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Relationships between nitric oxide biomarkers and physiological outcomes following dietary nitrate supplementation

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ABSTRACT

Dietary nitrate (NO_3^-) supplementation can increase nitric oxide (NO) bioavailability, reduce blood pressure (BP) and improve muscle contractile function in humans. Plasma nitrite concentration (plasma [NO_2^-]) is the most oft-used biomarker of NO bioavailability. However, it is unclear which of several NO biomarkers (NO_3^-, NO_2^-, S-nitrosothiols (RSNOs)) in plasma, whole blood (WB), red blood cells (RBC) and skeletal muscle correlate with the physiological effects of acute and chronic dietary NO_3^- supplementation. Using a randomized, double-blind, crossover design, 12 participants (9 males) consumed NO_3^--rich beetroot juice (BR) (~12.8 mmol NO_3^-) and NO_3^--depleted placebo beetroot juice (PL) acutely and then chronically (for two weeks). Biological samples were collected, resting BP was assessed, and 10 maximal voluntary isometric contractions of the knee extensors were performed at 2.5–3.5 h following supplement ingestion on day 1 and day 14. Diastolic BP was significantly lower in BR (-2 ± 3 mmHg, $P = 0.03$) compared to PL following acute supplementation, while the absolute rate of torque development (RTD) was significantly greater in BR at 0–30 ms (39 ± 57 N m s^{-1}, $P = 0.03$) and 0–50 ms (79 ± 99 N m s^{-1}, $P = 0.02$) compared to PL following two weeks supplementation. Greater WB [RSNOs] rather than plasma [NO_2^-] was correlated with lower diastolic BP ($r = -0.68$, $P = 0.02$) in BR compared to PL following acute supplementation, while greater skeletal muscle [NO_3^-] was correlated with greater RTD at 0–30 ms ($r = 0.64$, P=0.03) in BR compared to PL following chronic supplementation. We conclude that [RSNOs] in blood, and [NO_3^-] in skeletal muscle, are relevant biomarkers of NO bioavailability which are related to the reduction of BP and the enhanced muscle contractile function following dietary NO_3^- ingestion in humans.

1. Introduction

Nitric oxide (NO) is a signalling molecule that plays an essential role in regulating vasodilation [1], blood flow and tissue oxygen delivery [2], and skeletal muscle contraction [3,4]. Nitrate (NO_3^-), a molecule found in green leafy vegetables and beetroot, has been identified as a good source of NO via the reduction of NO_3^- to nitrite (NO_2^-) and to NO [5,6]. In 2006 and 2007, Larsen and colleagues reported that 3 days of dietary NO_3^- ingestion resulted in a significant reduction in resting blood pressure (BP) [7] and a reduced oxygen cost during exercise [8] in humans. These findings were then supported by other original research as well as meta-analysis [9–12].

In humans, NO_3^--reducing bacteria in the oral cavity are primarily responsible for the biochemical reduction of circulating NO_3^- to bioactive NO_2^- [13,14]. The swallowed NO_2^- then enters the systemic circulation, elevating plasma [NO_2^-], which is recognized as a key biomarker of NO bioavailability [15]. Despite being the most oft-used biomarker of NO bioavailability, the correlation between increased plasma [NO_2^-] and changes in physiological variables following dietary NO_3^- ingestion has been inconsistent. While some studies have reported that the increased plasma [NO_2^-] was significantly correlated with the reduced BP [10,16–18], and improved exercise performance [19], other studies found no relationship [20–23]. These results suggest that plasma [NO_2^-] may not necessarily be the most appropriate indicator of the physiological responses to dietary NO_3^- ingestion in humans.

In addition to increased [NO_3^-] and [NO_2^-], the concentration of S-nitrosothiols ([RSNOs]) in blood is also increased after dietary NO_3^- ingestion [24,25]. As a storage form of intravascular NO, RSNOs play important roles in smooth muscle relaxation [26] and vasodilation [27]. In addition to blood, recent research also suggests that skeletal muscle may play a central role in the production, storage, and metabolism of NO_3^- [28]. The [NO_3^-] in skeletal muscle has been found to be higher

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than that of blood and other organs in both rodents [29,30] and humans [28,31]. The existence of the NO_3^- reductases, xanthine oxidoreductase (XOR) and aldehyde oxidase (AO), in skeletal muscle implies that NO_3^- in skeletal muscle may serve as a source of NO [28]. Due to the high reactivity and short half-life of NO [32,33], the current consensus is that NO is produced when and where it is needed. This would suggest that, compared to increased NO biomarkers in the circulation, the local muscular NO_3^- and NO_2^- stores are more likely to regulate muscle contractile activity [23]. However, it is currently unclear which NO biomarkers (NO_3^-, NO_2^-, RSNOs) in which tissues (different blood compartments and skeletal muscle) are the most relevant biomarkers with which to reflect the physiological effects of dietary NO_3^- ingestion on BP and muscle contractile function.

Compared to acute NO_3^- ingestion, long-term NO_3^- supplementation has been reported to be more effective at altering exercise performance [10]. The increased exercise tolerance and improved muscle contractile function following chronic NO_3^- intake may derive from the altered expression of relevant proteins involved in mitochondrial respiration [34] and muscle Ca^{2+} handling [35,36] although it should be noted that physiological effects during exercise have been reported in the absence of changes in Ca^{2+} handling proteins and mitochondrial respiration efficiency [37,38] such that the underpinning mechanism remains controversial.

Therefore, the purpose of this study was to investigate the relationships between differences in key NO biomarkers in different blood compartments and skeletal muscle and differences in BP and muscle contractile function between placebo and dietary NO_3^- following acute and chronic supplementation. We hypothesised that in healthy adults, differences in BP would be more strongly correlated to the differences in [RSNOs] between PL and BR, while differences in muscle contractile function between PL and BR would be more closely related to differences in skeletal muscle [NO_3^-] and [NO_2^-] than to any NO biomarkers in blood.

2. Methods

Twelve healthy individuals (9 males, 3 females) who were not tobacco smokers or users of dietary supplements or antibacterial mouthwash, volunteered to take part in this study (Table 1). This study conformed to the ethics principles of the Declaration of Helsinki and was approved by the Ethics Committee of the University of Exeter, United Kingdom (Approval number: 21-10-20-B-03). Detailed experimental procedures, related benefits and potential risks were explained to all participants. All participants gave their written informed consent before the study commenced.

3. Experimental procedures

All participants were assigned to receive four different interventions (acute NO_3^--depleted placebo BR, acute NO_3^--rich BR, 2 weeks NO_3^--depleted placebo BR, and 2 weeks NO_3^--rich BR supplementation), in a double-blind and crossover design. The participants were informed that the purpose of the study was to investigate the relationships between differences in key NO biomarkers in blood and skeletal muscle and differences in resting BP and muscle contractile function between PL and BR following acute and chronic dietary supplementation. However, they were not aware of the experimental hypothesis, or which supplement they were consuming. The participants were also asked to refrain from tongue scraping, chewing gum and antibacterial mouthwash use throughout the study to preserve bacteria in the mouth, which is essential in reducing NO_3^- to NO_2^-. They were also asked to maintain their normal diet throughout the study but to record and replicate meals for 24 h before each lab visit to avoid any potential dietary confounders. During an initial screening visit, participants were fully familiarized with the relevant exercise tests to minimize any possible learning effects prior to the experimental lab visits.

Table 1
Participant Characteristics (n = 12, 9 males, 3 females).

Characteristics	Males	Females
Age (year)	25 ± 6	22 ± 2
Body Height (m)	1.74 ± 0.06	1.70 ± 0.05
Body Mass (kg)	74.6 ± 9.2	69.3 ± 5.0
Body Mass Index ($kg.m^{-2}$)	24.7 ± 2.6	24.0 ± 0.8
Resting SBP (mmHg)	105 ± 10	104 ± 1
Resting DBP (mmHg)	58 ± 9	57 ± 1
Peak power output (W)	263 ± 41	170 ± 47

3.1. Acute phase

All participants reported to the lab on two occasions separated by at least 3 days 'washout'. They were asked to avoid strenuous exercise, alcohol, and caffeine intake 24 h prior to each lab visit. All participants visited the lab in the morning (8–9 a.m.) in a rested and fasted state. Upon arriving at the lab, they were instructed to consume either two shots of NO_3^--depleted placebo BR (PL) (~0.08 mmol NO_3^-, Beet It; James White Drinks, Ipswich, UK) or NO_3^--rich BR (~12.8 mmol NO_3^-, Beet It; James White Drinks, Ipswich, UK) followed by a standardized breakfast (72 g oats and 180 ml semi-skimmed milk). Resting BP was measured, blood and skeletal muscle samples were collected, and maximal voluntary isometric contractions of the knee extensors were performed at 2.5–3.5 h post ingestion. The experimental procedure for this acute phase is illustrated in Fig. 1.

3.2. Chronic phase

All participants were asked to take two shots (one in the morning, and one in the evening) of either NO_3^--depleted placebo BR (~0.08 mmol NO_3^-) or NO_3^--rich BR (~12.8 mmol NO_3^-) each day for 2 weeks in a double-blind and crossover design, with at least a 7 day 'washout' period separating each dietary intervention. During this period, they were allowed to maintain their normal diet and physical activity; however, dietary and training diaries were kept throughout the intervention. All participants reported to the lab on 4 occasions over at least 5 weeks. They visited the lab in the morning (8–9 a.m.) in a rested and fasted state following 2 weeks BR supplementation. On experimental lab visit 1 (chronic phase), they were asked to consume 2 shots of the same supplement they had consumed during the past 2 weeks (either PL or BR). Blood and skeletal muscle samples were collected, and resting BP and muscle contractile function (identical to the acute phase described above) were assessed at 2.5–3.5 h post ingestion. The participants were then asked to return to the lab 24 h following supplement ingestion (experimental lab visit 2). On experimental lab visit 2, no additional supplements were given to participants, resting BP was measured, and skeletal muscle samples were collected. Participants were then provided with the alternate supplement to consume for 2 weeks after at least a 7 day 'washout' period, with the same biological samples and variables collected on experimental lab visit 3 and 4. The experimental procedure for this chronic phase is illustrated in Fig. 2.

4. Measurements

Resting BP Resting systolic BP (SBP), diastolic BP (DBP) and mean arterial pressure (MAP) were measured with an electronic sphygmomanometer (Dinamap Pro; GE Medical System, Tampa, FL) following 10 min supine rest at 2.5 h post ingestion. BP was measured 4 times with 1 min rest in between, with the last three measurements averaged to obtain a mean SBP, DBP, and MAP.

[NO_3^-], [NO_2^-] and [RSNOs] determination in plasma, WB and RBC Antecubital venous blood samples were drawn into two 6 ml lithium-heparin vacutainers (vacutainer 1 and vacutainer 2). 800 μl WB sample was extracted from vacutainer 1 and mixed with 200 μl NO_2^-

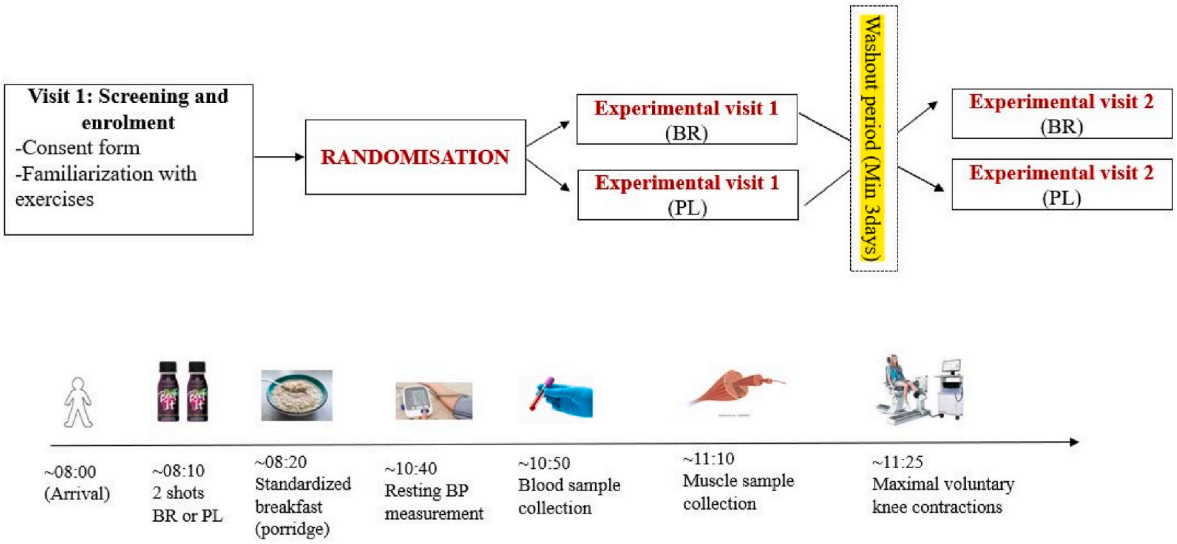

Fig. 1. Schematic diagram of the experimental protocol for acute supplementation
BR: beetroot juice; PL: placebo; BP: blood pressure.

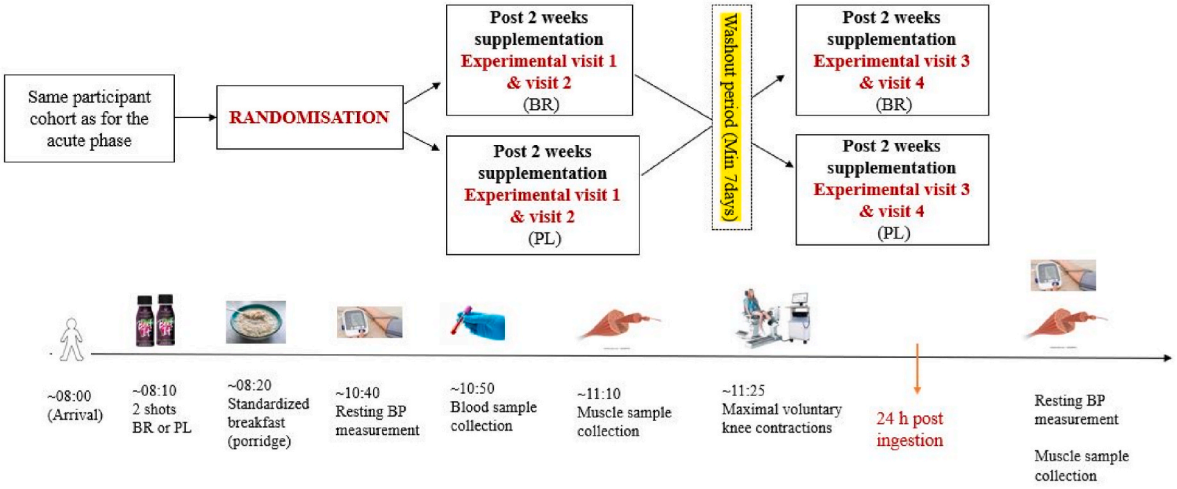

Fig. 2. Schematic diagram of the experimental protocol for chronic supplementation
BR: beetroot juice; PL: placebo; BP: blood pressure.

preservation solution. The NO_2^- preservation solution consists of 4.5 ml deionized water (dH2O), 890.9 mM potassium ferricyanide ($K_3Fe(CN)_6$), 118.13 mM N-ethylmaleimide (NEM) and NP-40 (octyl phenoxylpolyethoxylethanol) added in a 1:9 ratio. Vacutainer 2 was centrifuged at 3300 g and 4 °C for 7 min, within 3 min of collection. Plasma and 900 μl RBC were extracted separately, with 900 μl RBC mixed with 100 μl NO_2^- preservation solution. All blood samples were immediately frozen at −80 °C for subsequent [NO_3^-] and [NO_2^-] determination. Samples were mixed with cold ethanol in a 1:2 ratio (plasma) and a 1:1 ratio (WB and RBC) and were centrifuged at 13000 rpm at 4 °C for 15 min to precipitate proteins [39].

Two further blood samples were also drawn into 6 ml lithium-heparin vacutainers (vacutainer 3 and 4) at the same time. A 150 μl solution that consisted of 2.5 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM NEM, respectively, was added to vacutainer 3, and a 150 μl solution that consisted of 2.5 mM EDTA, 10 mM NEM and 10 mM potassium ferricyanide, respectively, was added to vacutainer 4 to block unreacted thiol groups and stabilize RSNOs in biological samples. A 1 ml WB sample was obtained from vacutainer 4 and mixed immediately with 4.0 ml hypotonic lysis solution that consisted of 2.5 mM EDTA, 10 mM NEM, and 10 mM potassium ferricyanide. The two vacutainers were then centrifuged at 3300 g for 7 min at 4 °C. The plasma from vacutainer 3 was extracted, while RBC (1 ml) from vacutainer 4 was immediately added to 4.0 ml hypotonic lysis solution before being frozen at −80 °C for subsequent [RSNOs] analysis. Tri-iodide solution that contains 2.0 g potassium iodide, 1.3 g iodine, 40 ml deionized water (dH_2O) and 140 ml acetic acid was used as the reagent solution to convert RSNOs to NO. Two 1 ml biological samples (plasma, WB, and RBC) were treated with 110 μl 5 % acidified sulfanilamide in 1 M Hydrochloric acid (HCl) and 110 μl 5 % acidified sulfanilamide in 1 M HCl with 0.2 % mercury chloride ($HgCl_2$), respectively. All samples were stored away from light and incubated under room temperature for 15 min before analysis. 500 μl processed samples were injected into the 50 ml purge vessel that contains 9 ml tri-iodide reagent solution to analyse RSNOs concentrations in different blood compartments using a Sievers gas-phase chemiluminescence nitric oxide analyser (Sievers, 280i NO analyser) [24]. The concentration of RSNOs was calculated by subtracting the peak area under the curve (AUC) of the sample processed with $HgCl_2$ from the AUC of the sample was not processed with $HgCl_2$.

Skeletal muscle [NO_3^-] and [NO_2^-] determination Skeletal

muscle was collected from vastus lateralis using a Bergstrom needle modified for manual vacuum [40]. After applying the local anaesthesia, a small incision (0.5–0.6 cm) was made through the skin and fascia on the participant's non-dominant leg prior to biopsy. Any visible adipose and connective tissue were removed immediately upon collection, and muscle samples were blotted with sterile gauze to remove blood before being stored at −80 °C. Muscle samples were processed with a series of NO_2^- preservation, homogenization, deproteinization and centrifugation procedures to obtain the supernatant [39]. A Sievers gas-phase chemiluminescence nitric oxide analyser (Sievers, 280i NO analyser) was used to measure [NO_3^-] and [NO_2^-] [39] and [RSNOs] [24] in plasma, WB, and RBC [41], and [NO_3^-] and [NO_2^-] in skeletal muscle [42].

Maximal voluntary isometric contractions Participants were asked to perform a series of 10 maximal voluntary isometric contractions of the knee extensor muscle group with their dominant leg. Participants were seated in the chair of a Biodex System 3 isokinetic dynamometer (Biodex Medical Systems, Shirley, NY) and firmly strapped across the shoulders, hip, and distal thigh to minimize upper body movement. The lateral femoral epicondyle was aligned to the rotational axis of the dynamometer, and the dynamometer lever arm was placed in line with the medial malleolus. Individual positioning of the seat height, knee joint angle, lever arm length and dynamometer head were recorded and replicated for subsequent tests.

A series of isometric contractions with 50 %, 75 %, 90 % and 100 % maximal effort were performed as a warm-up. The participants were then asked to perform 10 maximal voluntary isometric contractions for 2 s separated by 20 s rest with the instruction to 'kick as fast as you can, as hard as you can, especially fast'. A monitor with a cursor set on the highest peak slope of the torque-time curve (highest peak RTD) achieved was placed in front of participants to provide visual feedback throughout the test. The torque signal was recorded by PC utilizing Spike 2 Software (Cambridge Electronic Ltd., Cambridge, UK). The onset of contraction was determined at the time point when force exceeded 0 N m. The absolute rate of torque development (absolute RTD) was derived as the averaged slope of the torque-time curve over time intervals 0–30, 0–50, 30–50 ms relative to the onset of contraction. The peak torque, peak RTD and torque impulse were determined as the highest torque production, the peak slope of the torque-time curve, and the area under the torque-time curve, respectively for each contraction. The mean peak torque, mean peak RTD and mean torque impulse were calculated subsequently.

4.1. Statistical analysis

Statistical analyses were performed using SPSS version 28. Two-way repeated measures ANOVA was used to assess the differences in [NO_3^-], [NO_2^-], and [RSNOs] in plasma, WB, and RBC, [NO_3^-] and [NO_2^-] in skeletal muscle, resting BP, and muscle contractile function (mean peak torque, mean peak RTD, mean torque impulse and RTD) between conditions (PL vs BR) and across time (acute vs chronic). Significant main and interaction effects were analysed further, and least significant differences (LSD) post hoc tests were applied when there was a significant interaction effect. Data normality was assessed with Shapiro-Wilk. Pearson product moment correlation coefficient was used to assess the correlations between differences in key NO biomarkers in different blood compartments and skeletal muscle and differences in resting BP variables, and muscle contractile function between PL and BR following acute and chronic supplementation if the data was normally distributed; otherwise, a Spearman correlation coefficient was used. Results are presented as mean ± SD, and statistical significance was accepted at $P < 0.05$.

5. Results

No side effects other than discoloured urine and stools were reported by participants following either acute or chronic supplementation. Interaction effects were found between conditions and across time; therefore, the single effects of condition (PL vs BR supplementation) and time (acute, chronic, chronic+1-day) on key NO biomarkers in different blood compartments and skeletal muscle, resting BP, and muscle contractile function were analysed and reported, respectively.

5.1. Resting BP

There were no significant differences in SBP (acute: −4 ± 9 mmHg, $P = 0.17$; chronic: −2 ± 7 mmHg, $P = 0.32$) or MAP (acute: −3 ± 4 mmHg, $P = 0.07$; chronic: −1 ± 5 mmHg, $P = 0.44$) between PL and BR following acute and chronic supplementation. However, a significant difference in DBP was found between PL and BR following acute ingestion (−2 ± 3 mmHg, $P = 0.03$) but not 2 weeks of chronic supplementation (−1 ± 2 mmHg, $P = 0.15$). Similarly, there were no significant differences in SBP, DBP and MAP between acute, chronic, and chronic+1-day conditions following placebo (all $P > 0.05$, Fig. 3). However, compared to acute NO_3^- ingestion, significantly higher SBP, DBP and MAP were observed at 24 h following 2 weeks of BR supplementation (all $P < 0.01$) (Fig. 3).

5.2. [NO_3^-], [NO_2^-] and [RSNOs] in plasma, WB, and RBC

There were significant differences in plasma [NO_3^-] (acute: 407 ± 112 μM; chronic: 585 ± 138 μM), WB [NO_3^-] (acute: 342 ± 70 μM; chronic: 456 ± 63 μM) and RBC [NO_3^-] (acute: 256 ± 46 μM; chronic: 320 ± 39 μM) between PL and BR following acute and chronic supplementation (all $P < 0.01$). Similarly, significant differences in [NO_2^-] in plasma (acute: 0.26 ± 0.12 μM; chronic: 0.22 ± 0.14 μM), WB (acute: 0.23 ± 0.10 μM; chronic: 0.25 ± 0.11 μM), and RBC (acute: 0.06 ± 0.03 μM; chronic: 0.05 ± 0.03 μM) were also found between PL and BR following acute and chronic supplementation (all $P < 0.01$). Significant differences in [RSNOs] in WB (acute: 25 ± 17 nM; chronic: 24 ± 14 nM) and RBC (acute: 51 ± 37 nM; chronic: 37 ± 11 nM) were found between PL and BR, whereas no differences in plasma [RSNOs] (acute: 4.6 ± 14.6 nM, $P = 0.30$; chronic: −2.5 ± 6.9 nM, $P = 0.24$) were found between PL and BR after acute or chronic supplementation.

There were no significant differences in [NO_2^-] and [RSNOs] in plasma, WB, and RBC between acute and chronic interventions (both for placebo and BR supplementation, Fig. 4). However, compared to acute BR ingestion, [NO_3^-] in all three different blood compartments were significantly higher after chronic BR supplementation (Fig. 4).

5.3. [NO_3^-] and [NO_2^-] in skeletal muscle

Muscle biopsy samples were insufficient for two participants (one for acute supplementation and one for chronic supplementation), and so complete samples from 10 participants were included in this muscle [NO_3^-] and [NO_2^-] analysis. However, 11 participants were included in the correlation analysis between differences in muscle [NO_3^-] and differences in physiological responses between PL and BR, as the correlation analyses were run separately for acute and chronic supplementation. Significant differences in skeletal muscle [NO_3^-] (acute: 91 ± 46 μM, $P < 0.01$; chronic: 96 ± 95 μM, $P < 0.01$) were found between PL and BR following acute and chronic supplementation, while no differences in skeletal muscle [NO_2^-] (acute: 0.23 ± 0.96 μM, $P = 0.47$; chronic: −0.07 ± 0.94 μM, $P = 0.82$) were observed between PL and BR following acute or chronic supplementation ($P > 0.05$). No significant differences in skeletal muscle [NO_3^-] and [NO_2^-] were found between acute and chronic PL supplementation. Similarly, in the BR condition, no significant differences in muscle [NO_2^-] were found between acute, chronic, and chronic +1-day (Fig. 5). However, muscle [NO_3^-] was significantly lower at 24 h after chronic BR supplementation (108 ± 54 μM, $P < 0.01$) compared to acute (209 ± 54 μM) and chronic BR supplementation (221 ± 74 μM) (Fig. 5).

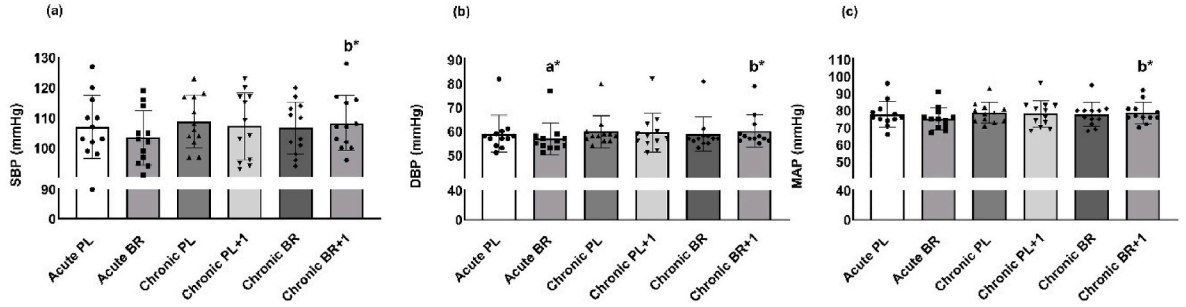

Fig. 3. The effects of acute and chronic beetroot juice supplementation on resting blood pressure in healthy adults (n=12). Mean ± SD in systolic blood pressure (SBP) (**panel a**), diastolic blood pressure (DBP) (**panel b**) and mean arterial blood pressure (MAP) (**panel c**) at 2.5 h after acute placebo (**Acute PL**), acute beetroot juice (**Acute BR**), chronic placebo (**Chronic PL**), chronic beetroot juice (**Chronic BR**) supplementation. Mean ± SD in SBP, DBP, and MAP at 24 h (**Chronic PL+1 and Chronic BR+1**) after Chronic PL and Chronic BR supplementation. Individual participant SBP, DBP and MAP responses to Acute PL, Acute BR, Chronic PL, Chronic PL+1, Chronic BR, and Chronic BR+1 were shown with dot plot in panel **a-c**, respectively. Significant differences in BP between Acute PL and Acute BR are shown with 'a*', significant differences in BP between Acute BR and Chronic BR+1 are shown with 'b*'.

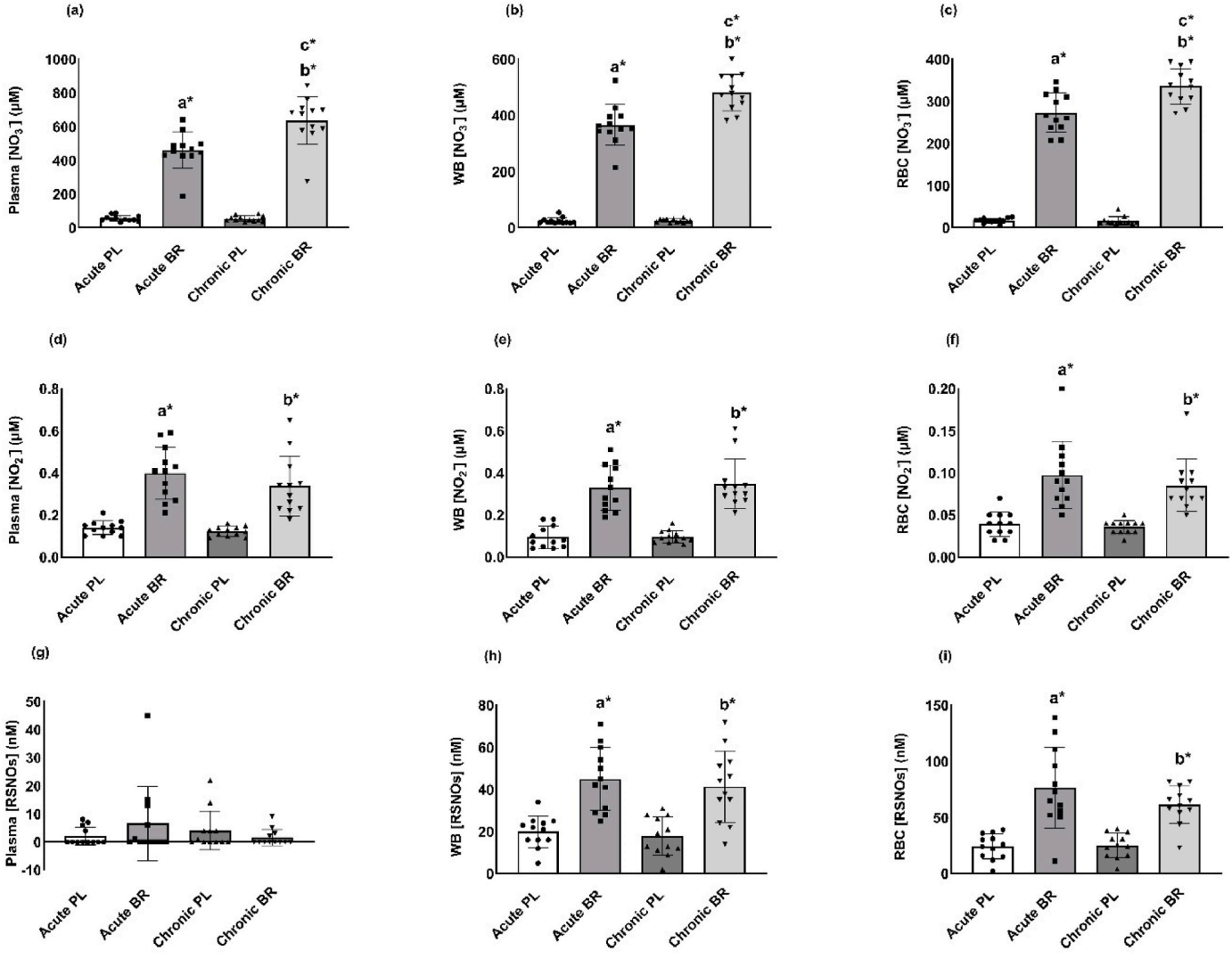

Fig. 4. The effects of acute and chronic beetroot juice supplementation on concentrations of NO biomarkers (NO_3^-, NO_2^- and RSNOs) in plasma, whole blood, and red blood cells in healthy adults (n=12). Mean ± SD in [NO_3^-] in plasma (**panel a**), whole blood (WB) (**panel b**) and red blood cells (RBC) (**panel c**); mean ± SD in [NO_2^-] in plasma (**panel d**), WB (**panel e**), and RBC (**panel f**); and mean ± SD in [RSNOs] in plasma (**panel g**), WB (**panel h**), and RBC (**panel i**). Data were collected at around 2.5 h following acute placebo (**Acute PL**), acute beetroot juice (**Acute BR**), chronic placebo (**Chronic PL**), and chronic beetroot juice (**Chronic BR**) supplementation. Individual participant [NO_3^-], [NO_2^-] and [RSNOs] in plasma, WB and RBC response to Acute PL, Acute BR, Chronic PL, and Chronic BR were shown with dot plot in panel **a-i,** respectively. Significant differences in [NO_3^-], [NO_2^-] and [RSNOs] in plasma, WB, and RBC between Acute PL and Acute BR are shown with 'a*'. Significant differences in [NO_3^-], [NO_2^-] and [RSNOs] in plasma, WB and RBC between Chronic PL and Chronic BR are shown with 'b*'. Significant differences in [NO_3^-] in plasma, WB, and RBC between Acute BR and Chronic BR are shown with 'c*'.

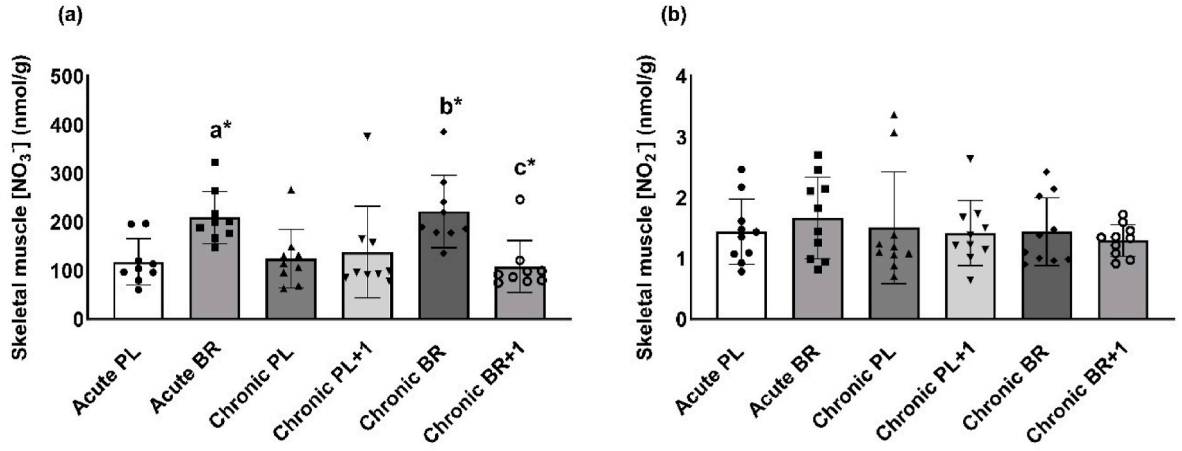

Fig. 5. The effects of acute and chronic beetroot juice supplementation on skeletal muscle [NO_3^-] and [NO_2^-] in human adults (n=10). Mean ± SD in skeletal muscle [NO_3^-] (**panel a**) and skeletal muscle [NO_2^-] (**panel b**) at 3 h following acute placebo (**Acute PL**), acute beetroot juice (**Acute BR**), chronic placebo (**Chronic PL**), and chronic beetroot juice (**Chronic BR**) supplementation. Mean ± SD in skeletal muscle [NO_3^-] and [NO_2^-] at 24 h (**Chronic PL+1 and Chronic BR+1**) after Chronic PL and Chronic BR supplementation. Individual participant muscle [NO_3^-] and [NO_2^-] responses to Acute PL, Acute BR, Chronic PL, Chronic PL+1, Chronic BR, and Chronic BR+1 were shown with dot plot in panel **a–b**, respectively. Significant differences in skeletal muscle [NO_3^-] between Acute PL and Acute BR and between Chronic PL and Chronic BR are shown with 'a*', 'b*', respectively. Significant differences in skeletal muscle [NO_3^-] between Chronic BR and Chronic BR+1 are shown with 'c*'.

5.4. Muscle contractile function

The data from one participant were excluded due to a technical issue during testing, meaning that the data from the remaining 11 participants were included in the muscle contractile function analysis. No significant differences in mean peak torque, mean peak RTD and mean torque impulse were found between PL and BR following acute or chronic supplementation. Similarly, no significant differences in absolute RTD at 0–30 ms (−23 ± 99 N m s^{-1}, $P = 0.47$), 0–50 ms (−51 ± 175 N m s^{-1}, $P = 0.36$) and 30–50 ms (−125 ± 291 N m s^{-1}, $P = 0.18$) were found between PL and BR following acute ingestion. However, significant differences in RTD at 0–30 ms (39 ± 57 N m s^{-1}, $P = 0.03$) and 0–50 ms

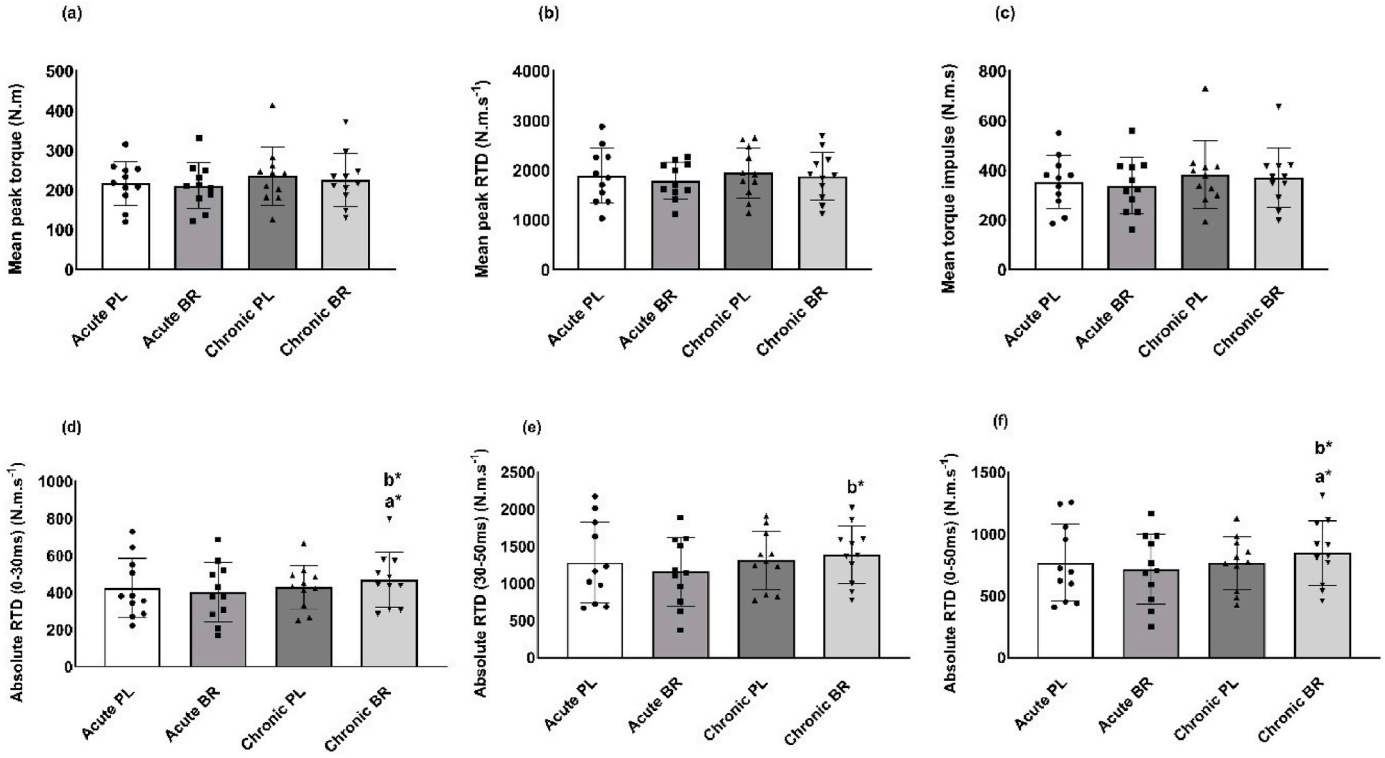

Fig. 6. The effects of acute and chronic beetroot juice supplementation on muscle contractile function in healthy adults (n=11). Mean ± SD in mean peak torque (**panel a**), mean peak rate of torque development (RTD) (**panel b**), mean torque impulse (**panel c**), RTD at 0–30 ms (**panel d**), 30–50 ms (**panel e**) and 0–50 ms (**panel f**) at 3 h after acute placebo (**Acute PL**), acute beetroot juice (**Acute BR**), chronic placebo (**Chronic PL**) and chronic beetroot juice (**Chronic BR**) supplementation. Individual participants mean peak torque, mean peak RTD, mean torque impulse and RTD data at 0–30 ms, 30–50 ms and 0–50 ms following Acute PL, Acute BR, Chronic PL, and Chronic BR are shown with dot plot in **panel a-f**, respectively. Significant differences in absolute RTD between Chronic PL and Chronic BR supplementation are shown with 'a*', and significant differences in absolute RTD between Acute BR and Chronic BR supplementation are shown with 'b*'.

(79 ± 99 N m s^{-1}, $P = 0.02$) were found between PL and BR after 2 weeks supplementation. In the placebo condition, no significant differences in RTD at 0–30 ms, 0–50 ms and 30–50 ms were found between acute and chronic supplementation (Fig. 6). However, in the BR condition, absolute RTD at 0–30 ms (acute: 402 ± 158 N m s^{-1}; chronic: 468 ± 148 N m s^{-1}; $P = 0.01$), 0–50 ms (acute: 716 ± 283 N m s^{-1}; chronic: 846 ± 260 N m s^{-1}; $P < 0.01$) and 30–50 ms (acute:1155 ± 463 N m s^{-1}; chronic: 1386 ± 389 N m s^{-1}; $P < 0.01$) were significantly higher after chronic BR supplementation than acute BR ingestion. (Fig. 6).

5.5. Correlation analysis

The correlation coefficients between differences in NO biomarkers in different blood compartments and skeletal muscle and differences in resting BP and RTD between PL and BR following acute and chronic supplementation are shown in Table 2 and Table 3, respectively. No significant differences in plasma [RSNOs] and skeletal muscle [NO_2^-] were detected between PL and BR following acute or chronic supplementation and therefore these variables were excluded from the correlation analysis.

5.6. Resting BP

There were no significant correlations between differences in BP and differences in [NO_2^-] in any of the blood compartments or [NO_3^-] in skeletal muscle (all $P > 0.05$) between acute PL and acute BR ingestion. However, the greater WB [RSNOs] was correlated with the lower DBP ($r = -0.68$, $P = 0.02$, Fig. 7) in BR compared to PL following acute ingestion. Differences in BP variables were not correlated to differences in NO biomarkers in blood between PL and BR after chronic supplementation (all $P > 0.05$).

5.7. Absolute RTD

No significant correlations were found between differences in [NO_3^-] and [NO_2^-] in any blood compartments and differences in absolute RTD at any time intervals (0–30 ms, 0–50 ms and 30–50 ms) between PL and BR following acute or chronic supplementation. However, the difference in RBC [RSNOs] was positively correlated to the differences in absolute RTD at 0–30 ms ($r_s = 0.63$, $P = 0.04$), 0–50 ms ($r_s = 0.63$, $P = 0.04$), and 30–50 ms ($r_s = 0.62$, $P = 0.04$) between PL and BR following acute ingestion. The greater absolute RTD at 0–30 ms was positively correlated with the greater skeletal muscle [NO_3^-] ($r = 0.64$, $P = 0.03$, Fig. 7)

Table 3
Correlation coefficients between differences in concentrations of NO biomarkers ([NO_3^-], [NO_2^-] and [RSNOs]) in plasma, whole blood, red blood cells and skeletal muscle and differences in resting blood pressure and muscle contractile function between chronic placebo and chronic beetroot juice supplementation in humans.

Correlation Coefficients	△SBP	△DBP	△MAP	△ RTD (0–30 ms)	△RTD (0–50 ms)	△ RTD (30–50 ms)
△ Plasma [NO_3^-]	r_s = −0.33 P = 0.29	r_s = −0.37 P = 0.24	r_s = −0.33 P = 0.30	r_s = 0.20 P = 0.53	r_s = 0.26 P = 0.42	r_s = −0.04 P = 0.91
△ WB [NO_3^-]	r = −0.16 P = 0.62	r = −0.14 P = 0.68	r = −0.15 P = 0.64	r = −0.45 P = 0.15	r = −0.48 P = 0.11	r = −0.14 P = 0.67
△ RBC [NO_3^-]	r = 0.07 P = 0.84	r = −0.19 P = 0.56	r = −0.03 P = 0.92	r = 0.08 P = 0.81	r = −0.24 P = 0.45	r = −0.05 P = 0.89
△ Plasma [NO_2^-]	r = 0.10 P = 0.75	r = −0.25 P = 0.44	r = −0.14 P = 0.67	r = 0.39 P = 0.24	r = 0.45 P = 0.17	r = 0.51 P = 0.11
△WB [NO_2^-]	r = 0.36 P = 0.26	r = −0.08 P = 0.80	r = 0.16 P = 0.63	r = 0.56 P = 0.07	r = 0.60 P = 0.05	r = 0.30 P = 0.37
△ RBC [NO_2^-]	r_s = −0.11 P = 0.75	r_s = −0.48 P = 0.12	r_s = −0.29 P = 0.36	r_s = 0.04 P = 0.90	r_s = −0.18 P = 0.58	r_s = 0.41 P = 0.18
△ WB [RSNOs]	r = 0.13 P = 0.70	r = −0.42 P = 0.17	r = −0.34 P = 0.29	r = 0.25 P = 0.44	r = 0.24 P = 0.45	r = 0.37 P = 0.24
△ RBC [RSNOs]	r = 0.55 P = 0.07	r = 0.28 P = 0.37	r = 0.52 P = 0.08	r = 0.26 P = 0.44	r = 0.22 P = 0.52	r = −0.09 P = 0.78
△ Skeletal muscle [NO_3^-]	/ /	/ /	/ /	**r = 0.64 P=0.03***	r = 0.50 P = 0.12	r = 0.21 P = 0.54

△: differences between chronic PL and chronic BR; SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial blood pressure; RTD: rate of torque development; WB: whole blood; RBC: red blood cells; RSNOs: S-nitrosothiols; NO_3^-: nitrate; NO_2^-: nitrite.

Table 2
Correlation coefficients between differences in concentrations of NO biomarkers ([NO_3^-], [NO_2^-] and [RSNOs]) in plasma, whole blood, red blood cells and skeletal muscle and differences in resting blood pressure and muscle contractile function between acute placebo and acute beetroot juice ingestion in humans.

Correlation Coefficients	△SBP	△DBP	△MAP	△ RTD (0–30 ms)	△RTD (0–50 ms)	△ RTD (30–50 ms)
△ Plasma [NO_3^-]	$r_s = -0.40$ $P = 0.20$	$r_s = -0.35$ $P = 0.26$	$r_s = -0.39$ $P = 0.21$	$r_s = 0.03$ $P = 0.94$	$r_s = 0.03$ $P = 0.94$	$r_s = 0.05$ $P = 0.89$
△ WB [NO_3^-]	**r_s=-0.65 P=0.02***	$r = -0.45$ $P = 0.14$	$r_s = -0.37$ $P = 0.23$	$r = -0.09$ $P = 0.80$	$r = -0.09$ $P = 0.80$	$r = -0.03$ $P = 0.94$
△ RBC [NO_3^-]	$r_s = -0.56$ $P = 0.06$	$r = -0.22$ $P = 0.49$	$r_s = -0.38$ $P = 0.23$	$r = 0.05$ $P = 0.88$	$r = 0.07$ $P = 0.85$	$r = 0.05$ $P = 0.88$
△ Plasma [NO_2^-]	$r_s = 0.03$ $P = 0.93$	$r = 0.06$ $P = 0.84$	$r_s = 0.09$ $P = 0.77$	$r = 0.46$ $P = 0.16$	$r = 0.41$ $P = 0.22$	$r = 0.45$ $P = 0.17$
△WB [NO_2^-]	$r_s = -0.31$ $P = 0.33$	$r = -0.10$ $P = 0.76$	$r_s = -0.16$ $P = 0.62$	$r = 0.61$ $P = 0.05$	$r = 0.53$ $P = 0.09$	$r = 0.53$ $P = 0.09$
△ RBC [NO_2^-]	$r_s = -0.05$ $P = 0.89$	$r_s = 0.20$ $P = 0.53$	$r_s = 0.15$ $P = 0.63$	$r_s = 0.27$ $P = 0.43$	$r_s = 0.27$ $P = 0.43$	$r_s = 0.21$ $P = 0.53$
△ WB [RSNOs]	$r_s = -0.58$ $P = 0.05$	**r=-0.68 P=0.02***	$r_s = -0.47$ $P = 0.13$	$r = 0.29$ $P = 0.40$	$r = 0.19$ $P = 0.58$	$r = 0.14$ $P = 0.69$
△ RBC [RSNOs]	$r_s = -0.15$ $P = 0.63$	$r_s = -0.21$ $P = 0.52$	$r_s = -0.09$ $P = 0.78$	**r_s=0.63 P=0.04***	**r_s=0.63 P=0.04***	**r_s=0.62 P=0.04***
△ Skeletal muscle [NO_3^-]	/ /	/ /	/ /	r = -0.38 P = 0.32	r = -0.37 P = 0.33	r = −0.46 P = 0.22

△: differences between acute PL and acute BR; SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial blood pressure; RTD: rate of torque development; WB: whole blood; RBC: red blood cells; RSNOs: S-nitrosothiols; NO_3^-: nitrate; NO_2^-: nitrite.

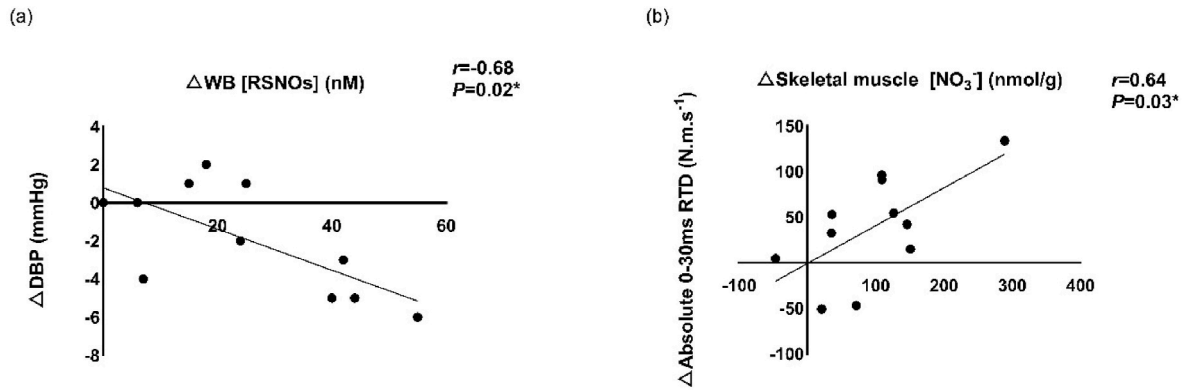

Fig. 7. Scatter plots showing the relationships between differences in NO biomarkers and differences in physiological responses for each individual between placebo and beetroot juice following acute and chronic supplementation in health adults (panel a: n=12; panel b: n=11). Differences (△) in whole blood [RSNOs] (△WB [RSNOs]) and differences in diastolic blood pressure (△ DBP) between acute PL and acute BR ingestion (**panel a**); differences (△) in skeletal muscle [NO_3^-] (△skeletal muscle [NO_3^-]) and differences in absolute rate of torque development at 0–30 ms (△ absolute 0–30 ms RTD) between chronic PL and chronic BR supplementation (**panel b**). Significant correlations are shown with '*'. Note: Sufficient skeletal muscle were collected from 11 participants after chronic BR supplementation, therefore, data from 11 participants were included in this correlation analysis.

in BR compared to PL following 2 weeks supplementation.

6. Discussion

We investigated the correlations between different NO biomarkers (NO_3^-, NO_2^-, and RSNOs) in different blood compartments and skeletal muscle and the physiological effects on BP and muscle contractile function following acute and chronic placebo and BR supplementation in healthy individuals. We found that compared to acute PL ingestion, a greater WB [RSNOs], rather than a greater [NO_3^-] and [NO_2^-], in different blood compartments was correlated with lower DBP following acute BR ingestion. A novel finding was that the difference in skeletal muscle [NO_3^-] was positively correlated with the difference in RTD at 0–30 ms between PL and BR following 2 weeks supplementation. To the best of our knowledge, this is the first study to have investigated the effects of acute and chronic dietary NO_3^- supplementation on skeletal muscle [NO_3^-] and [NO_2^-]. We found that chronic BR supplementation did not result in greater elevations in skeletal muscle [NO_3^-] and [NO_2^-] compared to acute BR ingestion, suggesting that muscle [NO_3^-] and [NO_2^-] are not influenced by the duration of dietary NO_3^- supplementation.

6.1. The effects of acute and chronic dietary NO_3^- supplementation on NO biomarkers in different blood compartments and skeletal muscle

Several previous studies have shown that [NO_3^-], [NO_2^-] and [RSNOs] are increased in different blood compartments [22,24,43,44], and that [NO_3^-] (but not [NO_2^-]) in skeletal muscle is increased, following acute dietary NO_3^- ingestion in healthy individuals [23,28]. However, until now, no studies had simultaneously examined the effects of acute and chronic dietary NO_3^- supplementation on the concentrations of NO biomarkers in different blood compartments and skeletal muscle in humans. We found that [NO_3^-] in three different blood compartments was significantly higher after 2 weeks of BR supplementation compared to acute BR ingestion. In contrast, there were no greater [NO_2^-] and [RSNOs] in plasma, WB, and RBC, or [NO_3^-] and [NO_2^-] in skeletal muscle following chronic BR supplementation compared to acute BR ingestion. No differences in plasma [RSNOs] or skeletal muscle [NO_2^-] were found between PL and BR following acute or chronic supplementation.

The number of studies that have investigated the effects of dietary NO_3^- ingestion on human skeletal muscle [NO_3^-] and [NO_2^-] is presently limited owing to technical challenges, and these have only considered short-term dietary NO_3^- ingestion [23,28,31,44]. In the present study, we found significantly higher skeletal muscle [NO_3^-] in BR than PL following both acute and chronic supplementation, with no significant difference between acute and chronic timepoints (~209 and 221 μM for acute BR and chronic BR, respectively). The higher skeletal muscle [NO_3^-] in BR compared to PL returned to the PL value at 24 h following 2 weeks of BR supplementation. Our results therefore indicate that longer dietary NO_3^- supplementation did not result in a greater storage of NO_3^- in human skeletal muscle and confirm that [NO_3^-] in blood and skeletal muscle shows similar pharmacokinetics [44], with [NO_3^-] returning to the PL (baseline) value at 24 h post NO_3^- ingestion.

No differences in skeletal muscle [NO_2^-] were found between PL and BR following acute or chronic supplementation, which is consistent with previous research in humans [23,28,44]. This contrasts with a study in rodents which reported a significant increase in muscle [NO_2^-] following 7 days supplementation with 1 g/l sodium NO_3^- dissolved in water [45]. The explanation for differences in skeletal muscle [NO_3^-] and [NO_2^-] responses to dietary NO_3^- ingestion between rodents and humans are not clear, although differences in methods of NO_3^- ingestion (bolus supplementation in humans vs. continuous supplementation with water in rodents), muscle sample collection and processing [46], and the proportion of muscle fibre types in these species [47,48], may be influential. Furthermore, the measurement of NO_2^- in muscle is technically challenging such that small changes in [NO_2^-] might not be detectable using current methodological approaches.

6.2. The effects of acute and chronic dietary NO_3^- supplementation on resting BP

Resting DBP was significantly lower in BR than PL, while no differences in SBP or MAP were observed following acute ingestion, whereas no significant differences in SBP, DBP or MAP were detected between PL and BR following chronic supplementation. These results do not support the notion that chronic NO_3^- ingestion would result in a greater BP-lowering effect compared to acute NO_3^- ingestion. Our results contrast with a previous study in young healthy adults in which significant reductions in SBP and MAP were found both acutely and following 15 days of NO_3^- supplementation, with the effects being greatest at day 15 [10]. The differences in baseline BP level may explain the contrasting results. Baseline BP levels have been reported to be negatively correlated with the peak changes in BP after dietary NO_3^- intake [9]. The group mean baseline BP (SBP: 104 ± 9 mmHg, DBP: 58 ± 8 mmHg) in the present study was much lower compared to the Vanhatalo et al. [10] study (SBP: 127 ± 6 mmHg; DBP: 72 ± 5 mmHg). In addition, a meta-analysis has indicated that reductions in SBP are associated with the daily dose of

NO_3^-, but not with supplementation duration [20]. The effects of acute and chronic dietary NO_3^- supplementation on BP need to be investigated in hypertensive individuals, in whom a greater fall in BP would be anticipated following dietary NO3- supplementation [9].

6.3. Correlation between differences in NO biomarkers in different blood compartments and differences in resting BP between placebo and dietary NO_3^- following acute and chronic supplementation

Negative correlations were found between differences in WB [RSNOs] and differences in DBP between PL and BR following acute ingestion. Plasma [NO_2^-], as the most oft-used biomarker of NO bioavailability, was not correlated with the BP-lowering effects of NO_3^- ingestion, a finding in line with some previous research [49], meta-analysis [20], and our recently published study [25]. NO_2^- and RSNOs, as the two storage forms of intravascular NO, have been considered as two important biomarkers of NO bioavailability due to their relatively long half-life and chemical stability [50,51]. However, the role of RSNOs as an NO biomarker may have been underestimated in the past because the determination of [RSNOs] is technically challenging and large variation has been reported in the literature [24,52].

In a recent study, we reported negative correlations between changes in RBC [RSNOs] and changes in SBP, DBP and MAP, while the lower BP was not correlated with plasma [NO_3^-] or [NO_2^-] after bolus BR ingestion (12.8 mmol NO_3^-) in healthy individuals [25]. In the present study, the greater WB [RSNOs] rather than RBC [RSNOs] was negatively correlated to the lower BP in BR compared to PL after acute ingestion. The pharmacokinetics profiles of [RSNOs] in different blood compartments following bolus ingestion of 12.8 mmol NO_3^- intake has been examined previously [25], with [RSNOs] in WB and RBC reaching their peaks at 2 and 4 h post BR ingestion, respectively. In the present study, we collected blood samples and determined [RSNOs] at around 2.5 h after BR ingestion, when [RSNOs] in RBC would not have peaked. Although the lower BP was correlated with greater WB [RSNOs] in the present study and with RBC [RSNOs] in our previous study [25], presumably due to differences in the timing of sample collection, the two studies are consistent in showing that blood [RSNOs] is better correlated with BP reduction than is the more commonly measured plasma [NO_2^-]. We suggest that more attention needs to be given to blood RSNOs when investigating the BP responses to dietary NO_3^- supplementation.

6.4. The effects of acute and chronic dietary NO_3^- supplementation on muscle contractile function

There were no significant differences in mean peak torque, mean peak RTD and mean torque impulse between PL and BR following acute or chronic supplementation. Similarly, no significant differences in absolute RTD at any time intervals (0–30 ms, 0–50 ms, and 30–50 ms) were found between PL and BR following acute ingestion. Transcutaneous electrical nerve stimulation (TENS) is used to induce muscle action by stimulating muscular and nervous cells [53]. Haider and Folland [54] observed an increased RTD at 10–50 ms (increased by 8–14 %) and peak force (increased by 7 %) following acute dietary NO_3^- ingestion when femoral nerve stimulation was applied. However, in that study, no improved explosive and maximum voluntary force were found following dietary NO_3^- ingestion when the femoral nerve stimulation was not applied [54]. In our study, participants performed 10 muscle contractions without application of nerve stimulation, which may account for the lack of improvements in RTD following acute BR compared to PL ingestion. In rodents, a greater mean contractile force following NO_3^- ingestion was detected in fast twitch (but not slow twitch) muscles [35]. We harvested muscle samples from the vastus lateralis, a mixed muscle containing ~40 % type I muscle fibres [55]. Kadach and colleagues (2023) [23] found a 7 % increase in the mean muscle torque production during the first 90 s of 5 min of all-out maximal voluntary isometric contractions of the knee extensors (3 s muscle contraction and 2 s rest) following acute dietary NO_3^- ingestion. In this present study, however, participants were asked to complete 10 maximal voluntary isometric muscle contractions separated by 20 s rest, an exercise protocol which is likely to enable better muscle recovery and, therefore, maximum type II muscle fibres recruitment for each contraction. It is possible that differences in experimental protocol and consequent differences in muscle fibre recruitment influenced the effects of dietary NO_3^- ingestion on muscle contractile function.

Although no greater absolute RTD was found in acute BR compared to acute PL ingestion, absolute RTD at 0–30 ms and 0–50 ms were significantly greater by 7–9 % in BR than PL after 2 weeks supplementation. Measurement of RTD is recommended in muscle force production due its high sensitivity to acute and chronic changes in neuromuscular function [56]. Previous studies reported improved ATP hydrolysis, force production and greater mean contractile force in both rodents [35] and humans [57,58] after longer-term NO_3^- intake. For example, Hernández et al. (2012) [35] reported a 35 % increase in force production after 7 days of dietary NO_3^- supplementation in rodents, which was accompanied by increased Ca^{2+} handling protein expression (calsequestrin and dihydropyridine receptor) in type II skeletal muscle. However, in humans, Whitfield et al. (2017) [38] did not find altered content of Ca^{2+} handling proteins following 7 days BR ingestion, despite a significant improvement in muscle force production. Inter-species differences, including muscle fibre type distribution, might account for this disparity. Although the question of whether dietary NO_3^- ingestion alters the expression of Ca^{2+} handling proteins is unresolved, the current study adds to the available evidence to indicate that enhanced muscle contractile function, is more likely to occur following longer-term compared to acute dietary NO_3^- supplementation.

6.5. Correlation between differences in NO biomarkers in different blood compartments and skeletal muscle and differences in rate of torque development between placebo and dietary NO_3^- following acute and chronic supplementation

Although absolute RTD was not significantly different between acute PL and acute BR, the differences in RTD at 0–30 ms, 0–50 ms and 30–50 ms were positively correlated to the difference in RBC [RSNOs] between acute PL and BR ingestion. No significant correlations were found between differences in RTD and differences in any other NO biomarkers (NO_3^- and NO_2^-) in plasma, WB, RBC, or skeletal muscle between PL and BR following acute ingestion. Notably, following 2 weeks supplementation, the greater skeletal muscle [NO_3^-] was significantly correlated with the greater RTD at 0–30 ms in BR compared to PL. Skeletal muscle has been described as a NO_3^- 'reservoir' due to its much higher [NO_3^-] compared to blood [28,29]. In addition, NO_3^- reductases, such as XOR, AO, and mitochondrial amidoxime-reducing component, are found both in rodent [29,59] and human skeletal muscle [28,60], suggesting that the NO_3^- reduction pathway is active in skeletal muscle. NO has a very short half-life and can typically diffuse only a few μm, although this is tissue-dependent [32,61]. Following dietary NO_3^- ingestion, a significant decrease in muscle [NO_3^-] was reported in the exercised leg, but not in the contralateral non-exercised leg [23]. Similarly, in rodents, acute high-intensity exercise led to a significant reduction in muscle [NO_3^-] and a transient increase in muscle [NO_2^-] [30]. These results strongly suggest that NO_3^- stored in skeletal muscle might be an important source of NO during exercise. The present study adds to this evidence base by showing that compared to 2 weeks PL, a greater muscle [NO_3^-] is related to a greater RTD at 0–30 ms following 2 weeks of NO_3^- supplementation.

Although we did not find greater elevations in skeletal muscle [NO_3^-] and [NO_2^-] following 2 weeks of BR supplementation compared to acute ingestion, longer-term NO_3^- supplementation might have altered muscle protein expression to permit improvements in muscle contractile function. Exposure of skeletal muscle to NO modulates several physiological actions, including excitement-contraction coupling [57] and post-translational modification of myofibrillar proteins [62,63]. In

addition, transnitrosylation of the ryanodine receptor (RyR) also plays a role in enhancing Ca^{2+} release by increasing the possibility of the Ca^{2+} channels being in the open state, and therefore increasing intracellular [Ca^{2+}] and RTD [64]. The RyR response to NO is likely to be dose-dependent because it contains multiple sites for nitrosylation [65] and future studies should seek to elucidate the dose-response relationship between dietary NO_3^- ingestion, skeletal muscle [NO_3^-] and [NO_2^-], and modifications to muscle contractile function. We did not measure [RSNOs] in skeletal muscle in the present study. However, given the emerging evidence that RSNOs in blood is correlated with the BP-lowering effects of dietary NO_3^- supplementation, it is possible that RSNOs in muscle also play an important role in enhancing muscle contractile function.

7. Conclusion

We investigated the relationships between differences in NO biomarkers in different blood compartments and skeletal muscle and differences in the physiological response to acute and chronic PL and dietary NO_3^- supplementation in healthy adults. Consistent in part with our hypothesis, we found that the greater WB [RSNOs], rather than the oft-used [NO_3^-]and [NO_2^-] in plasma, was correlated with the lower DBP in BR compared to PL following acute ingestion. In muscle, greater RBC [RSNOs] in BR compared to PL was found to be correlated with the differences in absolute RTD at 0–30 ms, 0–50 ms and 30–50 ms between PL and BR following acute ingestion. In addition, we found a positive correlation between greater skeletal muscle [NO_3^-] and greater RTD at 0–30 ms following 2 weeks of BR supplementation compared to PL. To the best of our knowledge, this study is the first to examine the effects of longer-term dietary NO_3^- ingestion on skeletal muscle [NO_3^-] and [NO_2^-] in humans. We did not detect greater elevations in muscle [NO_3^-] and [NO_2^-] after chronic BR compared to acute supplementation, with skeletal muscle [NO_3^-] having returned to PL value at 24 h following chronic NO_3^- supplementation. We conclude that RSNOs in blood and NO_3^- in skeletal muscle are highly relevant biomarkers of NO bioavailability, which are linked to the reduction in BP and the improvement in muscle contractile function, following dietary NO_3^- ingestion in humans.

CRediT authorship contribution statement

Chenguang Wei: Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anni Vanhatalo:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Matthew I. Black:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jamie R. Blackwell:** Methodology, Investigation, Formal analysis. **Raghini Rajaram:** Validation, Project administration, Investigation, Data curation. **Stefan Kadach:** Methodology, Investigation, Data curation. **Andrew M. Jones:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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