulline/malate promotes aerobic energy production in human exercising muscle. Br J Sports Med 2002; 36 (4): 282-9
61. Sureda A, Cordova A, Ferrer MD, et al. L-Citrulline-malate influence over branched chain amino acid utilization during exercise. Eur J Appl Physiol 2010; 110 (2): 341-51
62. Larsen F, Weitzberg E, Lundberg JO, et al. Effects of dietary nitrate on oxygen cost during exercise. Acta Physiol 2007; 191: 55-66
63. Larsen FJ, Weitzberg E, Lundberg JO, et al. Dietary nitrate reduces maximal oxygen consumption while maintaining work performance at maximal exercise. Free Radic Biol Med 2010; 48 (2): 342-7
64. Larsen FJ, Schiffer TA, Borniquel S, et al. Dietary inorganic nitrate improves mitochondrial efficiency in humans. Cell Metab 2011; 13 (2): 149-59
65. Bloomer RJ, Smith WA, Fisher-Wellman KH. Glycine propionyl-L-carnitine increases plasma nitrate/nitrite in resistance trained men. J Int Soc Sports Nutr 2007; 4: 22
66. Bloomer RJ, Tschume LC, Smith WA. Glycine propionyl-L-carnitine modulates lipid peroxidation and nitric oxide in human subjects. Int J Vitam Nutr Res 2009; 79 (3): 131-41
67. Smith WA, Fry AC, Tschume LC, et al. Effect of glycine propionyl-L-carnitine on aerobic and anaerobic exercise performance. Int J Sport Nutr Metab 2008; 18 (1): 19-36
68. Bloomer RJ, Williams SA, Canale RE, et al. Acute effect of nitric oxide supplement on blood nitrate/nitrite and hemodynamic variables in resistance trained men. J Strength Cond Res 2010; 24 (10): 2587-92
69. Jones AM, Carter H. The effect of endurance training on parameters of aerobic fitness. Sports Med 2000; 29 (6): 373-86
70. Hauk JM, Hosey RG. Nitric oxide therapy: fact or fiction? Curr Sports Med Rep 2006; 5 (4): 199-202
71. Wu G, Morris M. Arginine metabolism: nitric oxide and beyond. Biochem J 1998; 336: 1-17
72. Wu G, Bazer FW, Davis TA, et al. Arginine metabolism and nutrition in growth, health and disease. Amino Acids 2009; 37 (1): 153-68
73. Buford T, Kreider R, Stout J, et al. International society of sports nutrition position stand: creatine supplementation and exercise. J Int Soc Sports Nutr 2007; 4: 6
74. Minuskin ML, Lavine ME, Ulman EA, et al. Nitrogen retention, muscle creatine and orotic acid excretion in traumatized rats fed arginine and glycine enriched diets. J Nutr 1981; 111 (7): 1265-74
75. Alvares TS, Meirelles CM, Bhambhani YN, et al. L-arginine as a potential ergogenic aid in healthy subjects. Sports Med 2011; 41 (3): 233-48
76. Rimando AM, Perkins-Veazie PM. Determination of citrulline in watermelon rind. J Chromatogr A 2005; 1078 (1-2): 196-200

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77. Curis E, Nicolis I, Moinard C, et al. Almost all about citrulline in mammals. Amino Acids 2005; 29 (3): 177-205
78. Rabier D, Kamoun P. Metabolism of citrulline in man. Amino Acids 1995; 9 (4): 299-316
79. Mehta S, Stewart DJ, Levy RD. The hypotensive effect of L-arginine is associated with increased expired nitric oxide in humans. Chest 1996; 109 (6): 1550-5
80. Romero MJ, Platt DH, Caldwell RB, et al. Therapeutic use of citrulline in cardiovascular disease. Cardiovasc Drug Rev 2006; 24 (3-4): 275-90
81. Rouge C, Des Robert C, Robins A, et al. Manipulation of citrulline availability in humans. Am J Physiol Gastrointest Liver Physiol 2007; 293 (5): 1061-7
82. Hartman WJ, Torre PM, Prior RL. Dietary citrulline but not ornithine counteracts dietary arginine deficiency in rats by increasing splanchnic release of citrulline. J Nutr 1994; 124 (10): 1950-60
83. van de Poll MC, Soeters PB, Deutz NE, et al. Renal metabolism of amino acids: its role in interorgan amino acid exchange. Am J Clin Nutr 2004; 79 (2): 185-97
84. Sureda A, Tauler P, Aguiló A, et al. Antioxidant supplementation influences the neutrophil tocopherol associated protein expression, but not the inflammatory response to exercise. Cent Eur J Biol 2007; 2 (1): 56-70
85. Tidball JG. Inflammatory processes in muscle injury and repair. Am J Phys Reg Integr Comp Physiol 2005; 288 (2): R345-R53
86. Wagenmakers AJ. Muscle amino acid metabolism at rest and during exercise: role in human physiology and metabolism. Exerc Sport Sci Rev 1998; 26: 287-314
87. Daussin FN, Zoll J, Ponsot E, et al. Training at high exercise intensity promotes qualitative adaptations of mitochondrial function in human skeletal muscle. J Appl Physiol 2008; 104 (5): 1436-41
88. Lundberg JO, Weitzberg E, Gladwin M. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov 2008; 7 (2): 156-67
89. Lundberg JO, Weitzberg E. NO generation from inorganic nitrate and nitrite: Role in physiology, nutrition and therapeutics. Arch Pharm Res 2009; 32 (8): 1119-26
90. Zeegers MP, Selen RFM, Kleinjans JCS, et al. Nitrate intake does not influence bladder cancer risk: the Netherlands cohort study. Environ Health Perspect 2006; 114 (10): 1527-31
91. Tricker AR, Preussmann R. Carcinogenic N-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential. Mutat Res 1991; 259 (3-4): 277-89
92. McKnight GM, Duncan CW, Leifert C, et al. Dietary nitrate in man: friend or foe? Br J Nutr 1999; 81 (5): 349-58
93. Lundberg JO, Giovoni M. Inorganic Nitrate is a possible source for systemic generation of nitric oxide. Free Rad Bio Med 2004; 37 (3): 395-400
94. Tannenbaum SR, Weisman M, Fett D. The effect of nitrate intake on nitrite formation in human saliva. Food Cosmet Toxicol 1976; 14 (6): 549-52
95. Duncan C, Dougall H, Johnston P, et al. Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. Nat Med 1995; 1 (6): 546-51
96. Webb AJ, Loukogeorgakis S, Okorie M, et al. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. Hypertension 2008; 51: 784-90
97. Bergstrom M, Hultman E. Energy cost and fatigue during intermittent electrical stimulation of human skeletal muscle. J Appl Physiol 1988; 65 (4): 1500-5
98. Reid MB. Role of nitric oxide in skeletal muscle: synthesis, distribution and functional importance. Acta Physiol Scand 1998; 162 (3): 401-9
99. Bertelli A, Conte A, Ronca G, et al. Protective effect of propionyl carnitine against peroxidative damage to arterial endothelium membranes. Int J Tissue React 1991;13 (1): 41-3
100. Bueno R, Alvarez de Sotomayor M, Perez-Guerrero C, et al. L-carnitine and propionyl-L-carnitine improve endothelial dysfunction in spontaneously hypertensive rats: different participation of NO and COX-products. Life Sci 2005; 77 (17): 2082-97
101. Calo LA, Pagnin E, Davis PA, et al. Antioxidant effect of L-carnitine and its short chain esters: relevance for the protection from oxidative stress related cardiovascular damage. Int J Cardiol 2006; 107 (1): 54-60
102. Bloomer RJ, Fisher-Wellman KH, Tucker PS. Effect of oral acetyl L-carnitine arginate on resting and postprandial blood biomarkers in pre-diabetics. Nutr Metab 2009; 6: 25
103. Podoprigora GI, Nartsissov YR, Aleksandrov PN. Effect of glycine on microcirculation in pial vessels of rat brain. Bull Exp Biol Med 2005; 139 (6): 675-7
104. Anand I, Chandrashekhan Y, De Giuli F, et al. Acute and chronic effects of propionyl-L-carnitine on the hemodynamics, exercise capacity, and hormones in patients with congestive heart failure. Cardiovasc Drugs Ther 1998; 12 (3): 291-9
105. Andreozzi GM. Propionyl l-carnitine: intermittent claudication and peripheral arterial disease. Exp Opin Pharmacol 2009; 10 (16): 2697-707
106. Grimble GK. Adverse gastrointestinal effects of arginine and related amino acids. J Nutr 2007; 137 (6): 1693S-701
107. Rogers MA, Vaughan TL, Davis S, et al. Consumption of nitrate, nitrite, and nitrosodimethylamine and the risk of upper aerodigestive tract cancer. Cancer Epidemiol Biomarkers Prev 1995; 4 (1): 29-36
108. Katan MB. Nitrate in foods: harmful or healthy? Am J Clin Nutr 2009; 90 (1): 11-2
109. Gilchrist M, Winyard PG, Benjamin N. Dietary nitrate–good or bad? Nitric Oxide 2010; 22 (2): 104-9
110. Hathcock JN, Shao A. Risk assessment for carnitine. Regul Toxicol Pharmacol 2006 Oct; 46 (1): 23-8
111. Karlic H, Lohninger A. Supplementation of L-carnitine in athletes: does it make sense? Nutrition 2004; 20 (7-8): 709-15
112. Maiorana A, O'Driscoll G, Taylor R, et al. Exercise and the nitric oxide vasodilator system. Sports Med 2003; 33 (14): 1013-35
113. Blair ML. Sex-based differences in physiology: what should we teach in the medical curriculum? Adv Physiol Educ 2007; 31 (1): 23-5

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114. De Souza MJ, Toombs RJ, Scheid JL, et al. High prevalence of subtle and severe menstrual disturbances in exercising women: confirmation using daily hormone measures. Hum Reprod 2010; 25 (2): 491-503
115. Warren MP, Perlroth NE. The effects of intense exercise on the female reproductive system. J Endocrinol 2001; 170 (1): 3-11
116. Zeni Hoch A, Dempsey RL, Carrera GF, et al. Is there an association between athletic amenorrhea and endothelial cell dysfunction? Med Sci Sports Exerc 2003; 35 (3): 377-83

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CONFERENCE PRESENTATION

I

55th Annual Meeting of the American College of Sports Medicine. Indianapolis (USA), 2008. Abstract published in Med Sci Sports Exerc, 2008; 40 (5): S401.

Effect of L-Arginine Enriched Diet on Running Economy of Elite Tennis Players

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High plasmatic L-Arginine levels increase Nitric Oxid (NO) production. It has been suggested that NO increase could elicit an increase on human effort running economy during physical exercise.

PURPOSE: To determine whether L-Arginine supplementation modifies the different parameters related with running economy during a running test on a treadmill.

METHODS: Nine elite male tennis players (mean ± DS; age: 18.2 ± 3.7 years) ingested 3 different diets along 3 consecutive days with a wash-out period of 4 days in between training sessions. Treatment 1 (T_1) Control: 5.5 ± 0.3 gr·day^{-1} of L-Arginine; treatment 2 (T_2): 9.0 ± 1.1 g·day^{-1} of L-Arginine; and treatment 3 (T_3): 20.5 ± 0.3 gr·day^{-1} of L-Arginine. The day after every treatment, plasma and urine nitrate levels from all subjects were determined. Subjects also performed an incremental submaximal test (initial speed of 10 km·h^{-1}, load increase of 1 km·h^{-1}·4 min^{-1} inishing the test one stage after having achieved the anaerobic threshold). Throughout the test, VO_2 and HR were monitored, and at the end of each stage a [La^-] sample was taken. To compare the different physiological parameters measured a repeated measures ANOVA was used. The significant level was of $P < 0.05$.

RESULTS: No significant differences were observed in any of the cardiorespiratory and metabolic parameters between T_1, T_2 and T_3, neither in urine (T_1: 0.37 ± 0.12 μmol·L^{-1}; T_2: 0.37 ± 0.10 μmol·L^{-1}; T_3: 0.37 ± 0.06 μmol·L^{-1}) nor plasma (T_1: 30.4 ± 5.8 μmol·L^{-1}; T_2: 31.9 ± 7.2 μmol·L^{-1}; T_3: 31.3 ± 7.9 μmol·L^{-1}) nitrate levels.

CONCLUSION: The oral supplementation of 20 gr·day^{-1} of L-Arginine during the days before a physical effort does not elicit improvements in the running economy measured by means of VO_2 , [La^-] and HR.

CONFERENCE PRESENTATION

II

17th Annual Meeting of the Society for Free Radical Biology and Medicine. Orlando (USA), 2010. Abstract published in Free Rad Biol Med, 2010; 49 (1): S109.

Acute administration of inorganic nitrate reduces VO_{2peak} in endurance athletes

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Nitrate (NO_3) and nitrite (NO_2) have been known as undesired molecules in the food chain with potentially dangerous effects, or as inert oxidative end products of endogenous nitric oxide (NO) metabolism. However, from research performed over the past decade, it is now apparent that NO_3 and NO_2 are physiologically recycled in blood and tissues to form NO and other bioactive nitrogen oxides.

In a randomized, double-blind crossover study we assessed the effect of a single oral dose of inorganic NO_3 on the cardio-respiratory and metabolic response. Moreover, we investigated the influence of NO_3 supplement on plasma levels of NO_3 and NO_2 over time. In this investigation 11 endurance athletes participated. They ingested 10 $mg \cdot kg^{-1}$ of NO_3 or placebo three hours before cycling exercise at different intensities. Results showed that plasma NO_3 and nitrite (NO_2) increased 86.9 ± 8.4 % and 12.3 ± 3.2 % ($P < 0.05$) three hours after supplementation at rest. At low-moderate intensities of exercise NO_3 supplementation had no affect on the cardio-respiratory and metabolic parameters, while, at higher intensities VO_2 was significantly reduced (NO_3: 4.64 ± 0.35 $L \cdot min^{-1}$; 4.82 ± 0.33; placebo: $P = 0.010$). This reduction of VO_2 occurred without changes in the time to exhaustion (NO_3: 416 ± 32; placebo: 409 ± 27 seconds) and maximal power (NO_3: 416 ± 29; placebo: 410 ± 28 W). In conclusion, these findings suggest that acute dose of NO_3 could play a important role in control of cellular respiration during conditions of oxygen limitation such as those during strenuous exercise.