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Could intramuscular storage of dietary nitrate contribute to its ergogenic effect? A mini-review

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ABSTRACT

Extensive research performed over the past 10 years has resulted in dietary nitrate being considered a nutritional supplement that can improve exercise performance. However, there is still limited insight in the metabolic fate of dietary nitrate following the appearance of nitrate and nitrite in the circulation. Recent observations in humans suggest the storage of nitrate in skeletal muscle tissue. This short review discusses the possibility of nitrate being stored and utilized in human skeletal muscle tissue, and why confirming this may increase our understanding of how the nitrate-nitrite-NO pathway improves exercise performance. Further insight in skeletal muscle nitrate storage and metabolism may provide answers to current gaps in knowledge, such as the ergogenic benefit of acute vs multiday dietary nitrate supplementation, as well as the suggested muscle fiber-type specific effects on exercise performance. In this mini-review, specific questions that need further exploration are also discussed.

1. Introduction

It is now well established that, apart from being metabolites of the nitric oxide synthase (NOS) pathway, both nitrate and nitrite can also serve as precursors of nitric oxide (NO) through the nitrate-nitrite-NO pathway (Fig. 1). Following digestion and absorption of nitrate-rich food, plasma nitrate concentrations rise, followed by nitrate being actively taken up by the salivary glands and concentrated in saliva [1]. Approximately 20% of the nitrate (re-)entering the oral cavity through the salivary glands is reduced to nitrite by commensal bacteria residing on the dorsal part of the tongue [2]. Nitrite is subsequently swallowed, absorbed into the circulation from the intestines, and transported to other parts within the body where further reduction to NO and other nitrogen oxides is reported to occur through various pathways [3–5]. Evidence of this entero-salivary path of nitrate has been provided by prohibiting subjects from swallowing their saliva following the ingestion of dietary nitrate, effectively attenuating the characteristic rise in plasma nitrite concentrations [6]. Although increases in plasma nitrate and nitrite concentrations following the ingestion of dietary nitrate have since been reported by multiple studies [7,8], there is still limited insight in the eventual metabolic fate of the ingested nitrate and nitrite *in vivo*. Research performed in animal models in the early 1980's provided some insight using radioactively labeled nitrate and nitrite, showing that both anions are swiftly distributed through the systemic circulation to many organs following administration [9]. Piknova *et al.* were eventually the first to publish groundbreaking work performed in rodents, showing that nitrate is not only present in blood, but also in liver and skeletal muscle tissue [10]. In line with those findings, we soon after showed that nitrate is also present in human skeletal muscle tissue [11]. We observed that post absorptive nitrate concentrations in skeletal muscle tissue are higher than circulating plasma nitrate concentrations. Moreover, ingestion of a nitrate bolus increased both plasma and skeletal muscle nitrate concentrations [13]. Wylie *et al.* [12], recently confirmed and extended on our observations by showing higher basal concentrations of both nitrate *and* nitrite in muscle compared to plasma, as well as significant increases in muscle and plasma nitrate concentrations following the ingestion of nitrate-rich beetroot juice. Together, these findings suggest that skeletal muscle tissue may be capable of buffering nitrate originating from exogenous (dietary nitrate) and endogenous (as a metabolite of the L-arginine and NOS pathway) sources. In light of these recent observations, this review discusses the possibility of skeletal muscle tissue serving as an endogenous nitrate reservoir in humans. Specifically, notions regarding the localization of nitrate (and nitrite) stores in skeletal muscle tissue are presented, as well as the mechanisms that may be required to benefit from such local storage. Finally, the possible storage of nitrate (and nitrite) in other organs is placed in context of the health benefitting effects observed following dietary nitrate intake.

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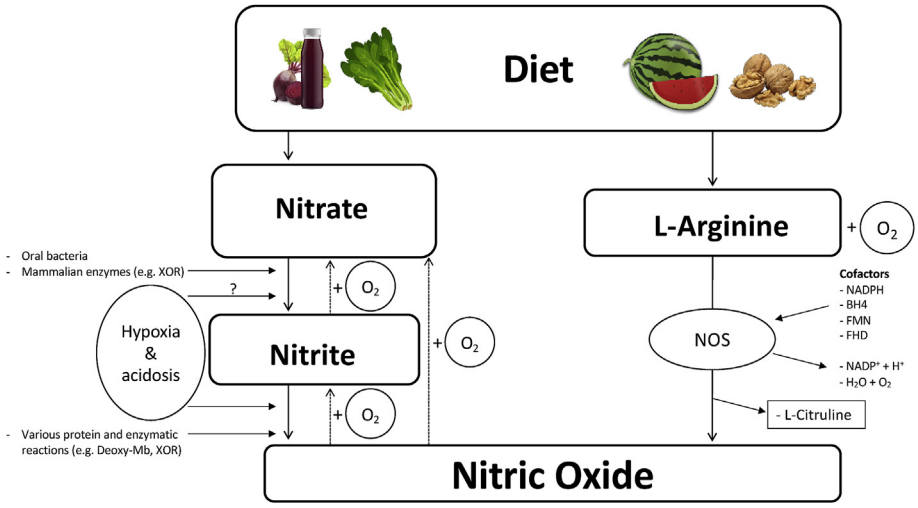

Fig. 1. The nitrate-nitrite- NO pathway, and the oxygen-dependent NOS pathway with cofactors that catalyze the two-step oxidation of L-arginine to NO. Abbreviations: XOR, Xanthine oxidoreductase; Deoxy-Mb, deoxy myoglobin; NADPH, nicotinamide adenine dinucleotide phosphate; BH4, tetrahydrobiopterin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

2. Skeletal muscle nitrate content

Recent observations by us [11] and others [12] indicate that nitrate is present in human skeletal muscle tissue. The nitrate concentrations measured in muscle in the post absorptive state have been observed to exceed concentrations measured in plasma up to 4-fold both in young [11,12] as well as older individuals [11]. The fact that ~3-fold higher nitrate concentrations in muscle compared to blood were previously also observed in rodents [10] implies that nitrate may preferentially be stored in skeletal muscle tissue. However, it is still unclear what the exact role of nitrate storage in muscle could be. One suggestion could be that such storage allows for a faster local NO production through the nitrate-nitrite-NO pathway. The established regulatory role of NO in various processes taking place in skeletal muscle tissue, such as force production, vasodilation, respiration and glucose homeostasis, indicate the functionality that a local nitrate buffer can have assuming it will enhance local NO bioavailability [13]. The latter likely becomes more relevant under (extreme) exercise conditions in which NOS-dependent NO production is limited, such as (local) hypoxia and acidosis. Speculating even further, as some of the performance enhancing effects associated with nitrate supplementation are believed to result from increased blood flow [14], it could be hypothesized that nitrate is stored in close proximity of blood vessels. The likelihood of this notion is supported by the fact that several enzymes suggested to be capable of reducing nitrate and nitrite to NO are present in, or close to blood vessels, including deoxygenated myoglobin and xanthine oxidoreductase (XOR) [3,15]. However, it is questionable whether such localization of a nitrate store close to blood vessels would allow the generated NO to facilitate certain intramuscular effects that have been attributed to nitrate. Effects such as improvements in sarcoplasmic reticulum Ca^{2+} handling (suggested to enhance skeletal muscle contractility), and a reduction in mitochondrial uncoupling due to proton leak (suggested to account for a reduced oxygen cost) have indeed been observed following dietary nitrate ingestion [16,17]. The highly diffusive nature of NO, and the intracellular signal transduction of NO through the second messenger cGMP may play an important role in facilitating these intracellular processes [13]. However, another explanation could be that nitrate reservoirs may be present in different subcellular locations. A good example of such specific localization is the local storage of glycogen, which in human and rodent skeletal muscle tissue has been shown to be present in the intramyofibrillar, intermyofibrillar, and subsarcolemmal space, each with its specific functional roles [18]. Intramyofibrillar storage and local reduction of nitrate to NO would, for example, allow for the local nitrosylation of ryanodine receptor type 1 (RyR1) cysteine residues [19]. The nitrosylation of RyR1 has indeed been proposed as a possible explanation for the improved contractile function observed following dietary nitrate ingestion by enhancing Ca^{2+} release [20]. A recent finding that may increase the likelihood of this notion is that of a nitrate transporter known as sialin, which was shown to be present in human skeletal muscle tissue [12]. Similar to the role that sialin fulfills as an electrogenic $2NO_3^-/H^+$ cotransporter in the enterosalivary nitrate path [21], sialin nitrate transporters present in skeletal muscle may facilitate the active uptake of nitrate from the circulation for intramyofibrillar, intermyofibrillar, and subsarcolemmal storage and utilization. Furthermore, as NOS has been shown to be present in human skeletal muscle tissue and to generate NO through L-arginine oxidation [12], it is likely that endogenously produced nitrate also contributes to skeletal muscle nitrate content [10]. Clearly, although these recent observations in humans indicate the existence of a skeletal muscle nitrate buffer, further studies are required to provide insight in the endogenous production, uptake from dietary nitrate sources, and exact subcellular localization of nitrate in human muscle.

3. Could nitrate storage in muscle be fiber type specific?

The fact that it is currently unclear where nitrate is buffered in skeletal muscle tissue leaves room for speculation to what extent the localization may be associated with the pharmacodynamic effects observed following dietary nitrate ingestion. There is a growing notion that dietary nitrate might preferentially exert beneficial effects on activities that strongly rely on type II muscle fiber recruitment [22]. This is mainly based on key studies by Ferguson *et al.* [14] and Hernandez *et al.* [17] with exercising rodents following dietary nitrate supplementation, showing increased blood flow and enhanced muscle contractility primarily in type II skeletal muscle fibers. These type II muscle fibers are characterized by low oxygen availability and anaerobic energy production. In contrast, type I muscle fibers have high capillary and mitochondrial density and greater oxidative capacity, facilitating aerobic energy production [23]. As such, these rodent studies [14,17] did not seem to fit the paradigm that dietary nitrate primarily benefits endurance-type exercise performance by modulating mitochondrial function (e.g. increasing P/O ratio by reducing ADP/ATP translocase expression) and thus oxygen dependent ATP resynthesis which would rely heavily on type I fiber recruitment [8,16,24]. Notably though, nitrite has been observed to show greater reduction to NO in low oxygen conditions [25], underlining why type II muscle fibers and activities that mainly require type II muscle fiber recruitment may benefit most from nitrate ingestion [22]. In accordance with this line of reasoning, observations in athletes suggest substantial performance benefits following dietary nitrate ingestion during high-intensity intermittent-type exercise activities, as well as during exercise under hypoxic conditions

[26,27]. We showed that a multiday dietary nitrate ingestion protocol improved repeated high intensity intermittent-type running performance in trained soccer players [26], which has been confirmed by others [28,29]. Furthermore, a study performed by Hoon et al. [30] nicely illustrated, using the blood flow restriction procedure, that dietary nitrate ingestion can improve exercise performance in a setting of local hypoxia. In that study, an increased exercise tolerance was observed following dietary nitrate ingestion, and this effect was suggested to result from greater NO bioavailability, most likely improving performance of the type II muscle fibers. An interesting question that arises from this explanation is whether the short half-life of NO and nitrite (milliseconds and several minutes, respectively) would require them to be reduced/produced in close proximity to the muscle fibers? Buffering of a stable precursor like nitrate close to or even within the fiber might be a solution, as this may allow confining the effect of the bioactive nitrite and NO to the desired area. In line with the above, it could thus be hypothesized that nitrate is primarily stored in the vicinity of, or even preferentially within type II skeletal muscle fibers. While this is still speculation, it could support previous findings. For example, our own observations suggest lower nitrate concentrations in skeletal muscle tissue of older healthy males, when compared with younger healthy males [11]. This may be attributed to the relative lower type II versus type I muscle fiber mass in older compared with younger individuals [31]. Extending this hypothetical coupling between skeletal muscle nitrate content and type II fiber content/activity further may also support the convincing body of evidence showing a lack of performance improvements in highly trained endurance athletes following dietary nitrate ingestion [32–34]. It has been proposed that the specific adaptations to endurance training leading to an improved oxidative capacity in such athletes may attenuate the occurrence of low oxygen conditions within the exercising muscle [32]. In addition, highly endurance-trained athletes typically have a muscle fiber composition towards more oxidative, type I muscle fibers, and their exercise pattern relies more heavily on the recruitment of type I muscle fibers [35], which may be less sensitive to nitrate [14,17,22]. Gaining further insight in the potential fiber-type specific storage and utilization of nitrate may, therefore, improve our understanding of physiological differences between populations that differ in the responsiveness to dietary nitrate supplementation (e.g. age, training status, disease state). As with skeletal muscle glycogen and lipid storage, quantification of fiber-type specific storage of nitrate may be achievable through biochemical and histochemical analytical techniques, using both animal and human skeletal muscle tissue [18]. Efforts will be required to set up and optimize such methods, as muscle fiber-type specific nitrate and nitrite content analyses have not yet been reported.

4. Skeletal muscle nitrate loading

With nitrate potentially being buffered in skeletal muscle tissue, it is important to consider what this could mean for current nitrate supplementation strategies. Although research over the past years has tried to define the most effective supplementation strategy with regard to nitrate source and dose, there is still limited insight in the optimal duration of dietary nitrate supplementation to maximize the proposed ergogenic properties [33,34]. While many studies have used a multiday supplementation approach, other studies have assessed the capacity of ingesting a single bolus of nitrate on exercise performance [36–39]. Interestingly, many of the exercise performance benefits observed following multiday supplementation have also been reported following ingestion of a single nitrate bolus. Indeed, improvements in parameters such as oxygen efficiency, fatigue resistance, and time trial performance have been observed within 4 h following ingestion of a single bolus of dietary nitrate [7,36]. It, therefore, remains unclear if and, if so, what the exact benefit would be of a multiday nitrate supplementation regimen. There have been some suggestions that ergogenic effects resulting from chronic dietary nitrate supplementation may be due to physiological adaptations at the myocellular level. For example, reductions in oxygen consumption following 3 days of dietary nitrate ingestion have been associated with changes in mitochondrial function that would unlikely manifest following acute ingestion [16]. However, reductions in oxygen requirement following multiple days of dietary nitrate ingestion have also been reported without measurable changes in mitochondrial function in human skeletal muscle tissue [40]. Although this does not exclude the possibility that certain adaptations at the muscle level may specifically result from multiday supplementation, the available body of evidence has been unable to pinpoint the mechanisms that would explain greater exercise performance improvements induced by chronic dietary nitrate supplementation. Nonetheless, there is a general belief that prolonged ingestion of dietary nitrate may result in greater performance benefits than ingestion of a single bolus. The latter is supported by specific studies that comprehensively compared acute and multiday supplementation regimens [38,41], and was also part of the conclusion of a recent meta-analysis [42].

As an alternative to inducing structural adaptations at the muscle level, the goal of a multiday nitrate supplementation regimen could be to allow a more sustained increase in plasma and muscle nitrate and nitrite concentrations. Based on our previous observations [34] and findings by others [33,38], plasma nitrate concentrations are indeed higher following a multiday nitrate ingestion regimen when compared with a single bolus. Interestingly, recent observations in rodents suggest that daily ingestion of dietary nitrate can also gradually increase nitrate concentrations in skeletal muscle tissue [43]. In that study, a low dietary nitrate diet showed a depletion of skeletal muscle nitrate content when compared with a standard, nitrate containing diet. In contrast, high dietary nitrate supplemented conditions showed increases in skeletal muscle nitrate contents when compared to the standard diet. In fact, a 'nitrate depletion' (via a 7-day low-nitrate content diet) and subsequent 'nitrate loading' strategy (via 7 days of a high-nitrate content diet) resulted in significantly higher nitrate concentrations in skeletal muscle tissue than when the high nitrate diet was not preceded by a low-nitrate period [43]. This suggests that skeletal muscle nitrate storage may show signs of super-compensation following depletion, perhaps similar to what has been reported previously for glycogen storage [44,45]. Furthermore, the study in rodents by Gilliard *et al.* [43] also provided some insight into the possible differences between acute and multiday nitrate supplementation strategies. The researchers observed that skeletal muscle nitrate content in rodents was higher after 7 days of dietary nitrate ingestion when compared with the content observed following acute nitrate ingestion [43]. Thus, although both acute and multiday dietary nitrate supplementation strategies strongly affect skeletal muscle nitrate contents, chronic nitrate supplementation may result in a greater and more sustained increase in intramuscular nitrate concentrations. This concept is visually illustrated in Fig. 2. Given the limited data available though, it is currently unknown to what extent various factors such as storage capacity, half-life of nitrate stored in skeletal muscle tissue, as well as the amount and source of dietary nitrate provided may impact the effectiveness of acute vs multiday nitrate ingestion on skeletal muscle nitrate contents. Interestingly, the recent study by Wylie *et al.* only observed a reduction in skeletal muscle nitrate content when exercise was performed with elevated nitrate stores from dietary nitrate supplementation [12]. Based on this, it would be interesting to determine whether increases in skeletal muscle nitrate content beyond a certain threshold are associated with effective local utilization of nitrate and specific physiological effects. Future studies could provide more insight by comprehensively assessing the impact of different nitrate supplementation regimens (e.g. source and dose) on muscle tissue nitrate contents. Observed changes in plasma and skeletal muscle nitrate concentrations may give some indication of the added benefits of a multiday supplementation protocol, especially if these changes can be associated with enhanced exercise performance effects.

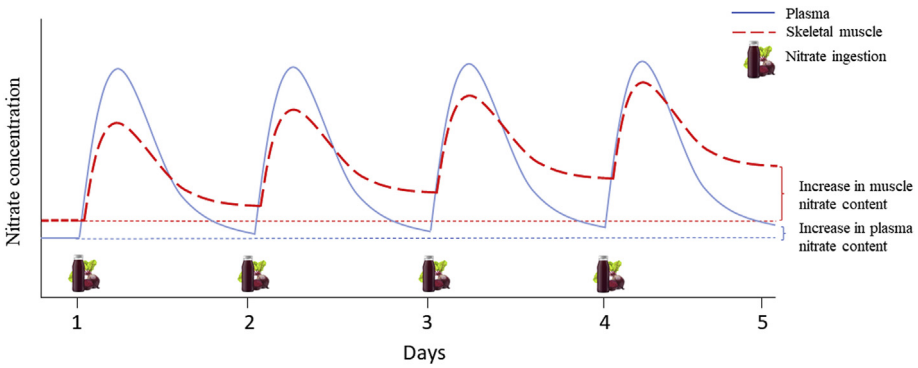

Fig. 2. Visual representation of the theoretical concept of changes in plasma and skeletal muscle nitrate content following acute and multiday dietary nitrate supplementation.

5. What about skeletal muscle nitrite?

Although in our previous study we measured nitrate concentrations in human skeletal muscle tissue, our analysis approach did not allow us to detect nitrite in skeletal muscle tissue in the post absorptive state, as well as following a single bolus ingestion of dietary nitrate [11]. However, the recent study by Wylie *et al.* [12] used a nitrite preserving solution and showed basal skeletal muscle nitrite concentrations to be in the range of ~4 nmol/g skeletal muscle tissue. Ingestion of a 12.8 mmol dietary nitrate bolus did not significantly increase these concentrations. Although the reason for this is currently unclear, it may suggest that significant changes in muscle nitrite concentrations require a different supplementation strategy (e.g. higher dose, or multiday supplementation), or conditions that strongly stimulate the nitrate-nitrite-NO pathway such as hypoxia or acidosis due to exercise. For example, a study in rodents showed a decrease in muscle nitrate and a transient increase in muscle nitrite concentrations in exercised skeletal muscle tissue, without prior ingestion of dietary nitrate [46]. The authors proposed the increase in nitrite to result from nitrate to nitrite reduction within skeletal muscle tissue, which was most likely stimulated by the hypoxic and metabolically acidic environment created by the exercise stimulus [46]. The recent study by Wylie *et al.* assessed this in healthy, young human participants following acute ingestion of nitrate-rich beetroot juice [12]. In contrast to what was observed in rodents [46], muscle nitrite concentrations showed no changes following high-intensity exercise performed both with, as well as without prior dietary nitrate intake. Furthermore, the dietary nitrate intervention also failed to significantly improve high-intensity exercise time to exhaustion. Therefore, it remains unclear whether human skeletal muscle nitrite concentrations will change as a result of exercise, and whether such potential changes can be associated with improvements in exercise performance. Future research will need to elucidate this while also taking factors into account that may modulate these effects, such as supplementation duration, as well as exercise type and duration.

Apart from nitrite being generated from nitrate reduction in skeletal muscle, it may be that nitrite itself is also buffered in skeletal muscle tissue. Local storage of nitrite would in fact minimize the reduction steps required to increase NO bioavailability. However, the short half-life of nitrite in blood (~110 s), as well as the reported bioactivity of nitrite as a signaling molecule [47] might arguably favor nitrate to serve as a more stable precursor for the nitrate-nitrite-NO pathway in skeletal muscle tissue. As already discussed, this would only seem useful if nitrate can be locally reduced, or transported towards a nearby site where nitrate reduction can take place. Interestingly, similar to what has been observed in rodent skeletal muscle tissue [10], the nitrate reducing enzyme XOR has been shown to be present in human skeletal muscle and suggests similar nitrate reducing capabilities in humans [12,48]. As such, future work should establish whether human skeletal muscle tissue is capable of not only buffering, but also reducing nitrate to nitrite locally. In line with what has been done in rodents by Piknova *et al.* [46], this could be done by combining *in vivo* measurements that quantify metabolism of ingested nitrate with *in vitro* measurements of nitrate and nitrite reduction activity in human skeletal muscle tissue. Furthermore, the collected muscle samples could also be used to assess possible changes in mitochondrial function, in line with the studies by Larsen *et al.* [16] and Whitfield *et al.* [40]. However, the *in vivo* measurements may prove challenging when trying to distinguish nitrite formed from nitrate reduction, and nitrite formed as a metabolite of the L-arginine and NOS pathway. The use of ^{15}N labeled nitrate may provide a solution, as this has previously been applied to track the metabolism of exogenous nitrate [49]. This approach may allow further quantification of the functional connection between intramuscular nitrate storage, the subsequent *in vivo* reduction of nitrate to nitrite and NO, and ultimately the physiological effects of increased NO bioavailability.

6. Nitrate utilization and storage in other organs

While this review has primarily focused on discussing the potential role of nitrate storage in skeletal muscle tissue, storage of nitrate may be equally relevant in other organs. Several studies propose nitrite to be an effective precursor of NO in different organs in humans [50,51]. However, the (basal) content of nitrate (and nitrite) in different organs has currently only been assessed in rodents [10]. Piknova *et al.* [10] and Gilliard *et al.* [43] previously showed that skeletal muscle nitrate concentrations prior to and following multiple days of supplementation can be 17-fold higher than the nitrate concentrations measured in internal organs (based on concentrations measured in liver). Based on these findings of 'selective' nitrate storage in different tissues, it could be speculated that human skeletal muscle tissue may be capable of storing more nitrate as compared to other human organs, supporting the notion of a nitrate buffering role for skeletal muscle tissue. Yet, these findings remain to be confirmed in humans by determining nitrate content not only in skeletal muscle but also in other human organs. Interestingly, organs such as the liver, kidneys, intestines, and to a lesser extent the heart have instead been reported to exhibit nitrate to nitrite reductase activity [52]. As with skeletal muscle tissue, the exact role of a local nitrate-nitrite-NO pathway system in these organs remains to be determined. It is not unlikely that the presence of this system may allow NO bioavailability to be increased during ischemia and/or hypoxia, perhaps stimulating cytoprotective effects (i.e. hypoxic vasodilation and attenuating ischemia-reperfusion injury) [50,51]. Nitrate stored close to or even within the organ may then represent a stable precursor pool for the nitrite and NO used during hypoxic conditions. Alternatively, some organs, as has been suggested for the liver, may not show signs of storage and may instead only be capable of enzymatically reducing nitrate and nitrite present in the circulation [10]. It seems that further assessment of the role of nitrate and nitrite in different organs is

necessary and could increase our knowledge of the possible health benefits of dietary nitrate. This will however require delicate research, as extremely high concentrations of NO have also been associated with inflammation resulting from a reaction between NO and superoxide [53]. It is however unclear whether such toxic amounts of NO produced by iNOS present in myeloid cells can be associated with nitrate stored and utilized in muscle and/or in other organs. Nonetheless, though future research into the storage and/or utilization of dietary nitrate *in vivo* may reveal opportunities for dietary nitrate to aid in the prevention and perhaps even treatment of diseases associated with defective NO production, it is of key importance to also explore possible health related risks, such as those posed under particular redox conditions.

7. Conclusion

There is currently limited insight in the exact mode of action underlying the ergogenic effects of dietary nitrate supplementation. There is also very little known about the metabolism of dietary nitrate following ingestion and the subsequent appearance of nitrate and nitrite in the circulation. Skeletal muscle is now being proposed as a possible site of nitrate buffering, and the available data supports the idea of skeletal muscle tissue as a nitrate reservoir. The capacity to store nitrate in skeletal muscle and/or the ability to increase such storage may soon be considered an important factor modulating the effectiveness of dietary nitrate supplementation to improve performance. However, the exact (intramuscular) location of this nitrate reservoir in skeletal muscle is still unclear, and we can currently only speculate it to be in close proximity of blood vessels and/or being more prominent in type II when compared to type I skeletal muscle fibers. The relevance of muscle as such a nitrate buffer strongly depends on the ability of muscle to reduce the stored nitrate to nitrite. Although evidence of nitrate reduction has been observed in rodent muscle and in several human organs, it is currently unknown whether reduction of nitrate to bioactive nitrite and NO occurs in human skeletal muscle tissue. Further work is needed to establish whether the storage of nitrate in human skeletal muscle tissue (and other organs) is as relevant as we think it may be.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2020.03.025.

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