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Nitric Oxide/Arginine: Is Cardiovascular Modulation Effects in Athletes Supplementation?

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Abstract

Dietary nitrates and L-Arginine have been increasingly recognized to play a promising role as sport dietary supplementation, getting more and more popular as ergogenic aids, namely, substances used with regard to performance enhancement. Inorganic nitrate (NO3-) is abundant in numerous foodstuffs and is convertible into nitrite (NO2-) following ingestion. Nitrite, in turn, can be metabolized into nitric oxide (NO), one of the most widespread signaling molecules, taking part in virtually every cellular and organic functions, most notably blood flow regulation and vasodilation, mitochondrial respiration, platelet function and metabolic homeostasis.

L-Arginine is a conditionally essential amino acid that has been target of considerable attention as the main precursor of NO, revealing other promising potential effects on growth hormone (GH) release, promotion of creatine synthesis, all leading to possible exercise tolerance and muscle efficiency benefits, impairment of O_2 uptake (cost of submaximal exercise) and rise in workout duration before fatigue. Accordingly, the purpose of this review is to provide an overview on the accumulating evidence concerning not only the importance of NO and its associated precursors in exercise and sports performance, but also the inherent cardiovascular modulation during workout, as well as ascertain the possible benefits and hazards, never despising the crucial role ascribed to sports nutrition professionals.

Keywords: Nitric oxide; L-Arginine; Nitrates (nitrate/nitrite); Sports/dietary supplementation; Ergogenic aids; Exercise tolerance/performance; Muscle efficiency; O_2 uptake; Fatigue; Blood pressure/flow; Cardiovascular modulation; Mitochondrial respiration; Growth hormone

Introduction

Elite athletes are inevitably engaged in multiple strenuous exercise training sessions, continuously striving to improve both workout capacity and performance [1,2]. Thence, this makes them quite prone to the adverse consequences of high-intensity training, such as high rates of protein catabolism, pro-inflammatory profile, muscle damage, soreness, chronic oxidative stress and immune suppression [3]. Despite the undeniable importance of a balanced and highly nutritive diet, there is growing evidence that some supplements can contribute to optimal nutrition [3].

A nutritional supplement that enhances exercise capacity and is consumed before, during or after training, is known to have an ergogenic effect [2,4]. Ergogenic aids are substances that enhance energy production, use or recovery, providing athletes with a competitive advantage [5].

Athletes are uniquely vulnerable to advertisements regarding dietary supplements, sports nutrition foods and ergogenic aids, which are portrayed as having many different benefits, but are not as well studied as prescription or over-the-counter drugs, often coming into scrutiny by legal authorities for their claimed benefits and safety concerns [1,2,6]. It is unconditionally true that many athletes feel pressured to use supplements to maintain a competitive advantage over their supplement using-peers, so, physicians should give careful advice in order to prevent serious health risks [7].

Whereas approximately 50% of general population report taking some sort of dietary supplements, the percentage of athletes through supplementation ranges from seventy-six to one hundred, depending on the type of sport in question [7]. In what concerns trained subjects, 45% seem to seek for physicians' approval/recommendation, before beginning any kind of supplementation. In fact, primary care physicians working with athletes should always inquire about drugs/supplements consumption and a thorough knowledge on this topic may help the establishment of rapport with these athletes [6]. Therefore, physicians usually evaluate these products by focusing mainly in four variables: mechanism of action/physiologic basis, available research, adverse effects and legality [7]. It is hoped that the more knowledge and awareness concerning ergogenic substances by physicians, the better education and health care for athletic population [6].

Historically, many different substances have been used as ergogenic aids in humans [6]. The primary aim of ergogenic aids consumption is to improve exercise performance, muscle strength and delay the onset of fatigue [2]. Anabolic steroids (testosterone derivatives), amino acids and their derivatives, particularly, whey protein, carbohydrates (frequently packed with proteins, constituting what we call "gainers"), amphetamines, antioxidants, eritropoietin, multivitamins, magnesium, caffeine, among others, represent the principal choices made with regard to improving exercise performance, whether isolated or in different associations [7].

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Recently, nitric oxide (NO) has emerged as a promising ergogenic aid by supplementation, leading to a revolution in pharmacological and physiological knowledge [8]. Although NO is, *per se*, unstable, rapidly exerting its benefits due to its high affinity to various heme-containing proteins, determining several actions and regulating its biological short half-life, it has no role as a supplement, requiring the ingestion of other compounds that influence internal NO production systems [8,9]. Despite constituting only one of many vasodilator substances produced by the endothelium, it is worthwhile emphasizing the emerging importance of mitochondrial actions interrelated to NO, including mitochondrial respiration, as well as its essential role in vascular and metabolic control [8,10,11].

The purpose of this article is to provide an overview of the accumulating research evidence concerning the role played by NO and its forming compounds in the physiological responses to exercise and sports performance. Taking into account the myriad of organic processes in which NO is involved, particularly, at cardiovascular level, this article will also contemplate cardiovascular repercussions of NO enhancement during workout, as well as explore the possible hazards or ergogenic properties inherent to nitrates/arginine/NO supplementation.

Pathways of No Production and Its Physiological Importance

NO, a labile and short half-life molecule present in many biological fluids, is one of the most widespread signaling molecules, participating in virtually every cellular and organic function in body [12,13]. It is a critical regulator of vascular homeostasis and takes part in myointimal hyperplasia reduction [10,14,15]. Furthermore, this lipid soluble gas is synthesized at several locations in the body, being responsible for important functions, such as neuroprotection, synaptic plasticity, regulation of blood flow (more specifically, relaxation and contraction of vascular smooth muscle cells), muscle contractility, glucose and calcium homeostasis, mitochondrial O_2 respiration and biogenesis (through cGMP pathways, angiogenesis, thrombosis, among others, which are important determinants of the impact in muscle fatigue and performance, explaining an expected improvement in muscle efficiency [8,12,16-18].

On the other hand, NO rapidly gets oxidized to nitrate by oxyhemoglobin and to nitrite in plasma by copper-storage plasma protein ceruloplasmin [12]. Being one of the smallest and simplest biologically active molecules with enormous versatility and importance in the body, NO is a potent endogenous vasodilator responsible for increasing blood perfusion via shear stress and contributes to changes in blood flow during dynamic exercise and post-exercise recovery [10,12].

In addition to this, NO inhibits leukocyte adhesion, platelet aggregation, superoxide generation, expression of vascular cell adhesion molecules and monocyte chemotactic peptides, smooth muscle proliferation and endothelin-1 (a vasoconstrictor) release [19]. There seems to be a role for NO in hepatic gluconeogenesis regulation, once high levels of NO are expected to increase the release of glucose from liver under conditions of inducible NO synthase (iNOS) induction [20]. NO also reduces glycolysis in hepatocytes by decreasing levels of some key enzymes, like glucokinase and glyceraldehyde dehydrogenase [20]. Moreover, NO is known to be a high affinity inhibitor of cytochrome oxidase activity, thus, by reducing "slippage" of mitochondrial proton pumps, it might enhance oxidative phosphorylation efficiency, as well as modulate muscle contractile function [21].

NO synthesis is known to occur through at least two physiological pathways: NO synthase (NOS) dependent and independent [22]. Nitric oxide can be generated solely through L-Arginine oxidation (in which L-Arginine is the main precursor), being oxidized to NO and L-Citrulline in equimolar concentrations, in a reaction catalyzed by a family of NO synthase enzymes (NOS), resulting in the endogenous production of nitrate (NO_3^-) and nitrite (NO_2^-) [23]. This NOS-dependent pathway is dependent upon the availability of several substrates and cofactors, such as nicotinamide adenine dinucleotide phosphate, flavine mononucleotide, tetrahydrobiopterin – BH_4, flavin adenine dinucleotide (FAD), haem and calmodulin [24,25]. NOS activity is also dependent on the rate of electron transfer, L-Arginine and oxygen availability [24].

Three isoforms of NOS 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 Ca2+/Calmodulin [22]. nNOS is inducible at gene transcription level, being Ca^{2+} independent and expressed by muscle activity, aging process, as well as by macrophages and other tissues in response to inflammatory mediators [22].

NO_3^- is found in healthy individuals' plasma, in concentrations ranging from 20 to 40 *micro*M, during fasting conditions [9]. NO_3^- is also ingested orally, predominantly in green-leafy vegetables (the effective $t_{1/2}$ for plasmatic NO_3^- after ingestion is calculated to be 5.7-6.7 h), being rapidly absorbed across the upper GI tract and bypassing first-pass metabolism with closely 100% bioavailability [9]. Up to 2/3 of the absorbed NO_3^- is excreted unexchangeably in urine and most of the remaining is concentrated in salivary glands of NO_3^-, by sialin, a $2NO_3^-/H^+$ cotransporter, being then secreted into the oral cavity as NO_3^- - rich saliva, at concentrations up to 10-fold higher than plasma (entero-salivary circulation) [9]. NO_3^- can be seen as a "pro-drug" for bioactive NO_2^-, through action of bacterial NO_3^- reductases [9]. In what concerns NO_2^-, its conversion to NO is known to increase acidosis and hypoxia environments in which L-Arginine/eNOS pathway is dysfunctional, representing a complementary "back-up" system when conventional NO generation is compromised [9].

There is emerging body of evidence reporting a relationship between NO and AMPK expression and activity in cells, which cooperatively promotes glucose and fatty acid oxidation [20]. AMPK is known to regulate NO production, by phosphorylating eNOS at position Ser^{1177} [20]. On the other hand, NO can modulate AMPK activity through two mechanisms: a change in gene expression and AMPK activation via peroxynitrite [20].

About NOS-independent pathway, it is catalyzed by a wide range of proteins (particularly, deoxyhemoglobin) present in plasma and other tissues, leading to one-electron reduction of nitrite to NO [23]. It has been suggested that this alternative pathway may complement the L-arginine–NOS–NO pathway in conditions resembling skeletal muscle physiology during exercise, by enabling NO production in conditions of low pH (NOS function is compromised) and low oxygen availability in which NOS activity (which is oxygen dependent) may be reduced [8,17,23]. Furthermore, a portion of NO_3^-, produced through the reaction between NO and oxyhaemoglobin, and a portion of NO_2^-, produced through NO oxidation by ceruloplasmin, can be recycled back to NO via the $NO_3^- \rightarrow NO_2^- \rightarrow NO$ pathway, which functions as an escape mechanism to potentiate NO production across a range of physiological conditions, including exercise [25].

Physical Exercise as a Physiological Modulator

Firstly, it is important to understand that the initial vascular response to exercise is capillary recruitment, followed by important changes in total muscle blood flow, being NO implicated in both [26]. NO is increased in response to acute exercise sessions and can be increased as an adaptation to regular training. Therefore, well-trained subjects may have higher resting circulating NO than sedentary ones [27]. There is good evidence that NO is required for normal increases in skeletal muscle glucose uptake, during contraction/exercise, in rodents and humans, and, effectively, it may be playing an essential role in skeletal muscle glucose uptake regulation during exercise [10,28].

NO is also involved in the regulation of contractile force, either directly by gating the sarcoplasmic reticulum calcium release channels, or indirectly by modulating mitochondrial oxidative phosphorylation [29,30]. Despite the still actual need to determine the downstream signalling pathway involved, a rise in glucose uptake into muscle cells and even translocation by GLUT-4 are two possible underlying mechanisms described [29,30]. Localization of NOS both in mitochondria and along contractile fibers accentuates the role of NO in respiratory and contractile functions of skeletal muscles, which also takes part in muscle repair and helps tolerate heavy exercise [29]. Currently, satellite cell activation, an essential aspect to muscle tissue growth, is thought to be initiated by increased NO production in skeletal muscle [26]. Furthermore, mitochondrial function is essential for skeletal muscle metabolism, producing adenosine triphosphate (ATP) synthesis from different sources (carbohydrates, lipids, proteins…) [31]. It has been largely stated that either NO or physical exercise play an important role in mitochondrial biogenesis in a variety of tissues, regulating expression of multiple transcription factors, such as PGC-1α and mtTFA [31].

Interestingly, NO production occurring at vascular levels is directly related to a rise in shear stress, while the increased blood flow induced by exercise provokes itself a rise in shear stress, creating, thus, a relationship between exercise, increased blood flow and endogenous NO production [10]. NO endogenously produced by skeletal muscle among other cell types has the potential to inhibit calpain activity and cytoskeletal proteolysis, yet possible repercussions in body composition are still inconclusive [32,33].

During basal metabolism, NOS-dependent pathway is the major one for mitochondrial biogenesis, while, during physical exercise, NOS-independent via may play a role [31]. Highly trained subjects are likely to have high NOS activity, which might minimize the nitrate-nitrite-NO pathway importance for NO production, and are expected to have greater skeletal muscle capillarization, perhaps compensating for any possible hipoperfusion of metabolically active tissue during exercise and, therefore, reducing the requirement for NO production through nitrite reduction [23]. However, it is important to emphasise that highly trained subjects may have higher nitrite plasma levels than lesser trained or even sedentary ones, explaining the possible reduction in a standard dose of nitrate's response [23].

A fundamental tenet of human exercise physiology is a predictable oxygen cost for a given submaximal work rate [21]. VO_2 work rate during submaximal exercise is considered to be largely independent of age, health status, aerobic fitness and training status and its steady-state increase is linearly related to the increase in external work rate [21,22]. It is also known that tolerance to high-intensity training is a function of VO_{2max} and submaximal exercise economy [21].

Endurance exercise performance is a function of the fractional utilization of VO_{2max} and exercise efficiency and there is evidence that VO_2-work rate relationship during submaximal exercise is independent of factors such as age, health status, aerobic fitness, and training status [22,23]. About low-intensity endurance exercise, in which skeletal muscle remains well oxygenated and pH does not fall significantly, it would not obligatorily originate NO production from nitrite [23].

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 stress, which obviates the need for ongoing functional dilatation [8]. That is why training performed by competitive athletes seems to have a greater effect on improving the NO system than NO supplementation [8].

Interestingly, repetitive exercise over weeks, with large muscle mass activation, results in a systemic response in endothelial NO activity [8]. Therefore, aerobic physical training has shown systematically an upregulation of antioxidant enzymes, with the subsequent rise in NO bioavailability and physical performance's improvement [31].

Alternatives That Potentiate No Bioavailability … To Go Farther

Inorganic nitrate is present in numerous foodstuffs, being particularly abundant in green leafy vegetables (lettuce, spinach, rocket, celery, cress) and beetroot [23]. Following ingestion, nitrate (NO_3^-) is converted to nitrite (NO_2^-) by oral commensal bacteria and stored in the organism, functioning as a circulating "reservoir" for "on demand" NO production [23]. Nitrite and nitrate are also added to cured and processed meat to "fix" color and delay spoilage, but there has been some controversy about the safety of ingested nitrite, due to the potential for nitrosamines formation and the underlying risk of carcinogenesis [9,23].

Nitrate and nitrite are the main substrates to produce NO via the NOS-independent pathway, which seems to complement the endogenous NO production, especially during ischaemia and acidosis, in which oxygen availability is low and the NO synthases poorly operate [22].

Arginine is the most abundant carrier of protein nitrogen in animals and, particularly extracellular Arginine, not intracellular, is the main determinant of NO production in endothelial cells [19,34]. L-Arginine, a conditionally essential amino acid (2-amino-5-guanidinovaleric acid) that serves functions of relevance to athletes, is the standard NO donor in supplementation and its typical dietary intake is nearly 3.5-5 g per day [16,35,36]. L-Arginine is considered a semi-essential amino acid once the body is usually capable of producing it in sufficient amounts and is classified as glucogenic, due to being metabolized into α-ketoglutarate and entering acid cycle [24,32]. Therefore, plasma Arginine concentrations are maintained mostly by protein catabolism (85%) or by synthesis from other amino acids, only 5-15% originating from *de novo* synthesis [37,38].

Arginine is found in a wide variety of protein-rich foods, both plants and animal sources, and normal plasma L-Arginine concentrations depend on age, being homeostasis primarily achieved by catabolic processes [35]. Interestingly, the liver is capable of synthesizing considerable amounts of L-Arginine, yet this is completely reutilized in the urea cycle, being crucial to its normal function [22]. Urea is synthesized from Arginine to enable the body to remove ammonia excess, which is toxic to cells, clarifying the determinant effect of L-Arginine in the recovery from hard training [38-40].

It is known that an L-Arginine infusion at rest increases plasma insulin, glucagon, growth hormone, prolactin and catecolamine concentrations [22]. In turn, NO may mediate the effect of insulin in stimulating glucose transport in adipocyte tissue and skeletal muscle, by increasing levels of glucose transporter 4 (GLUT4) at cells surface [20]. Curiously, brown adipocyte development has been mentioned as a chronic effect of physiological levels of NO *in vivo* [41].

After oral administration, L-Arginine is extensively metabolized by Arginases in the gut wall and liver, increasing blood flow due to the provision of nitric oxide, providing oxygen and nutrients [22,35,38]. It is also involved in several regulatory cycles (urea, NO, ornithine) and it is convertible into a few other bioactive molecules, such as creatine and agmatine, important in body homeostasis [10]. Arginine also acts as an allosteric activator of N-Acetylglutamate synthase, which generates N-Acetylglutamate from Glutamate and Acetyl-CoA [42].

The dietary interest of this amino acid has substantially increased in the last years due to the importance of L-Citrulline (an essential intervenient in urea cycle in liver) as its precursor, which bypasses hepatic metabolism and is not a substrate for arginase enzymes [8]. That is why NO production depends mostly on the efficient recycling of Arginine-derived citrulline and less on exogenous supply, consisting systemic administration of L-Citrulline in a more efficient way to elevate extracellular L-Arginine levels [8,37]. Furthermore, L-Citrulline has been indicated as a secondary NO donor, being converted to L-Arginine, mainly in the kidney [8,22,24].

Lysine is an amino acid that competes with L-arginine for entry into cells, also inhibiting Arginase activity [8]. Arginases are enzymes that compete with NOS for L-Arginine and participate in the fifth and final steps of the urea cycle [8]. Under normal feeding conditions, the total amount of dietary L-Arginine should not be more than 150% greater than the amount of Lysine (L-Arginine: Lysine ‹ 2.5) [8].

In some cell types, reduced glutathione (GSH) seems to be necessary for NO synthesis, also stimulating Arginine turnover and increasing NOS activity [43]. Glutathione is a low molecular weight water-soluble tripeptide composed of cysteine, glutamic acid and glycine, being an important antioxidant and playing a major role in endogenous metabolic products detoxication [43]. Intracellular glutathione exists in two forms: oxidized disulfide (GSSG) and reduced (GSH) [43]. Therefore, combining L-Citrulline with GSH may augment NO production, already suggested by some authors, albeit oral GSH supplementation's effectiveness in association with L-Citrulline still has not been clearly delineated [44].

Even though it is widely recognized that L-Arginine is oxidized to NO by the action of NOS enzymes, the presence of endogenous analogs of L-Arginine: ADMA and L-NMMA (monomethylarginine) can competitively compromise NOS activity [45]. Nevertheless, some authors defend the hypothesis of L-Arginine paradox, which postulates that exogenous L-Arginine supplementation improves NO-mediated biological effects, despite high endogenous concentration [46]. Indeed, this paradox refers to specific situations in which L-Arginine supplementation appears to stimulate NOS activity, even when endogenous levels are found in a physiological range [10]. Actually, physiological L-Arginine concentrations are enough to saturate eNOS, thus, supplementary L-Arginine does not promote increased enzyme activity [10]. Hence, eNOS activity is linked to L-Arginine transport and extracellular levels of L-Arginine and ADMA are responsible for modulating eNOS activity [45]. One plausible explanation for this paradox is the presence of an endogenous inhibitor of NOS towards higher L-Arginine levels and the ratio of L-Arginine over ADMA is one determinant of NO production by NOS [38].

Outcomes Concerning Performance

In fact, the wide majority of studies on L-Arginine supplementation were taken in trained populations, whether through acute (minutes/hours/some days before training) or chronic (during weeks) regimes. Although there seems not to be a trend concerning the type of exercise test implemented, the training stimulus most frequently described are undoubtedly cycle ergometer, isokinetic dynamometer, resistance training and running. For instance, among 26 studies reporting protocols of L-Arginine Supplementation, only 6 did not report any advantageous performance benefit. Among those, the protocols of exercise training were quite heterogeneous (1 cycle ergometer, 2 isokinetic exercise, 1 resistance training and 1 km and 5 km run). Curiously about the three studies describing running performance tests, namely, 1, 5 and 31 km, only the 31 km run report some benefit inherent to supplementation, making it possible to speculate about the minimal running distance needed for L-Arginine consumption to show any advantage.

Considering the 20 studies showing more consistent supplementation benefits, by realising that 9 of them consisted of cycling training and 2 based on 1-repetition maximum (RM) bench press test, it is possible to infer some heterogeneity inherent to the variables analysed. Therefore, some of the variables we cannot ignore are the dose of L-Arginine administered, the relationship between supplementation and exercise stimulus, as well as via of administration, which was almost generally the oral one. In order to better understand the magnitude of this heterogeneity, it is worth noting that 7 g of L-Arginine before each resistance exercise bout seems not to influence strength performance, as well as muscle blood volume and oxygenation, while 10 g of L-Arginine seem to proportionate measurable benefits.

There is reason to believe that bolus Arginine ingestion, as well as potentiation of nitrate-nitrite-NO pathway, by increasing NO synthesis and muscle flow, may have a potential ergogenic effect, influencing muscle anabolism, physiology, and, consequently, exercise performance [23]. Concerning 6g-L-Arginine supplementation in strength performance, there is good evidence supporting strength and power progresses, improving nutrient uptake and ATP utilization, but muscle growth uplift is still under controversy [26]. L-Arginine supplementation may also reduce superoxide anions released by the endothelium [10].

All in all, the vast majority of studies focusing on the effects of the promising association between L-Arginine supplementation and physical performance agree on positive outcomes, describing improvements in training performance status with greater tolerance (increased time to exhaustion) [31]. Thus, there seems to be supportive evidence in humans of a beneficial role for *per os* loading of Arginine in athletes' performance, particularly weightlifters, improving strength and muscle workout capacity [10]. However, at this time, guidelines concerning Arginine supplementation in Weightlifters are just still speculative.

In what concerns nitrate supplementation, there are several ways of consumption, such as beetroot juice, spinach, sodium or potassium nitrate as acute regimes in trained subjects. Between the 34 studies describing consistent protocols of nitrate supplementation, only 7 report no significant benefit at all. Considering the 27 studies reporting some advantage apparently nitrate-related, 12 of them are

based on cycle ergometer protocols, a relevant predominance among other extremely heterogeneous trainings such as rowing, apneic series performance, yo-yo tests and maximal treadmill cardiopulmonary exercise testing.

Focusing on molecular instances, NO may play a lesser role in exercise blood flow during endurance performance, compared with a more central role during resistance-type exercise [47]. One of the reasons for the lack of performance improvements following dietary nitrate ingestion in endurance-trained athletes may be due to higher baseline levels of NO [8]. NO is only responsible for a small proportion of the increase in muscle blood flow during exercise [47]. It is, likewise, suggested that the increase of blood pressure derived from NO synthesis may improve recovery processes in the post-training period [8].

Based on existing data, NO_3^- supplementation appears to improve metabolic efficiency and exercise tolerance in healthy humans, but its influence on VO_{2max} may be dependent on the exercise modality and/or the training status of the subjects [8].

Underlying Mechanisms

In view of their diverse chemical properties, dietary factors regulate constitutive and inducible NO production, likely through different mechanisms: changes in intracellular concentrations of NOS substrates and cofactors, alterations in NOS expression and kinetic properties [12].

Some studies argue that 15 days of nitrate supplementation may gradually increase $VO_{2\ max}$ through NO-mediated changes in skeletal muscle local perfusion, effects on cardiac output, and also increased mitochondrial mass as a consequence of elevated NO availability [22]. Moreover, the total body O_2 cost of exercise following dietary supplementation seems to be related to a reduced ATP cost of muscle force production, perhaps consequent to reduced cross-bridge cycling or sarcoplasmic reticulum Ca^{2+}-ATPase activity [23,48]. An alternative explanation for the coincident reductions in steady-state VO_2 is that nitrate supplementation simultaneously improves muscle oxygenation and mitochondrial efficiency [23]. Another possible speculation consists of an increased inhibition of cytochrome C oxidase by NO, which might be sensed by cells as mild hypoxia, with the consequent activation of signalling mechanisms that result in downregulation of adenine nucleotide translocase (ANT), a protein involved in mitochondrial proton conductance, and improved mitochondrial efficiency, enforced by the lack of increase in lactate concentration [17,23]. In fact, NO can combat a decreased NO production during hypoxic conditions, maintaining or even improving exercise performance [14].

The acute effects on the VO_2 response to moderate-intensity exercise are maintained over 15 days of supplementation, with no indication of either a reduced sensitivity to supplementation or an increasing effect with time [22]. The possible mechanisms by which VO_{2max} may be increased following 15 days of nitrate supplementation include NO-mediated changes in local perfusion in skeletal muscle and possible effects on cardiac output. Alternatively, it could be linked to increased mitochondrial mass as a consequence of elevated NO availability [22].

To make matters even more plotting, some authors argue that dietary nitrate supplementation not only enhances blood flow to contracting muscle, but may also increase muscle function during normal movement, which is consistent with the effects of nitrate on muscle efficiency being, at least, partially explained by extramitochondrial mechanisms [23]. Dietary nitrate supplementation increases plasma nitrite concentration and reduces resting blood pressure [23].

Curiously, NO modulates the mechanical behaviour of skeletal muscle cells through a decrease in calpain-mediated cytoskeletal proteolysis [32]. Calpain proteins are a family of Ca^{2+}-activated non-lysosomal proteases known to cleave muscle proteins, three of them being well characterized in skeletal muscle: μ-calpain, m-calpain and n-calpain [32]. Thus, NO inhibition of calpains may provide a potential therapeutic approach to protect from skeletal muscle injury and that is why NO production is known to increase dramatically in injured skeletal muscle, despite many uncertainties still fogging the role of NO in recovery after muscle injury [32]. It has been suggested that a possible contribution to tissue reconstruction is NO activation after injury exerts an effect on the expression of myogenic regulatory factors (MFRs, like myogenin) that occur during recovery [32]. Still focusing at cellular level, NO seems to potentiate the proliferation of satellite cells, present in skeletal muscle fascicles, exerting a positive stimulus to muscular hypertrophy.

The mechanism underpinning the observed benefits of NO_3^- supplementation on exercise capacity is believed to be a reduction in the oxygen cost of exercise, as a consequence of a reduced energy cost of contraction or enhanced mitochondrial efficiency. There is also evidence that NO_3^- supplementation can improve the efficiency of muscular work by extending exercise duration tolerance during submaximal exercise, originating VO_{2max} reduction, an effect possibly related to a reduced O_2 cost of mitochondrial ATP resynthesis and/or to a lower ATP cost of muscle contraction [25,49]. Some mechanistic studies have disclosed that NO_3^- may also reduce the ATP cost of muscle force production, as well as improve mitochondrial respiration efficiency [25]. However, nitrate supplementation possible repercussions into performance have to be considered systematically across a range of exercise intensities [44]. For instance, higher exercise intensities are likely to result in a greater degree of skeletal muscle hypoxia, which would be expected to facilitate NO production through NO_2^- reduction [44].

Chronic exposure of cells to NO has been shown to result in cGMP-mediated activation of regulatory protein sirtuin (SIRT1), which upregulates transcriptional and nuclear respiratory factors, involved in the coordination of mitochondrial fusion and fission events [22]. However, whether NO-cGMP-induced mitochondrial biogenesis is manifested in human skeletal muscle in vivo following dietary nitrate intervention still remains to be determined [22]. In what concerns nitrite, it is known to modulate mitochondrial function, structure and density; contrariwise, mitochondrial proteins act as nitrite reductases and tissues with higher mitochondrial oxygen consumption demonstrate greater nitrite reductase activity [50].

Concerning exogenous Arginine, itsaf induces both ARG1 and ARG2 expression and the more Arginine consumed, the more it will be destroyed [37]. There is evidence that 8-week L-Arginine supplementation associated with physical training was effective in promoting greater physical capacity, with up-regulation of proteins related to mitochondrial biogenesis (mtTFA, and PGC-1α) and electron transport chain [31].

Tolerance to Physical Exercise and Ergogenic Potential

Dietary NO_3^- increases time to task failure at high intensity exercise and reduces the time needed to achieve a certain distance in time trials [9]. NO_2^- plasma levels have recently been referred as an important factor for exercise tolerance in healthy subjects, suggesting the potential of NO_3^- supplementation for improving exercise tolerance, by rising NO_2^- plasma concentration [25].

Furthermore, there seems to be a dose-response relationship between NO3- intake and its ergogenic effects, while the association with oxygen consumption is not as evident [9]. The effects of NO_3^- seem to be present both after acute and chronic administration, which may indicate that separate mechanisms are in play, since it is unlikely that alterations in protein expression would occur already 1-2 h after acute administration [9]. There is evidence about the role of NO in exercise-induced vasodilation by the increased levels of plasma and urinary markers of NO in humans: nitrate, nitrite and cGMP, but the contribution of NO to vasodilation may vary depending on the type of exercise [10].

The efficacy of acute nitrate supplementation will depend on several factors, such as age, health, diet, fitness/ training status, intensity, duration, nature of the exercise task and whether the exercise is performed in normoxia or hypoxia [23]. Acute nitrate intake may rapidly influence vascular tone and peripheral tissue oxygenation, but more time may be necessary to permit changes in mitochondrial and contractile proteins to influence exercise performance [23]. Some studies report that nitrate supplementation is associated with a moderate improvement in constant load time to exhaustion, but did not significantly affect time trial or graded exercise testing performance [51].

Actually, training status of subjects is an important factor linked to the ergogenic effect of NO and the absence of ergogenic effects of increased NO availability in endurance-trained athletes may be explained by the physiological and metabolic adaptations derived from chronic physical training. Still considering endurance performance, there seems to be gains in endurance and running/cycling economy, for untrained and moderately trained athletes, but not elite ones [26].

The duration of continuous maximal exercise for which nitrate appears to be ergogenic is in the range of 5-30 min [23]. There is limited evidence that nitrate is beneficial for longer duration exercise performance, at least when administered acutely [23]. This may be related to the lower intensity of such exercise and the associated reduced likelihood of the development of local matching of perfusion to metabolic rate in muscle [23]. Whether nitrate supplementation may be ergogenic during very high-intensity continuous or intermittent exercise has not been systematically evaluated.

In what concerns chronic L-Arginine supplementation, it has been linked to a positive influence in hemodynamics and increment in VO_{2max}, and exercise capacity, likely due to the rise in NO production, lessening both plasma lactate concentration and heart rate [28]. However, in healthy individuals, with normal NO production, L-Arginine supplementation seems to have little impact on aerobic exercise capacity, contrarily to individuals with reduced basal NO production [28]. L-Arginine supplementation seems to have no effect on post-exercise muscle hyperemia [47]. Interestingly, many studies have shown that L-arginine supplementation significantly improved physical performance with greater tolerance to physical exercise [31].

In respect to specifically highly trained endurance athletes (VO_{2max}: 60-70 mL/kg per minute), several studies have reported no ergogenic effect of short-term supplementation on exercise performance. The studies showing negligible effects of nitrate in elite athletes who have used acute (2-3 h preceding performance) or short-term (3 day) supplementation protocols. [23]

In contrast, mechanistic studies, arguing that nitrate alters muscle contractility and mitochondrial proteins, employed longer (3-7 day) supplementation periods. This raises the possibility that longer-term supplementation and/or higher nitrate doses may be required to improve performance in elite athletes [23].

The literature appears consistent in showing that 2-6 days (or up to 15 days) of supplementation can increase indices of performance during high-intensity constant work-rate exercise and maximal incremental exercise. The effects of acute supplementation on performance are less consistent, with some studies showing a positive effect and others showing no effect [23].

Supplementation … From a Practical Point of View

Nitrate intake, which has been positively associated with exercise capacity in humans, particularly in the form of beetroot products, varies greatly between different cultures, being important to establish the dose effect ranges and the duration of effects [17,23,27]. Furthermore, the efficacy of acute nitrate supplementation will depend on several factors, some intrinsic to athletes, as age, health, diet, fitness/training, status (including muscle fiber type proportions, capillarization, and baseline plasma nitrite levels), some related to training characteristics: nature, intensity, duration, as well as whether exercise is performed in normoxia or hipoxia [23]. In fact, although acute nitrate intake may rapidly influence vascular tone and peripheral tissue oxygenation, more time may be necessary to produce changes in mitochondrial and contractile proteins, and, consequently, influence exercise performance [23,27]. As much as 5-9 mmol of nitrate per day, an amount possibly achievable with normal diet, has shown favorable effects on physiological responses to exercise and there is no evidence of greater benefits with further nitrate intake, even because very high nitric oxide concentrations favor cell cycle arrest and apoptosis and excessive NO production is known to be detrimental to tissues and cardiovascular function [12,23,52].

Moreover, a careful view of NO-stimulating supplements' composition shows that, in many circumstances, they contain a great variety of molecules (creatine, carbohydrates, amino acids, vitamins, minerals…), which may be, total or partially, responsible for the ergogenic effect found, as the amounts of NO ingredients are frequently very low and incapable of inducing NO changes [8].

To enhance performance, athletes are already using arginine (known as a potent hormone secretagogue) supplementation, in an attempt to stimulate GH levels, and, subsequently, potentiate IGF-1, thus, increasing muscle mass [28,53]. In fact, GH response to amino acids is affected by sex and training status and repercussions on exercise-induced GH response will be slight [54]. Even though GH release during aerobic exercise is clearly related to exercise intensity and duration, the use of specific amino acids for the purpose of stimulating GH release to promote greater gains in muscle mass and strength and to alter body composition is not recommended [54].

L-Arginine supplementation, in combination with Glutamate and Aspartate, is known to be effective at reducing blood levels of ammonia, as well as blood lactate, during exercise [8]. In male studies, it has been shown that it could enhance the respiratory response, by significantly increasing speed in pulmonary oxygen consumption at the onset of moderate intensity endurance cycle exercise after 6 g-day of L-Arginine supplementation, during 14 days [8]. Higher doses of dietary L-Arginine (>10 g) are ineffective at increasing blood flow and its exogenous route bioavailability is nearly 60% [4,8]. Furthermore, 6 g-day of L-Arginine supplementation seem to be well tolerated and oral root of administration has recently become quite popular [24].

Acute 6 g -L-Arginine supplementation was able to increase

muscular blood flow during recovery from sets of resistance exercise, with no increase in strength performance and no significant change in peripheral blood flow [24]. Resistance exercise is also known to transiently increase circulating levels of GH, by suppressing the secretion of somatostatin (an hormone that inhibits GH release), but the relevance of the greater increase in GH following Arginine supplementation is not clear [16,55]. Therefore, it still seems premature recommending nutritional supplements containing L-Arginine as an ergogenic aid to increase muscle strength during resistance training [24].

In what concerns trained runners, L-Arginine supplementation stimulates neither the production of GH, insulin and IGF-1, nor the reduction of cortisol and does not enhance their exercise performance [55]. Therefore, nutritional supplements based on L-Arginine should not be recommended to induce hormonal changes and improve exercise performance in trained runners [55]. On the other hand, during cycling, L-Arginine supplementation has been associated to an increase in physical working capacity, before reaching neuromuscular fatigue [26].

Active athletes are known to have increased protein needs and, in fact, beneficial effects of arginine supplementation on muscle strength may be minimal in young healthy men who are already eating plenty of protein [7,35]. However, there are other factors that may minimize the effect of dietary L-Arginine, like the physiological and metabolic adaptation derived from chronic training and high L-Arginine–Lysine ratio [8].

To sum up, both L-Arginine and exercise increase NO production by skeletal muscle [28]. Due to the multiple roles attributable to L-Arginine, its supplementation is likely to result in increased metabolism via pathways other than NO synthesis, thus, other effects via NO-independent mechanisms may also play a role [24].

Focusing on Safety Profile

Actually, nitrate supplements present as a low risk intervention that may endurance exercise performance [51]. In turn, inorganic NO_3^-, in doses achievable through diet, seems to improve metabolic and mechanical efficiency during exercise in healthy subjects, due to several mechanisms involving mitochondria, muscle and vasculature [9].

Even though L-Arginine supplementation has been frequently linked to metabolic diseases, only a few studies have been evaluating the effects of associating L-Arginine supplementation and physical workout on health outcomes [31]. In what concerns L-Arginine, clinical results about bioavailability and safety of *per os* L-Arginine loading suggest a need for further testing on the reduction of ammonia levels induced during high-intensity training [10]. Interestingly, despite the results achieved by now, it is only possible to speculate about the insight mechanisms involved yet. By now, administration of Arginine loading to athletes with determination of plasma ammonia levels after exercise has not been performed yet [10].

Although supplementation with L-Arginine or L-Citrulline does not lack in undesirable effects, particularly gastrointestinal disturbances, such as nausea, vomiting or diarrhoea, there is great inter-individual variability in the tolerance to these amino acids [8]. Other possible side effects in healthy subjects are allergic reactions, including anaphylaxis, suggesting that supplementation should be avoided by atopic individuals [10].

Cardiovascular Repercussions

Within the cardiovascular system, basal endothelial NO release plays a critical role in sustaining cardiovascular health, by exerting vasodilator, anti-platelet, anti-proliferative and anti-leucocyte phenotype [9]. Therefore, the decline in NO production or availability has been implicated in several cardiovascular disease processes [50]. In recent years, dietary nitrate has emerged as a promising therapeutic agent for the treatment of hypertension, lowering both systolic and diastolic blood pressures, with a compensatory rise in heart rate [22,28,46].

There is no doubt that endothelial and NO dysfunction is a hallmark of cardiovascular disease, namely hypertension, obesity, diabetes and malnutrition [56]. Exercise training, in part via increased capacity for NO production, is known to retard atherosclerosis [26].

It appears that L-Arginine is a limiting factor for NO synthesis in patients at risk for atherosclerosis, but not for healthy subjects and some studies associate improvement in exercise tolerance of patients with underlying pathologies to antiatherogenic (inhibiting atherosclerotic plaques' progression) and vasodilatory properties of Arginine, via optimization of coronary and/or peripheral blood flow [4,10]. Actually, there is reduced bioavailable nitric oxide in patients with both cardiovascular risk factors and vascular disease manifestations and decreased L-Arginine/eNOS pathway activity is thought to contribute to pathology [9].

Arginine supplementation has been shown to improve NO-dependent endothelial relaxation in patients with major cardiovascular risk factors (hypercholesterolemia, smoking, hypertension, diabetes, obesity, insulin resistance, aging) and many ordinary cardiovascular disorders [12]. L-Arginine supplementation before resistance exercise seems to have no significant effect on central/peripheral arterial stiffness, as well as no effect on hemodynamic and vascular responses to resistance exercise [47].

Bioavailability of NO is known to be reduced in obese subjects, due to reduced synthesis and increased oxidation [41]. Conversely, physiological levels of NO derived from endothelial cells activates guanylyl cyclase, which, in turn, generates cGMP in vascular smooth muscle cells and platelets, thereby, promoting fatty acid and glucose oxidation and reducing the deposition of white fat tissue [41]. Therefore, L-Arginine, as well as its precursor, L-Citrulline, constitutes promising nutrients for the enhancement of white adipose tissue loss in obese individuals [41]. In addition to this, $NO_3^- \rightarrow NO_2^- \rightarrow NO$ pathway has been linked even to brain perfusion improvement and inhibition of cancer cell [10].

Even though NO, either from endogenous (eNOS derived) or exogenous sources, plays a pivotal role in cardioprotection against myocardial ischemia and reperfusion injury, the underlying mechanisms still remain unclear [11]. In fact, a wide spectrum of cardioprotective maneuvers operates in an NO-dependent manner: beta-adrenergic antagonism, L-type calcium channels inhibition, sarcolemmal and mitochondrial k_{ATP} channels activation, cycloxigenase-2 activation with production of cytoprotective prostanoids, proapoptotic proteins inhibition and antioxidant effects [11]. Moreover, NO has been shown to increase diastolic distensibility and has also been implicated in the negative inotropic effects derived from selective ET-B1 receptor stimulation, collectively with prostaglandins, independently of the direct actions of each endothelial agent [57].

Interestingly, in what concerns non-healthy individuals, there is also evidence of endothelial function improvement by increasing plasma L-Arg levels by supplementation [24]. Nevertheless, some studies report acute cardiovascular modulation, particularly associated

with high doses of intake (30 g maximum), such as hypotension with compensatory tachycardia, reduced peripheral arterial resistance and increase in cardiac output [10]. Beyond this, it is important to safeguard that L-Arginine supplementation is not recommended for post-infarction survivors [10].

Furthermore, L-Arginine stimulates NO biosynthesis, which leads to reduction of oxidative stress, thus, it makes sense that endothelial dysfunction, which leads to decreased bioavailability of NO, is a disadvantageous factor for patients with arterial hypertension, as well as endogenic ADMA (assymetric dimethylarginine), which has become a marker of cardiovascular risk [58]. It cannot be excluded that L-Arginine, by lowering oxidative stress, probably restrains indirectly the increase in vasoconstrictors' concentrations, like ET-1, secreted under influence of reactive-oxygen species (ROS) [58].

With regard to hypercholesterolemic patients, the elevation in ADMA levels found in plasma seems to be possibly overcome with supplemental L-Arginine, avoiding the consequent impaired endothelial NO synthesis, and thence, endothelial dysfunction [12,45,59].

During ischaemia, nitrite appears to confer cytoprotection through modulating mitochondrial function, by its reduction to NO, which, in turn, competes with oxygen, and, consequently, inhibits mitochondrial respiration [50]. Indeed, mitochondria are pivotal players in NO-dependent protection [11] and nitrite is a selective donor of NO in hypoxic/ischaemic tissues, compensating for the diminuished NOS activity [50]. Thus, nitrite, through exogenous dietary/pharmacological administration, has the potential to become promising in the next years' prevention and treatment approaches to cardiovascular disease [50].

Conclusion

In conclusion, whatever the aims athletes want to achieve and regardless of the widespread availability of sports drinks, nutrition foods and ergogenic aids, it is strongly recommended that any athlete considering to consume this kind of substances should firstly seek for advice from a sports nutrition professional.

In short, the research of nitrate as an ergogenic aid is still in its infancy, with the bulk of studies reporting favorable outcomes, namely, a small benefit to performance [51]. Further research is undoubtedly needed to investigate the influence of dietary NO_3^- supplementation on the VO_{2max}. Nevertheless, increasing plasmatic NO_2^- levels via dietary supplementation with NO_3^- may represent a practical and cost-effective intervention to restore NO homeostasis and improve exercise tolerance in these populations [25].

There is also clearly a need for more studies to verify if L-Arginine enhances strength, power accomplishment and muscular recovery associated with increases in NO production in healthy subjects [10]. On the other hand, training adaptations achievable by athletes throughout their careers seems to be much more meaningful and robust than any possible increments obtained by NO enhancement.

There is still a long path to grub: elucidating the sports and environments where nitrate may trigger most benefits, refining knowledge on desired and safe quantity, timing and quality of supplementation [51].

Nitrates/Arginine/NO-triangular plot will, certainly, deserve a major spot in many fertile areas of future investigation. Anyway, athletes' priorities must be safe training, healthy eating and efficient recovery. It is wise to minutely analyze the cost-benefit ratio of ergogenic aids, keeping in mind its potential illegality, always staying on guard against false promises.

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