Spleen contraction and Hb elevation after dietary nitrate intake

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INTRODUCTION

During apnea, diving mammals exhibit several responses to maintain vital physiological functions based on the available oxygen ($O_2$) stored in the lung, blood, and other tissues. In humans, at least two such responses are evident: the cardiovascular diving response (diving response) (7, 23) and the hematomorphic response resulting from spleen contraction (47). The diving response, characterized by selective vasoconstriction, bradycardia, and reduced cardiac output, may reduce $O_2$ cost by a combination of greater reliance on anaerobic metabolism in peripheral tissues and a reduced myocardial $O_2$ consumption (11, 23).

The spleen is known to serve as a dynamic reservoir for erythrocytes in many mammals, including humans (26, 54). Sympathetic nerve fibers densely innervate the spleen (45), and the organ can be recruited upon sympathoexcitation during various types of physiological stress to elevate blood $O_2$ storage and transportation (54). Spleen contraction has been found to be induced by apnea (26), exercise (55), and hypoxia (36) and to be modified by hypercapnia (44), resulting in a more powerful contraction during apnea compared with during simulated high altitude (36). In humans, the splenic reservoir can contain 200–250 mL of blood (54), with double the hemoglobin (Hb) concentration of normal blood (37). A decrease in spleen volume typically increases the total amount of circulating erythrocytes between 3% and 6%, with some individuals responding with up to 10% increases (44). The expulsion of stored erythrocytes may temporarily increase the circulating $O_2$ storage and CO$_2$ buffering capacity, thus improving performance in both normoxic and hypoxic conditions in humans. For example, the contraction of the spleen has been found to prolong voluntary apneic duration across apnea series (9, 47), and spleen volume is found to correlate with competitive apneic diving performance (50).

Ingestion of dietary nitrate ($NO_3^-$) has been shown to increase plasma nitrite ($NO_2^-$) and nitric oxide (NO) concentrations through bioconversion in the $NO_3^-$-NO$_2^-$-NO enterosalivary pathway (29). It has been shown that 6 days of dietary nitrate consumption improved exercise tolerance and reduced $O_2$ consumption (6). Since then, many studies using different exercise modalities have indicated positive effects (27, 31, 38, 57), whereas others show a negligible or a lack of effect on exercise performance (12, 46, 52, 53). Proposed mechanism of action includes changes to muscle energy metabolism (5), improved mitochondrial efficiency (33), improved muscle contractility

NEW & NOTEWORTHY

This is the first study to examine changes of spleen volume and circulating Hb following dietary $NO_3^-$ supplementation. After dietary $NO_3^-$ ingestion, the spleen volume at rest was reduced and Hb was elevated. The spleen contains a dynamic red blood cell reservoir, which can be mobilized and facilitate oxygen transport during various types of physiological stress. This study has revealed an additional, previously unexplored mechanism possibly contributing to the ergogenic effects of dietary $NO_3^-$.

apnea; arterial oxygen saturation; beetroot; breath-holding; spleen contraction

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function (24), and blood flow (21). Detailed reviews on the ergogenic effects of NO$_3^-$ supplementation on exercise performance are beyond the scope of this study but have been published elsewhere (29, 41).

With respect to voluntary apnea, similar disunity of studies also exists. For example, acute NO$_3^-$ supplementation has been reported by several studies to attenuate the reduction in arterial oxygen saturation (SaO$_2^-$) across the apnea and prolonged apneic duration (14, 18, 40), whereas two studies did not find such an effect (10, 51). Although these studies did not measure O$_2$ uptake, a previous study has reported a decrease in O$_2$ uptake at rest following NO$_3^-$ ingestion (33), thus implying a reduction in basal metabolic activity that could be beneficial during apnea.

None of the studies investigating performance and physiological responses to exercise in apneic and eupneic protocols included measurements related to the hematological effects of the spleen, and it is currently unknown if NO$_3^-$ supplementation influences splenic contraction and the associated Hb increase. Notably, administration of the NO donor drug nitroglycerin has been shown to have vasodilatory effects on the splanchnic vasculature in human (28) and reduce blood perfusion to the spleen in dogs (13, 16). Elevations in NO bioavailability could infer the vascular and contractile properties of the spleen. Therefore, our objective was to investigate whether dietary NO$_3^-$ supplementation influences spleen volume reduction and associated increase in Hb during apnea.

METHODS

Participants. Eight healthy and recreationally active volunteers (5 men and 3 women) of means ± SD age = 24 ± 1 yr, height = 178 ± 3 cm, body mass 74 ± 5 kg, and vital capacity = 5.3 ± 1.3 L participated in the study. Most participants had no experience of breath-holding, whereas two had some experience but were not currently in training. Participants gave their written informed consent after receiving written and oral information about the study procedures and potential risks of participating in the study. The protocol was approved by the regional ethical committee at Umeå University, Sweden, and complied with Swedish laws and the Declaration of Helsinki for research involving human subjects.

Nitrate supplementation. Participants were assigned in a double-blinded, randomized, crossover, and weighted design to consume an oral dose of 70 mL of concentrated NO$_3^-$ -rich beetroot juice (BR) or placebo (PL) 2 h before the apnea tests on different days, with at least a 24-h washout period. The BR contained ~5.0 mmol NO$_3^-$ and PL contained ~0.003 mmol NO$_3^-$ (James White Drinks Ltd., Ipswich, UK). BR is indistinguishable from PL, as it is identical in color, taste, smell, and texture (32). PL is made by filtering NO$_3^-$ -containing BR through a Purolite A520E ion-exchange resin that selectively removes NO$_3^-$ (32). The tests were identical except for the measurement of anthropometric data collected during the first visit.

Experimental protocol. Participants were instructed to arrive at the laboratory at least 2 h postprandially, adequately hydrated, and without having consumed caffeine, nicotine, or alcohol within 12 h. Participants were also asked to refrain from strenuous exercise or breath-hold activities within 12 h. Participants were also instructed to mimic their prearrival routine for the second visit. After collecting anthropometric data, participants rested for 10 min in the prone position. The participant’s head rested on a stable platform with their arms resting on its sides in a forward position. Aligning with previous studies (18, 48), the apnea protocol consisted of the following steps: before each apnea, subjects were given a 2-min countdown; at 30 s, the headrest platform was removed in order for the subject to hold the head straight upward facing down and give a nose clip; the final 10 s were counted and, without prior hyperventilation, subjects exhaled fully and took a deep, but not maximal last inspiration and started the apnea. This procedure has been shown to result in a lung volume of ~85% of vital capacity (49) and avoids overfilling of the lung and reduces the risk of syncope caused by impeding venous return (3). Participants performed a 2-min (with time cues) and a maximal duration apnea (without time cues)—separated by 2 min of eupneic rest with the head-rest platform. In case peripheral oxygen saturation (SpO$_2^-$) decreased below 65%, during either of the apneas, the subjects would be told to resume breathing to avoid hypoxic syncope. After each apneic episode, the nose clip was removed. Laboratory temperature was 21 ± 1°C. Participants were tested at the same time on both occasions and instructed to perform similar levels of activity and diet on the day of testing.

Measurements. Peripheral oxygen saturation (SpO$_2^-$) and heart rate (HR) were continuously monitored (Biox 3700e, Ohmeda, Madison, WI). Spleen maximal diameters were measured via ultrasonic imaging (M-Turbo ultrasound system, FUJIFILM Sonosite Inc, Bothell, WA) every minute beginning 3 min before first apnea, between the apneas, and for 10 min after the maximal apnea. Capillary blood samples were collected at 2 min before the first apnea (baseline), directly after the maximal apnea, and 10 min after the maximal apnea, and analyzed in triplicate via a portable hemoglobin analyzer (Hemocue AB, Angelholm, Sweden).

Analysis. Comparative effects between BR and PL on the submaximal and maximal apneas were based on changes in physiological variables and apneic duration. Baseline values for HR and SpO$_2^-$ were obtained from a 30-s period starting 1 min before the first apnea. SpO$_2^-$ nadir was defined as lowest SaO$_2^-$ measured after the apnea. The reduction in HR during apnea was defined as the drop in HR from baseline to the minimum (5 s) value of HR.

Spleen volume was calculated using the Pilström equation ([Lπ (WT - T$^3$)/3] (48) based on the diameters of the spleen obtained using triaxial ultrasonic imaging to determine maximal length (L), thickness (T), and width (W).

Mean preapneic spleen volumes were calculated from the two measurements before the first apnea (2 and 1 min before the first apnea). Spleen volume changes arising from apneas and recovery were obtained by comparing baseline values with values obtained directly after and during the last minute of the 2-min recovery period (17). Baseline Hb was collected 2 min before the first apnea. Changes in Hb were obtained by comparing baseline values with mean values obtained immediately after the submaximal and maximal apnea after recovery.

Statistics. All statistical analyses were calculated with SPSS (v. 19; IBM Statistics). Normality was investigated using the Shapiro–Wilk test. Changes in spleen size and Hb were assessed using one-way repeated-measures analysis of variance (RM ANOVA). Differences between supplemental conditions were tested by consideration of the interaction term (time × condition) derived from the two-way RM ANOVA. P values of the post hoc comparisons were corrected using Bonferroni correction for multiple comparisons. SpO$_2^-$ and HR were assessed using Wilcoxon matched-pairs signed rank test and paired Student’s t test, respectively, for comparison between BR and PL conditions. Data are presented as means ± standard deviation (SD). Statistical significance was accepted at P ≤ 0.05.

RESULTS

Maximal apnea duration. Maximal apnea durations were similar between PL (171 ± 26 s) and BR conditions (172 ± 33 s; P = 0.944).

Spleen volume. At baseline, mean spleen volume was reduced by 22.9% with BR (222.5 ± 74.5 mL), compared with PL (288.1 ± 102.8 mL; P = 0.046; Table 1; Fig. 1), whereas spleen volume was not different between PL and BR for the other test conditions (NS). A two-way RM ANOVA revealed
no time-condition interaction for spleen volume. Separate consideration of BR and PL spleen volume data at submaximal apnea, maximal apnea, and recovery revealed that the mean level of spleen volume was not different from baseline in BR measurements, whereas it was reduced by 25.5% (P = 0.002) and 37.2% (P = 0.027) from baseline after submaximal and maximal apnea PL measurements, respectively (Fig. 1; Table 2).

**Hemoglobin concentration.** At baseline, mean Hb concentration was increased by 3.0% from 145.4 ± 9.6 g/L with PL to 149.8 ± 7.3 g/L with BR (P = 0.015). Mean Hb concentration was not different between PL and BR at maximal apnea (P = 0.090) and at recovery (P = 0.247; Table 2). No significant time × condition interaction effect for Hb concentration was identified using two-way RM ANOVA. Separate analysis of BR and PL Hb concentration revealed that the mean levels of Hb concentration were increased from baseline both in the PL and BR measurements. Post hoc analysis of Hb measurements showed that Hb concentration increased from baseline to maximal apnea in both BR and PL, whereas Hb concentration at recovery was not different from baseline for BR or PL (NS; Table 2).

**Heart rate and arterial oxygen saturation.** Baseline HR was similar between PL and BR conditions (P = 0.156; Table 1). Mean reduction in HR during apneas was similar between conditions for both submaximal duration (PL = −43 ± 12 beats/min; BR = −39 ± 16 beats/min; P = 0.282) and maximal duration apneas (PL = −43 ± 14 beats/min; BR = −40 ± 11 beats/min; P = 0.249).

Baseline SpO2 was not different between PL and BR conditions (P = 0.070; Table 1). The resulting reduction in mean SpO2 nadirs following apneas was similar between conditions both at submaximal apnea (−5.0% ± 2.7% with PL and 5.1% ± 3.3% with BR; P = 0.911) and maximal duration apneas (−11.9 ± 4.6 with PL and 10.9% ± 4.8% with BR; P = 0.879). No subject demonstrated an SpO2 below 65%.

**DISCUSSION**

The principal finding was that dietary NO3− supplementation induced a reduction in spleen volume at rest. The reduction was concomitant with an increase in Hb concentration, suggesting that stored erythrocytes in the spleen are ejected into the circulation following the NO3−-derived volume reduction. This suggests that a single dose of NO3− has the potential to elicit splenodilative Hb elevation, which depending on the exercise modality may contribute to beneficial effects during short-term exercise and hypoxic conditions. This represents a novel mechanism possibly contributing to the previously observed ergogenic effects of dietary NO3− (6, 29, 34). The spleen volume reduction observed already at rest could help explain the observed apneic prolonging effects after NO3− ingestion (14, 18, 40), especially as there was no effect of dietary NO3− on the diving response.

Previous studies have found that spleen contraction in humans occurs during physiological stress such as intense exercise, apnea or apneic diving, high-altitude exposure, hypovolemic shock, induced conditions of hypoxia and hypercapnia, and low-dose epinephrine infusion (8, 20, 36, 43, 54). However, as the reduction in spleen volume was found in a resting condition at sea level, it cannot be explained by these factors. The mechanistic bases for the decreased spleen volume observed following dietary NO3− remain unclear. We speculate that it could be related to changes in vascular resistance associated with NO3− intake. Dietary NO3− via its reduction to NO2− and NO is known to promote systemic vasodilation by several mechanisms (59). In addition, NO2− has been shown to cause dose-dependent vasodilation in healthy participants (15). Interestingly, an acute dose of the organic nitrate nitroglycerin has been found to have a vasodilatory effect on the splanchnic circulation by reducing vascular resistance (28). Another study showed a 23% increase in splenic vein diameter following nitroglycerin administration (56). Furthermore, it has been previously shown that nitrates induce an initial vasodilative response followed by reflex vasoconstriction in the splanchnic and mesenteric circulations (1, 22).

Two studies in dogs have shown that the infusion of nitroglycerin can induce a significant reduction in blood perfusion to the spleen (13, 16). Given that the splenic artery is reflexively constricted after the initial vasodilative response upon NO3− ingestion, the decreased volume could potentially result from a passive collapse of the spleen following reduced splenic artery blood flow, potentially in combination with reduced splenic vein resistance. Alternatively, the sympathetic reflex discharge could induce an active contraction of the spleen itself without a change in splenic artery blood flow. Immunohistochemical staining has revealed contractile proteins within the walls of the arteries, veins, splenic capsule, and trabecula (42), and Palada et al. (39) showed that the spleen contraction during apnea was active rather than a passive collapse due to sympathetically mediated splenic arterial constriction, as suggested earlier (2).

Interestingly, a study by Andrew et al. (4) in rats reported increased intrasplenic microvascular pressure and increased
fluid extraversion into the systemic lymphatic system following infusion of a NO donor (S-nitroso-N-acetylpenicillamine). It cannot be ruled out that increased NO bioavailability from the NO\textsubscript{3}→NO\textsubscript{2}→NO pathway may enhance the arteriovenous flow and cause fluid efflux from the splenic vasculature into the lymphatic system in human. However, it is not known if an increased fluid extraversion may lead to reduced volume of the spleen.

As indicated by the current study, the spleen volume reduction during apnea is followed by an increase in Hb concentration, which was of a similar magnitude as found in previous studies (17, 47). The correlation between spleen contraction and Hb is strong, and it is estimated that ~60% of the change in Hb can be directly attributed to the emptying of the spleen's stored blood content. However, during the apneas after BR ingestion, Hb increased by a similar relative magnitude as during the PL apneas despite the difference in spleen volume at baseline. This finding might imply either that the spleen did not reach its minimum volume with BR or that erythrocytes were also released from other sources, where the liver is a likely candidate.

In some previous studies, it has been shown that the transient increase in the circulation of erythrocytes is associated with improved apneic performance, likely by increased O\textsubscript{2}-carrying capacity (47). Three previous studies have demonstrated increased apneic performance following NO\textsubscript{3} supplementation (14, 18, 40). In these studies, it was suggested that the improved performance could be related to a reduced metabolic rate following dietary NO\textsubscript{3} supplementation, although direct metabolic measurement was not done. The lack of change in maximal apneic duration in the present study is in accordance with the Schiffer et al. (51) study of well-trained apneic athletes. In addition, the present study did not show any effect on SpO\textsubscript{2} during the time-limited apnea, which is in contrast to the studies by Engan et al. (18) and Patrician and Schagatay (40).

One explanation for the lack of difference in apneic duration, or O\textsubscript{2} conservation (evidence by SpO\textsubscript{2} nadir), could be the general unexperienced nature of the current participants. It has been shown previously that participants with little/no training in apnea terminate the apnea due to psychological factors, rather than a physiological breaking point (7, 35). Thus, beneficial effects of NO\textsubscript{3} ingestion on apneic performance could only present in trained divers, who can override the psychological discomfort.

In agreement with findings by Engan et al. (18), no difference in HR reduction during apnea was found between PL and BR, thus inferring that the diving response was not affected by acute NO\textsubscript{3} ingestion. In contrast, Schiffer et al. (51) found a reduced diving response and concluded that oral administration of NO\textsubscript{3} could counteract the sympathetically induced constriction of vessels by NO-induced vasodilation. Unfortunately, continuous blood pressure measurement was not obtained in the current study. Several studies have shown reduced blood pressure at rest in healthy adults after NO\textsubscript{3} intake (25), and it cannot be ruled out that NO\textsubscript{3} ingestion influenced peripheral arterial resistance both at rest and during apneas. However, the lack of difference in HR reduction during apneas suggests that any potential vasodilation was not compensated by an increase in HR. Additionally, differences in supplementation routines and study protocols might have influenced the results.

It should also be noted that studies investigating exercise performance following dietary NO\textsubscript{3} ingestion have reported both positive and null effects (30). To the authors’ knowledge, no studies investigating the ergogenic effect of dietary NO\textsubscript{3} supplementation have included any measurement related to the hematological effect of the spleen. It is possible that the splanchnic response to NO\textsubscript{3} supplementation could help explain the previously observed effects on performance and possibly the discrepancy in results in both apneic and nonapneic endurance performances.

An interesting feature was that the spleen volume and Hb recovered to the individual BR and PL baseline levels following a 10-min resting period after the maximal apnea. It has been shown that a short break of a few minutes between series of apneas is not sufficient to allow the spleen volume to normalize to the initial levels. Previous studies have demonstrated that it takes at least 8 min before spleen volume returns to normal after an apnea series (9, 48). The fact that the spleen relaxation was not affected by NO\textsubscript{3} administration implies that the spleen can act as a dynamic blood reservoir even after an initial NO\textsubscript{3}-induced volume reduction. It should be noted that the reduction in spleen volume from baseline following apneas was evident during PL but not during BR. It appears likely that the potential for volume reduction during apneas was reduced following BR since the spleen volume was already reduced at baseline. It has been shown that the spleen contraction, and the associated increase in Hb, is maximized after three to five apneas, after which the spleen volume is not further reduced (48). However, it is not known if the maximal capacity for spleen volume reduction obtained during apnea is related mainly to the mechanical properties of the splenic capsule, to

**Table 2. Summary of results for spleen volume and hemoglobin concentration**

<table>
<thead>
<tr>
<th>Spleen volume, mL</th>
<th>Baseline</th>
<th>Submax Apnea</th>
<th>Max Apnea</th>
<th>Recovery</th>
<th>Within-Group (P Values)</th>
<th>Between-Group (P Values)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Condition</td>
<td>Time</td>
</tr>
<tr>
<td>PL</td>
<td>288.1 ± 102.8</td>
<td>214.6 ± 90.0*</td>
<td>181.0 ± 74.2*</td>
<td>300.7 ± 117.2</td>
<td>0.029</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>(P = 0.002)</td>
<td>(P = 0.026)</td>
<td>(P = 0.405)</td>
<td></td>
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<tr>
<td>BR Hb, g/L</td>
<td>222.5 ± 74.5</td>
<td>187.7 ± 95.7</td>
<td>176.4 ± 87.0</td>
<td>247.0 ± 96.0</td>
<td>0.164</td>
<td></td>
</tr>
<tr>
<td>PL</td>
<td>145.4 ± 9.6</td>
<td>151.5 ± 10.7*</td>
<td>145.2 ± 10.7*</td>
<td>148.2 ± 11.1</td>
<td>0.002</td>
<td>0.034</td>
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<tr>
<td></td>
<td>(P = 0.001)</td>
<td>(P = 0.943)</td>
<td>(P = 0.501)</td>
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<tr>
<td>BR</td>
<td>149.8 ± 7.3</td>
<td>154.8 ± 7.2*</td>
<td>148.2 ± 11.1*</td>
<td></td>
<td>0.002</td>
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<td></td>
<td>(P &lt; 0.001)</td>
<td>(P = 0.001)</td>
<td>(P = 0.01)</td>
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</table>

Values are means ± SD. BR, beetroot; Hb, hemoglobin; Inter, interaction; PL, placebo. Student’s t test (i.e., P value); One-way ANOVA (i.e., within-group) and two-way ANOVA (i.e., between-group) statistics are presented. *P < 0.05 from baseline (within-group effect).
neural or circulatory factors, or to a combination of these. It is likely that mechanical properties of the spleen set the ultimate limits for contraction, but it is difficult to know from experiments using single stimuli where this limit is; in apnea divers, with very large spleens, it was found that ~50% spleen volume reduction occurred after 2 min of apnea (50) whereas the largest relative reduction seen in our study was 37%, thus potentially leaving a margin to maximal contraction. It is also possible that a higher O2-carrying capacity resulting from elevated Hb at baseline following BR reduced the chemoceptive drive associated with apnea-induced hypoxia and is responsible for initiating the spleen contraction. Further studies are needed to determine factors that limit spleen contraction.

Mechanistic studies which have indicated that NO3 alters muscle contractility and mitochondrial functions have used a long (3–7 days) supplementation period. In contrast, some studies showing negligible effects of NO3 in elite athletes have used acute (2–3-h) or short-term (3-day) supplementation (29). It cannot be ruled out that longer-term supplementation and/or higher NO3 doses may influence the magnitude of spleen volume change, the diving response, and maximal apneic performance than short-term administration.

Plasma NO3 and NO2 were not measured after PL or BR supplementation. However, the dosing protocol was based on earlier reports which showed that 5 mmol NO3 consumption resulted in a 36% increase in plasma NO2 post ingestion (58). Although the small sample size may limit the generalizability of the results, the current study provides intriguing evidence of an additional means by which dietary NO3 could influence circulating Hb and potentially performance in endurance sports. Additional research is required to reveal the effect of NO3 on the mesenteric and splanchic circulations to clarify the responsible mechanisms, and the potential ergogenic effects during exercise and hypoxic conditions should be further studied.

CONCLUSION
After short-term dietary NO3 supplementation, we found a reduced spleen volume at rest compared with placebo. The concomitant increase in Hb suggests that oral ingestion of NO3 induces a spleen-related blood boosting. The acutely elevated Hb could temporarily enhance O2-carrying capacity and increase CO2-buffering capacity, with several effects on human performance. This is a novel finding of a mechanism possibly contributing to the ergogenic effect of dietary NO3.

ACKNOWLEDGMENTS
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GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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