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Reduction in blood pressure following acute dietary nitrate ingestion is correlated with increased red blood cell S-nitrosothiol concentrations

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ABSTRACT

Dietary nitrate (NO_3^-) supplementation can enhance nitric oxide (NO) bioavailability and lower blood pressure (BP) in humans. The nitrite concentration ($[NO_2^-]$) in the plasma is the most commonly used biomarker of increased NO availability. However, it is unknown to what extent changes in other NO congeners, such as S-nitrosothiols (RSNOs), and in other blood components, such as red blood cells (RBC), also contribute to the BP lowering effects of dietary NO_3^-. We investigated the correlations between changes in NO biomarkers in different blood compartments and changes in BP variables following acute NO_3^- ingestion. Resting BP was measured and blood samples were collected at baseline, and at 1, 2, 3, 4 and 24 h following acute beetroot juice (~12.8 mmol NO_3^-, ~11 mg NO_3^-/kg) ingestion in 20 healthy volunteers. Spearman rank correlation coefficients were determined between the peak individual increases in NO biomarkers (NO_3^-, NO_2^-, RSNOs) in plasma, RBC and whole blood, and corresponding decreases in resting BP variables. No significant correlation was observed between increased plasma $[NO_2^-]$ and reduced BP, but increased RBC $[NO_2^-]$ was correlated with decreased systolic BP ($r_s = -0.50$, $P = 0.03$). Notably, increased RBC [RSNOs] was significantly correlated with decreases in systolic ($r_s = -0.68$, $P = 0.001$), diastolic ($r_s = -0.59$, $P = 0.008$) and mean arterial pressure ($r_s = -0.64$, $P = 0.003$). Fisher's z transformation indicated no difference in the strength of the correlations between increases in RBC $[NO_2^-]$ or [RSNOs] and decreased systolic blood pressure. In conclusion, increased RBC [RSNOs] may be an important mediator of the reduction in resting BP observed following dietary NO_3^- supplementation.

1. Introduction

Hypertension, or high blood pressure (BP), is one of the most common cardiovascular diseases, and is linked to more than 7 million deaths per year [1]. A 5 mmHg reduction in systolic blood pressure (SBP) could decrease the mortality risk from cardiovascular diseases and stroke by 9% [1]. Dietary ingestion of nitrate (NO_3^-), a molecule found in high concentrations in leafy green vegetables and beetroot, has been reported to lower resting BP and is now considered a prophylactic for reducing the risk of developing hypertension and other cardiovascular diseases [2–4]. The pathophysiology of hypertension is complicated, and is related to high salt intake, obesity, insulin resistance and genetic factors [5]. However, lower nitric oxide (NO) bioavailability leading to endothelial dysfunction is recognized as an important contributory mechanism [6]. In humans, NO is produced via the oxidation of L-arginine in a reaction catalyzed by a family of nitric oxide synthase (NOS) enzymes. However, NO is also generated by the stepwise biochemical reduction of NO_3^- to nitrite (NO_2^-) and then NO [7], with this pathway likely to be favoured when tissue oxygen tension is low [7–9]. 'Cross-talk' between these two NO production pathways has been described in mice and rats [10,11]. Notably, reduced NOS activity and NO availability have been reported in hypertension [12,13]. It is therefore possible that augmenting the $NO_3^- \rightarrow NO_2^- \rightarrow NO$ pathway via dietary NO_3^- supplementation, for example through the ingestion of beetroot juice (BR), might enable better maintenance of NO homeostasis when NOS function is impaired.

In humans, NO_3^--reducing bacteria in the oral cavity are primarily responsible for the biochemical reduction of circulating NO_3^- to

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bioactive NO_2^- [14,15]. The swallowed NO_2^- then enters the systemic circulation, elevating plasma [NO_2^-] [16], which is recognized as a key biomarker of NO bioavailability [17]. An inverse relationship between increased plasma [NO_2^-] and decreased BP following NO_3^- ingestion has been reported [2,18–20]. However, a meta-analysis including 15 studies did not detect a significant correlation between changes in plasma [NO_2^-] and changes in BP [21]. This suggests that while an elevated plasma [NO_2^-] might be necessary for a decrease in BP to be observed following dietary NO_3^- intake, there is not a 'one-to-one' relationship, and the decreased BP might be more closely related to factors other than increased plasma [NO_2^-], *per se*. Red blood cells (RBC) are the main storage site of deoxyhaemoglobin, which is important for the reduction of NO_2^- to NO and thus for vasodilation in humans [22]. Following exposure to NO (in the form of an aqueous solution, or as a gas, or via NO donors), an increased NO concentration in both plasma and RBC has been reported along with NO-like bioactivity and vasodilation both *in vivo* and *in vitro* [23–25]. Therefore, it is possible that increased [NO_2^-] in other blood compartments, not only plasma, might mediate the anti-hypertensive effects of NO_3^- supplementation.

S-nitrosothiols (RSNOs) are a group of NO congeners formed by the S-nitrosation of non-protein thiols (for example, cysteine and glutathione) or protein thiols [26]. Following dietary NO_3^- ingestion, NO_3^- is rapidly reduced to NO_2^-, which is concentrated in the saliva and, on swallowing, leads to RSNOs formation in the acidic environment of the stomach [27–29]. RSNOs, as a storage form for intravascular NO, are important in smooth muscle relaxation [30], vasodilation [31], and inhibition of platelet aggregation [32]. In a hypertensive rodent model, the BP lowering effects of NO_3^- ingestion were attenuated after gastric pH was increased with a proton pump inhibitor [33]. Importantly, this was associated with lower plasma [RSNOs] but no change in plasma [NO_3^-] and [NO_2^-] [33]. Following NO_3^- ingestion in humans, plasma [NO_2^-] peaks after 2.5–3.0 h and returns to close to baseline values by 24 h [18,20,34]. However, 24 h following NO_3^- ingestion, BP was reported to remain decreased compared to baseline in grade I hypertensive humans [34], implying that some factor other than plasma [NO_3^-] and [NO_2^-] may be at least partly responsible for the BP lowering effects of dietary NO_3^- supplementation. However, it is unclear whether RSNOs contribute to the changes in BP observed following NO_3^- ingestion in humans.

It has been reported that [NO_3^-] and [NO_2^-] increase in whole blood (WB), RBC, and plasma, and that [RSNOs] increases in RBC and plasma, after BR supplementation in humans [35]. Although plasma [NO_2^-] is the most commonly used indicator of increased NO bioavailability following dietary NO_3^- supplementation [36,37], it is possible that increases in [NO_3^-], [NO_2^-] and/or [RSNOs] in other blood compartments (WB, RBC) may also mediate some or all of the diverse physiological effects of NO_3^- ingestion. In light of evidence that [RSNOs] play a role in BP regulation in rodents [33], and that [RSNOs] are elevated following BR ingestion in humans [35], we were interested in exploring possible relationships between changes in [RSNOs] and changes in BP following BR intake. Therefore, the purpose of this study was to investigate the relationships between changes in key NO biomarkers in three different blood compartments and changes in resting BP following acute BR supplementation. We hypothesized that, in healthy adult humans: (1) the peak change in RBC [NO_2^-] would be more strongly correlated with changes in BP than the peak change in plasma [NO_2^-]; and (2) the peak change in RBC [RSNOs] would be more strongly correlated with changes in BP than peak changes in either RBC or plasma [NO_2^-].

2. Methods

2.1. Participants

Twenty healthy individuals (12 males and 8 females) volunteered to participate in this study. The physical characteristics of the participants is shown in Table 1. Exclusion criteria included smoking, antibacterial mouthwash use, and current or recent consumption of NO_3^-, L-arginine or L-citrulline supplements. This study conformed to the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the University of Exeter, United Kingdom (approval number: 21-06-16-B-02). The detailed experimental procedures, related risks, and potential benefits were explained to all participants before written, informed consent was provided.

Table 1
Participant Characteristics (n = 19, 11 males, 8 females).

Characteristics	Males (Mean ± SD)	Females (Mean ± SD)
Age (year)	36 ± 13	42 ± 13
Body Height (m)	1.77 ± 0.05	1.63 ± 0.07
Body Mass (kg)	75.5 ± 12.6	65.5 ± 10.0
Body Mass Index ($kg.m^{-2}$)	24.0 ± 3.4	24.9 ± 5.1
Resting SBP (mmHg)	108 ± 9	119 ± 13
Resting DBP (mmHg)	64 ± 7	70 ± 9

2.2. Experimental procedures

Participants reported to the laboratory on consecutive days. On both occasions, they were required to arrive at the laboratory in the morning (7–8 a.m.) in a fasted state having avoided strenuous exercise for the previous 24 h, and caffeine and alcohol intake for the previous 6 h. To minimize the influence of habitual diet on study results, participants were provided with a list of NO_3^- rich foods and instructed to avoid consuming these for three days prior to the first laboratory visit and during the experiment. Participants were also asked to avoid tongue scraping, antibacterial mouthwash use, and chewing gum to prevent attenuation of commensal oral bacteria, which is essential in reducing NO_3^- to NO_2^-.

The full experimental protocol is illustrated in Fig. 1. Upon arrival at the laboratory, body mass and height were measured. Resting BP was measured using an electronic sphygmomanometer (Dinamap Pro; GE Medical System, Tampa, FL) after 10 min supine rest. BP was measured 4 times separated by 1 min, with the last three measurements averaged to obtain a mean SBP, diastolic blood pressure (DBP), and mean arterial pressure (MAP). A cannula (20 g Insyte-WTM cannula; Becton Dickinson, Madrid, Spain) was then placed in the antecubital vein and four 6 ml blood samples were collected. Participants then consumed two bottles of NO_3^- rich BR (140 ml) containing a total of 12.8 mmol NO_3^- (11.5 ± 0.9 mg NO_3^-/kg; Beet It Sport; James White Drinks, Ipswich, UK). The dose of 12.8 mmol NO_3^- was chosen based on previous studies which showed that this dose elevated blood [RSNOs] [35] and reduced BP [20]. The composition of the BR, including total betacyanins and polyphenol compounds, has been reported by our group previously [38,39]. The participants were then provided with a standardized low NO_3^- breakfast (72 g oats and 180 ml semi-skimmed milk). Following BR ingestion, resting BP was measured and four 6 ml venous blood samples were collected at 1, 2, 3, 4, and 24 h. Following the 4 h measurement time-point, the participants were free to leave and to return to the laboratory on the following morning. On the second day, resting BP was measured as described above and four 6 ml blood samples were collected by venepuncture.

2.3. Biochemical measurements

[NO_3^-] and [NO_2^-] determination. Venous blood samples were drawn into two lithium-heparin tubes. WB (800 μl) was extracted from one vacutainer and mixed with 200 μl NO_2^- preservation solution that consisted of 890.9 mM potassium ferricyanide ($K_3Fe(CN)_6$), 118.13 mM N-ethylmaleimide (NEM), Nonidet P-40 (octyl-phenoxylpolyethoxylethanol) added in a 1:9 ratio, and 4.5 ml deionized water (dH_2O) [40]. The other vacutainer was centrifuged at 3300 g (4 °C) for 7 min, within 3 min of collection. Plasma and 900 μl RBC (mixed with 100 μl NO_2^-

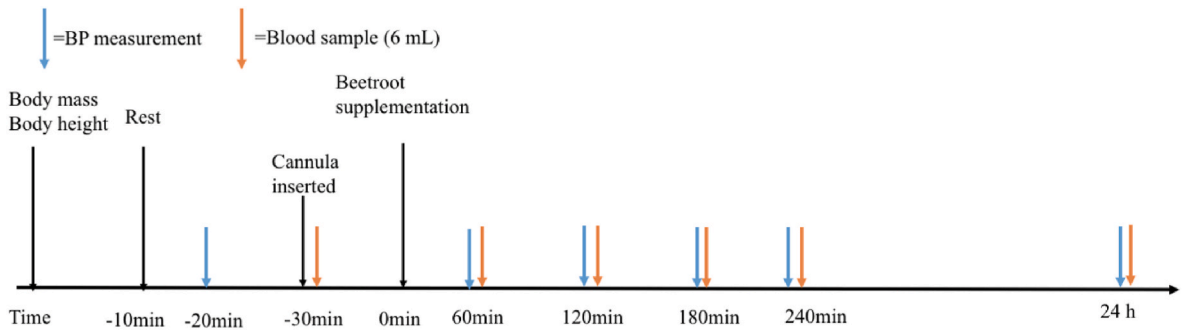

Fig. 1. Schematic diagram of the experimental protocol.

preservation solution) were collected. All blood samples were immediately frozen at −80 °C for later determination of [NO_3^-] and [NO_2^-]. Each sample was mixed with cold ethanol at a ratio 1:2 (sample/ethanol) and centrifuged at 13,000 rpm (4 °C) for 15 min to precipitate proteins [40]. The supernatant was then analyzed for [NO_3^-] and [NO_2^-] using a Sievers gas-phase chemiluminescence nitric oxide analyzer (Sievers, 280i NO analyzer), as described previously [20].

[RSNOs] and [mercury resistant signal] determination. Venous blood samples were collected into two lithium-heparin vacutainers. A 150 μl solution that consisted of 5 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM NEM was added to the first vacutainer. Similarly, 150 μl solution that consisted of 2.5 mM EDTA, 10 mM NEM and 10 mM ferricyanide was added to the second vacutainer. The WB sample (1.0 ml) was obtained from the second vacutainer and mixed immediately with 4.0 ml hypotonic lysis solution (containing EDTA, NEM, and ferricyanide at final concentrations of 2.5, 10, and 10 mM, respectively). The two vacutainers were then centrifuged at 3300 g for 7 min at 4 °C. The RBC sample (1.0 ml) from the second vacutainer was immediately added to 4.0 ml of hypotonic lysis solution and stored in Eppendorf tubes. The plasma was obtained from the first vacutainer and stored in Eppendorf tubes. All Eppendorf tubes were then stored at −80 °C until further analysis [41]. Two 1 ml biological samples were treated with 110 μl 5% acidified sulfanilamide in 1 M Hydrochloric acid (HCl) and 110 μl 0.2% mercury chloride ($HgCl_2$) and 5% acidified sulfanilamide in 1 M HCl, respectively. Tri-iodide solution that consists of 2.0 g potassium iodide, 1.3 g iodine, 40 ml dH_2O, and 140 ml acetic acid was used as the reagent liquid to convert RSNOs to NO. 500 μl treated samples were injected into the 50 ml purge vessel that contains 9 ml tri-iodide reducing solution to analyze [RSNOs]. [RSNOs] in different blood compartments was measured using a Sievers gas-phase chemiluminescence nitric oxide analyzer (Sievers, 280i NO analyzer), as described previously [35]. [RSNOs] was calculated by subtracting the peak area under the curve (AUC) of the sample treated with acidified sulfanilamide and $HgCl_2$ from the AUC of the sample only treated with acidified sulfanilamide. [Mercury resistant signal] was determined as the AUC of each sample treated with sulfanilamide and $HgCl_2$. Representative chemiluminescence signal traces for NO_3^-, NO_2^- and RSNOs are shown in Supplementary Fig. 1.

2.4. Statistical analysis

One-way ANOVA was used to analyze the changes in BP variables (SBP, DBP and MAP) and NO biomarkers ([NO_3^-], [NO_2^-], [RSNOs] and [mercury resistant signal]) in plasma, WB and RBC between baseline and different time-points (1, 2, 3, 4 and 24 h) following BR supplementation. Data normality was assessed using the Shapiro-Wilk test prior to analysis. Because the blood [NO_3^-], [NO_2^-], [RSNOs] and [mercury resistant signal] data were not normally distributed, Spearman's rank correlation coefficient was used to assess relationships between peak changes in individual NO biomarkers ([NO_3^-], [NO_2^-], [RSNOs] and [mercury resistant signal]) in different blood compartments (plasma, WB and RBC) and changes in resting BP variables (SBP, DBP and MAP) at the corresponding time-point. The strengths of the correlation coefficients between different NO biomarkers in plasma, WB and RBC and BP variables were assessed with Fisher's Z transformation. Statistical analyses were performed using SPSS.25. Results are presented as mean ± SD unless otherwise indicated, and statistical significance was accepted at $P < 0.05$.

3. Results

No side effects other than discoloured urine and stools were reported by the participants. The data from one participant were excluded due to a technical error in blood sample processing. Therefore, 19 subjects were included in the data analysis.

3.1. Resting BP

The group mean SBP and DBP were 111 ± 2 mmHg and 65 ± 2 mmHg, respectively (Table 1). Compared to baseline, the mean resting SBP was lower at 3 h (−5 ± 5 mmHg, $P < 0.01$), and mean DBP was lower at 1 h (−3 ± 4 mmHg, $P = 0.002$), 2 h (−3 ± 4 mmHg, $P = 0.004$), and 3 h (−3 ± 3 mmHg, $P = 0.002$) following BR ingestion. The mean MAP was also lower at 1 h (−3 ± 4 mmHg, $P = 0.02$) and 3 h (−3 ± 4 mmHg, $P = 0.002$) following BR ingestion. No significant effects on SBP (−1 ± 5 mm Hg, $P = 0.46$), DBP (0 ± 4 mmHg, $P = 0.60$), or MAP (0 ± 4 mm Hg, $P = 0.87$) were found at 24 h (Fig. 2).

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

At baseline, prior to BR ingestion, the mean values of WB [NO_3^-] (39 ± 23 μM) and RBC [NO_3^-] (31 ± 22 μM) were not significantly different ($P = 0.20$), but plasma [NO_3^-] (52 ± 27 μM) was significantly higher than WB [NO_3^-] ($P = 0.03$) and RBC [NO_3^-] ($P < 0.01$). The mean baseline WB [NO_2^-] (105 ± 25 nM) was significantly higher than plasma [NO_2^-] (92 ± 33 nM, $P = 0.048$) and RBC [NO_2^-] (67 ± 17 nM; $P < 0.01$). The increase in [NO_3^-] above baseline (i.e., Δ) following BR ingestion reached peak values at 1 h in plasma (675 ± 126 μM), WB (393 ± 94 μM), and RBC (277 ± 83 μM), and only plasma [NO_3^-] remained higher than baseline at 24 h. The increase in [NO_2^-] above baseline peaked at 3 h in the three different blood compartments (Δplasma: 384 ± 235 nM; ΔWB: 283 ± 152 nM; ΔRBC: 73 ± 68 nM, respectively) and [NO_2^-] in all blood compartments had returned to baseline at 24 h (Fig. 3).

3.3. [RSNOs] in plasma, WB, and RBC

At baseline, WB [RSNOs] and RBC [RSNOs] were not different (11 ± 6 nM vs. 15 ± 11 nM, respectively; $P = 0.20$) but both were significantly higher than plasma [RSNOs] (3 ± 5 nM; $P < 0.01$). Following BR ingestion, RBC [RSNOs] significantly increased compared to baseline at 1 h, reaching a peak change at 4 h (24 ± 29 nM), before declining to a value that was not different from the initial baseline at 24 h. No

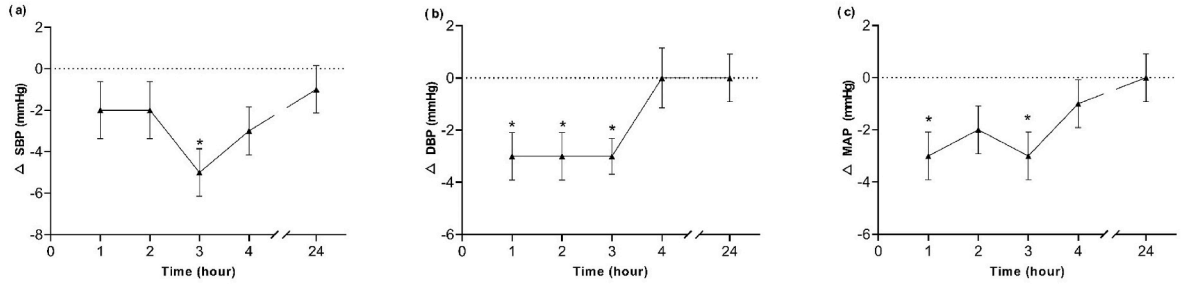

Fig. 2. Time course of changes in resting blood pressure relative to baseline. Mean ± SEM changes (△) in systolic blood pressure (△SBP) (**panel a**), diastolic blood pressure (△DBP) (**panel b**) and mean arterial pressure (△MAP) (**panel c**) at 1, 2, 3, 4, and 24 h following acute beetroot juice (~12.8 mmol NO_3^-) ingestion in healthy adults (n = 19). Significant differences ($P < 0.05$) between specific time points and baseline are shown with '*'.

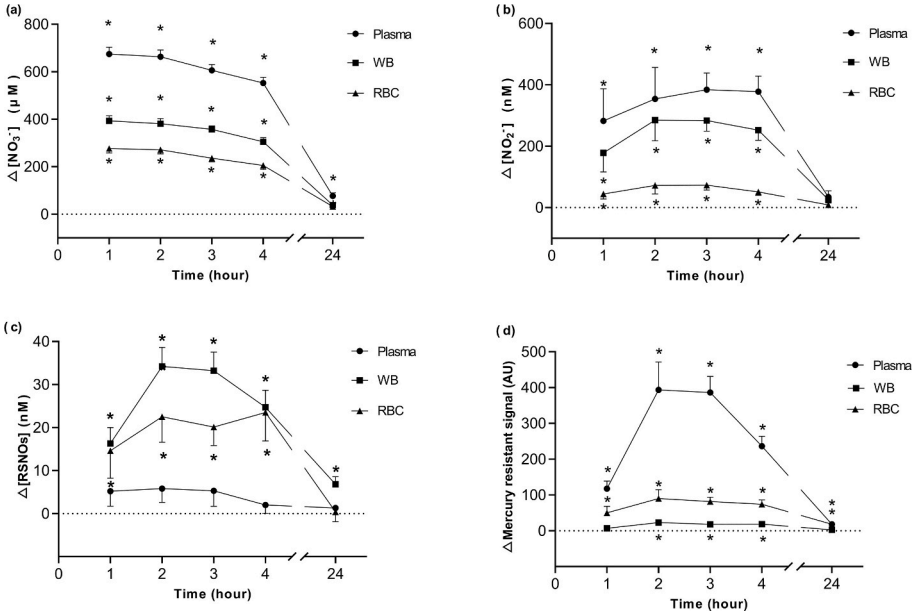

Fig. 3. Time-course of changes in the concentrations of NO congeners in different blood compartments relative to baseline. Mean ± SEM changes (△) in nitrate concentration (△[NO_3^-]) (**panel a**), nitrite concentration (△[NO_2^-]) (**panel b**), S-nitrosothiol concentration (△[RSNOs]) (**panel c**) and [mercury resistant signal] (e.g., including [RNNO]) (**panel d**) in plasma, whole blood (WB), and red blood cells (RBC) at 1, 2, 3, 4 and 24 h following acute beetroot juice (~12.8 mmol NO_3^-) ingestion. Closed circles represent plasma, closed squares represent whole blood (WB), and closed triangles represent red blood cells (RBC). Significant differences ($P < 0.05$) between specific time points and baseline are shown with '*'.

differences in RBC [RSNOs] were observed between 2, 3 and 4 h ($P > 0.05$). WB [RSNOs] showed a similar trend to RBC [RSNOs]; however, WB [RSNOs] reached a peak increase above baseline at 2 h (34 ± 19 nM) and remained elevated above baseline at 24 h. No changes in plasma [RSNOs] were detected at any time-point following BR ingestion ($P > 0.05$) (Fig. 3).

3.4. [Mercury resistant signal] in plasma, WB, and RBC

At baseline, the mercury resistant signal was not significantly different between plasma (21 ± 21), WB (11 ± 9) and RBC (17 ± 13). Following BR ingestion, [mercury resistant signal] in plasma and RBC were significantly increased from 1 h and reached peak changes at 2 h (Δ plasma: 393 ± 340; Δ RBC: 90 ± 108) and remained elevated at 24 h compared to baseline. [Mercury resistant signal] in WB started to increase and reached the peak change at 2 h (23 ± 24), before declining to value that was not different from the initial baseline value at 24 h following BR ingestion (Fig. 3).

3.5. Correlations between changes in [NO_3^-], [NO_2^-], [RSNOs], and [mercury resistant signal] in plasma, WB, RBC, and changes in resting BP variables

All correlation coefficients between changes in different NO biomarkers ([NO_3^-], [NO_2^-], [RSNOs], and [mercury resistant signal]) in plasma, WB, and RBC and changes in resting BP variables are shown in Table 2. There were no significant correlations between changes in [NO_3^-] in any of the blood compartments and changes in SBP, DBP and MAP ($P > 0.05$) following BR ingestion. Similarly, there were no significant correlations between changes in plasma [NO_2^-] or WB [NO_2^-] and changes in SBP, DBP and MAP. However, the increase in RBC [NO_2^-] was correlated with the decrease in SBP ($r_s = -0.50$, $P = 0.03$) (Fig. 4). No significant correlations were found between changes in [mercury resistant signal] in plasma, WB, and RBC and changes in resting BP variables (all $P > 0.05$). There were no significant correlations between changes in plasma [RSNOs] or WB [RSNOs] and changes in resting BP variables. However, there were significant negative correlations between the increase in RBC [RSNOs] and the decreases in SBP ($r_s = -0.68$, $P = 0.001$), DBP ($r_s = -0.59$, $P = 0.008$), and MAP ($r_s = -0.64$, $P = 0.003$; Fig. 4). Although significant correlations were found between changes in both RBC [RSNOs] and RBC [NO_2^-] and changes in resting SBP, there was no significant difference in the strength of the correlation coefficients ($Z = 0.791$, $P = 0.214$).

4. Discussion

In 2006, Larsen and colleagues reported that three days of dietary $NaNO_3$ supplementation resulted in a significant reduction in resting BP in healthy volunteers [42]. Following confirmation of these findings by other groups [3,18,19,43–45], dietary NO_3^- supplementation has emerged as a popular and promising strategy to prevent or treat hypertension [46]. In dietary NO_3^- research studies, plasma [NO_2^-] has

Table 2
Spearman's rank correlation coefficients between peak changes in NO biomarkers ([NO_3^-], [NO_2^-], [RSNOs] and [mercury resistant signal]) in plasma, whole blood, and red blood cells for each individual and corresponding changes in blood pressure variables following acute beetroot juice ingestion.

Correlation Coefficients	△SBP		△DBP		△MAP	
△Peak Plasma [NO_3^-]	r_s = 0.03	P = 0.92	r_s = 0.21	P = 0.38	r_s = −0.01	P = 0.98
△Peak WB [NO_3^-]	r_s = 0.18	P = 0.47	r_s = 0.13	P = 0.60	r_s = 0.12	P = 0.63
△Peak RBC [NO_3^-]	r_s = −0.15	P = 0.55	r_s = −0.29	P = 0.23	r_s = −0.29	P = 0.23
△Peak Plasma [NO_2^-]	r_s = −0.33	P = 0.16	r_s = −0.20	P = 0.42	r_s = −0.25	P = 0.30
△Peak WB [NO_2^-]	r_s = −0.39	P = 0.10	r_s = −0.02	P = 0.94	r_s = −0.31	P = 0.20
△Peak RBC [NO_2^-]	**r_s = -0.50**	**P = 0.030***	r_s = −0.20	P = 0.41	r_s = −0.42	P = 0.07
△Peak Plasma [RSNOs]	r_s = −0.31	P = 0.20	r_s = −0.20	P = 0.41	r_s = −0.30	P = 0.22
△Peak WB [RSNOs]	r_s = −0.32	P = 0.18	r_s = 0.05	P = 0.84	r_s = −0.16	P = 0.52
△Peak RBC [RSNOs]	**r_s = -0.68**	**P = 0.001***	**r_s = -0.59**	**P = 0.008***	**r_s = -0.64**	**P = 0.003***
△Peak Plasma [mercury resistant signal]	r_s = 0.20	P = 0.41	r_s = 0.07	P = 0.77	r_s = 0.04	P = 0.86
△Peak WB [mercury resistant signal]	r_s = 0.03	P = 0.90	r_s = −0.27	P = 0.26	r_s = −0.28	P = 0.25
△Peak RBC [mercury resistant signal]	r_s = −0.04	P = 0.87	r_s = 0.23	P = 0.34	r_s = 0.13	P = 0.59

Changes (△) in systolic blood pressure (△SBP), diastolic blood pressure (△DBP), mean arterial pressure (△MAP). Changes (△) in peak nitrate concentration ([NO_3^-]), nitrite concentration ([NO_2^-]), S-nitrosothiol concentration ([RSNOs]) and mercury resistant signal (e.g., [RNNO]) in plasma, whole blood (WB), red blood cells (RBC). Significant correlations ($P < 0.05$) are shown with '*'.

been used almost ubiquitously to reflect changes in NO bioavailability [2,17–19,42,45,47–49]. Although some studies have identified associations between increased plasma [NO_2^-] and decreased BP following dietary NO_3^- supplementation [2,19,20,34], others, including a meta-analysis of 15 studies, have not found a significant correlation between changes in these variables [21,50]. While these contrasting results might be related to inter-study differences in NO_3^- supplementation regimen, blood sampling and BP measurement time-points, and participant age and health status, it is also possible that plasma [NO_2^-] is not the most appropriate biomarker to quantify changes in NO availability and its relationship to changes in BP following NO_3^- supplementation. Consistent with this notion, the principal original finding of the present study was that the peak increase in RBC [RSNOs] (and, for systolic BP, the peak increase in RBC [NO_2^-]), but not the peak increase in plasma [NO_2^-], was significantly correlated with the decrease in resting BP following acute BR ingestion.

In the present study, we adapted a newly developed analytical procedure [35] to examine the pharmacokinetic profile of [RSNOs] in different blood compartments following dietary NO_3^- ingestion. This allowed us to compare the relationships between changes in different NO biomarkers (NO_3^-, NO_2^-, RSNOs) in different blood compartments (plasma, WB, and RBC) and changes in BP following acute dietary NO_3^- ingestion in healthy adults. We found that the peak increase in RBC [RSNOs] following NO_3^- ingestion was significantly correlated with decreased SBP, DBP and MAP at the corresponding time-point. In contrast, there were no significant correlations between the peak increases in NO biomarkers ([NO_3^-], [NO_2^-] or [RSNOs]) in plasma or WB and changes in BP. Both increased RBC [NO_2^-] and RBC [RSNOs] were correlated with decreased SBP, with there being no difference in the strength of these correlations. To the best of our knowledge, the present study is the first to indicate that the RBC may be the more important site, and that RSNOs may be the more important NO congener, in mediating the BP-reducing effects of NO_3^- supplementation.

4.1. Time course of [NO_3^-], [NO_2^-], [RSNOs], and [mercury resistant signal] changes in different blood compartments following acute BR ingestion

Plasma [NO_2^-] has been used almost exclusively as a biomarker of NO availability in previous dietary NO_3^- studies. Consistent with a

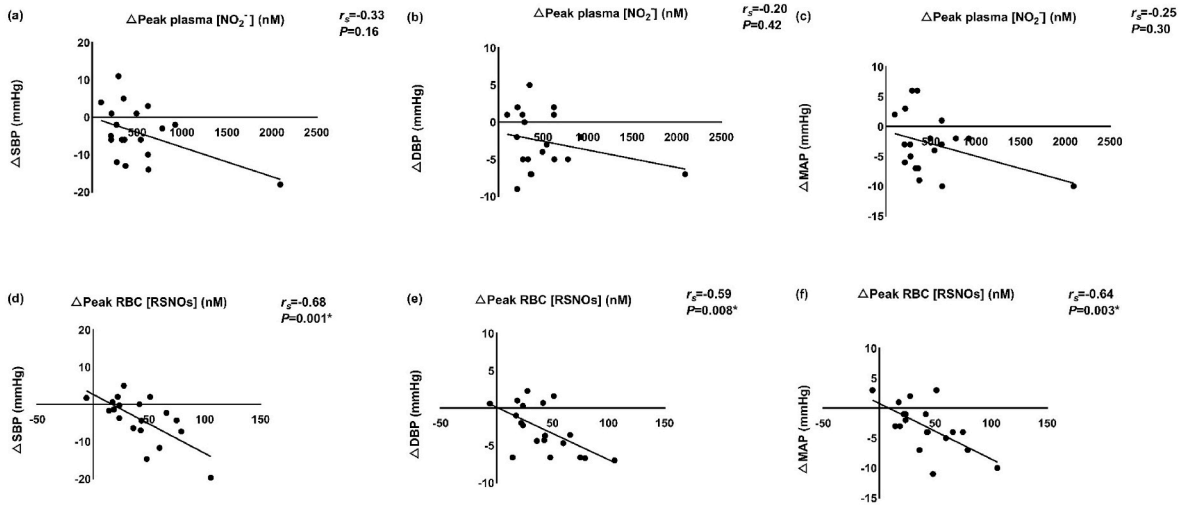

Fig. 4. Scatter plots showing the relationships between peak changes in plasma [NO_2^-] and RBC [RSNOs] for each individual and changes in resting BP at the corresponding time-point following acute beetroot juice supplementation in healthy adults (n = 19). Changes (△) in peak plasma nitrite concentration (△Peak plasma [NO_2^-]) and changes (△) in systolic blood pressure (△SBP) (**panel a**), diastolic blood pressure (△DBP) (**panel b**), and mean arterial pressure (△MAP) (**panel c**) at the corresponding time-point; Changes (△) in peak red blood cells S-nitrosothiol concentration (△peak RBC [RSNOs]) and △SBP (**panel d**), △DBP (**panel e**), and △MAP (**panel f**) at the corresponding time-point following acute beetroot juice ingestion (~12.8 mmol NO_3^-). Significant correlations ($P < 0.05$) are shown with '*'.

considerable body of previous research [18–20,42,51], [NO_3^-] and [NO_2^-] were significantly increased following BR ingestion, peaking at 1 and 3 h post BR ingestion, respectively, with the time-lag reflecting the dependence on the enterosalivary circuit for the reduction of NO_3^- to NO_2^- [14,16]. Plasma [NO_2^-] declined to the initial baseline level, while plasma [NO_3^-] remained elevated at 24 h. Similarly, [NO_3^-] and [NO_2^-] in WB and RBC were both increased following BR ingestion, also peaking at 1 and 3 h, respectively, before returning to baseline at 24 h. The [mercury resistant signal] in the three different blood compartments was significantly increased following BR supplementation, with values peaking at 2 h, and remaining elevated in plasma and RBC samples at 24 h. We also found that WB [RSNOs] and RBC [RSNOs] were significantly increased following BR ingestion, reaching their respective peaks at 2 h and 4 h, although it should be noted that the values were not significantly different between 2 h and 4 h. RBC [RSNOs] declined to a value that was not different to the initial baseline value at 24 h. Ghosh et al. [34] reported that the BP lowering effects of NO_3^- supplementation were maintained for 24 h in grade I drug free hypertensives. On the basis that plasma [NO_3^-] and [NO_2^-] had returned to baseline within that time frame [34], this implies that some other factor, including gastric RSNOs formation, may play a role in modulating BP. In contrast, we did not observe reduced BP, or elevated RSNOs, at 24 h following BR ingestion in healthy adult humans. Differences in health status (healthy adults vs. grade I hypertensives), supplementation dose (~12.8 mmol vs. ~3.5 mmol NO_3^-), and BP measurement (clinical BP measurement vs. 24 h ambulatory BP measurement) may explain these contrasting findings, with any of these factors potentially contributing to different [RSNOs] pharmacokinetics, NO bioactivity and BP responses to NO_3^- supplementation.

To our knowledge, this is the first study to report the pharmacokinetic response of [RSNOs] in plasma, WB and RBC following acute dietary NO_3^- ingestion. We identified no significant difference between baseline WB [RSNOs] and RBC [RSNOs], and both WB [RSNOs] and RBC [RSNOs] were increased at 1 h following BR ingestion. Unlike RBC and WB, there were no significant increases in plasma [RSNOs] at any time-point following BR ingestion. While this result is consistent with earlier reports [52,53], a recent study by Abu-Alghayth et al. [35] reported a significant increase in plasma [RSNOs] (median data: 104 nM) compared to baseline (12 nM) and placebo (11 nM) following BR supplementation. These contrasting results are likely explained by substantial differences in NO_3^- dose (12.8 vs. 44.8 mmol NO_3^- consumed over 30 h [20,35]) along with appreciable inter-individual response variability. It should be emphasised that our pharmacokinetic results are specific to the acute ingestion of BR containing 12.8 mmol NO_3^-. The effects of other NO_3^- doses and supplementation durations on the pharmacokinetic profile of [RSNOs] in different blood compartments requires further investigation.

4.2. Correlations between changes in [NO_3^-] and [NO_2^-] in different blood compartments and changes in resting BP following acute BR ingestion

NO is an endothelium-derived relaxing factor and a regulator of vasodilation in humans [54–56]. The quantitative measurement of NO is limited because it has a short half-life (a few ms) and high reactivity [57,58] such that NO can only diffuse a few μm *in vivo*. Plasma [NO_2^-] is an attractive and oft-used biomarker for NO due to its greater stability. We and others have previously reported significant correlations between increased plasma [NO_2^-] and reduced BP following dietary NO_3^- supplementation in humans [18–20,34,59]. For example, Vanhatalo et al. [19] reported significant correlations between increased plasma [NO_2^-] and reduced SBP and DBP following dietary NO_3^- supplementation ($r = -0.73$, $P < 0.05$ and $r = -0.57$, $P < 0.05$, respectively). However, a meta-analysis of 15 studies published between 2006 and 2012, involving a total of 254 participants, revealed that changes in plasma [NO_2^-] and BP following NO_3^- ingestion were not significantly correlated [21]. These results suggest that plasma [NO_2^-] may be a rather crude indicator of increased NO bioavailability and of the propensity to experience beneficial physiological effects following dietary NO_3^- supplementation.

Recently, Abu-Alghayth et al. [35] reported that not only plasma, but also RBC and WB [NO_3^-] and [NO_2^-] were increased following NO_3^- ingestion. It is unclear whether plasma or RBC play a more important role in the BP lowering effects of dietary NO_3^- supplementation. In a solution of 10 mM oxyhaemoglobin, NO is only able to diffuse approximately 1 μm [60] such that NO can only have biological effects close to its site of generation. Plasma is relatively close to smooth muscle cells in the vessel wall and, therefore, it is possible that NO congeners in plasma, via uptake into tissues and the actions of NO_2^- and NO_3^- reductases, play a more important role in vasodilation than RBC. In addition, NO in the circulation is oxidized to NO_3^- by oxyhaemoglobin: the high concentration of haemoglobin in RBC [61], coupled with the near-diffusion-limited rate of reaction between NO and oxyhaemoglobin [62], might prohibit NO from being exported from the RBC to influence vasodilation. A novel finding in the present study, however, was that the peak increase in RBC [NO_2^-], but not the peak increase in plasma [NO_2^-], was significantly correlated with decreased SBP following NO_3^- ingestion. This suggests that RBC may play an important role in the BP lowering effects of NO_3^- ingestion.

Brooks [63] first described the ability of deoxyhaemoglobin to act as a NO_2^- reductase. Cosby et al. [22] reported that exposure of deoxygenated RBC to NO_2^- produced NO and caused vasodilation, implicating the RBC as a key player in NO homeostasis. The unstirred layer around the RBC, the cell free zone, and the RBC submembrane are all diffusion barriers [64], which may serve to prevent oxidation of NO, thereby preserving NO bioactivity in the circulation. The results of the present study challenge the assumption that elevated plasma [NO_2^-], *per se*, is an important determinant of increased NO bioactivity following dietary NO_3^- ingestion and suggest that the measurement of other, perhaps more appropriate, blood biomarkers, including RBC [NO_2^-], should be considered in studies relating dietary NO_3^- intake to BP.

4.3. Correlations between changes in [RSNOs] in different blood compartments and changes in resting BP following acute BR ingestion

While the increases in plasma [NO_2^-] and RBC [NO_2^-] following BR ingestion were significantly correlated with one another in the present study, only the increased RBC [NO_2^-] was correlated with decreased SBP. However, it is unclear how NO generated in the RBC can be transported to smooth muscle cells given the high concentrations of intravascular haemoglobin which would be expected to scavenge NO and attenuate its ability to diffuse from the endothelium. In this regard, it is of interest that previous research found that protein disulfide isomerase (PDI), a protein primarily found in the endoplasmic reticulum, can be S-nitrosylated when RBC are exposed to NO_2^- under ~50% oxygen saturation [65]. The S-nitrosylated PDI was reported to be released from the RBC surface and to transfer NO into the endothelium, thereby leading to vasodilation [65]. RSNOs have also been considered a storage form for NO and have been shown to be important in functions such as the inhibition of platelet aggregation [32], smooth muscle contraction [30], and vasodilation [31]. Stamler et al. [26] proposed that RSNOs may serve as a relatively stable mediator of NO bioactivity by minimizing the reaction of NO with reactive oxygen species and therefore increasing the half-life of NO in the circulation. Compared to NO_2^- and NO, RSNOs have a longer half-life *in vivo*, are more chemically stable [66,67], and do not react with either deoxyhaemoglobin or oxyhaemoglobin [68]. These properties may enable RSNOs to diffuse further, and therefore exert effects on the vasculature.

The formation of RSNOs *in vivo* is complicated and influenced by factors such as stomach pH [69,70] and the concentration of free thiols [71]. Following dietary NO_3^- ingestion and the reduction of NO_3^- to NO_2^- by the oral microbiota [72], NO_2^- is further reduced to NO and reactive nitrogen species (RNS) upon exposure to the acidic milieu of the stomach [73]. RSNOs are then formed by the reaction of RNS with thiol

groups [52,74].

Both NO_2^- and RSNOs may serve as measures of NO bioavailability and have the potential to modulate NO bioactivity. Whether NO_2^- or RSNOs are more instrumental in the BP lowering effects of NO_3^- supplementation remains unclear, in part due to the challenges involved in RSNOs measurement, as is evident in the large variations in RSNOs values reported in the literature [75]. In the present study, we used a recently established analytical procedure to detect [RSNOs] in different blood compartments [35]. We observed a significant increase in RBC [RSNOs] following acute BR ingestion and found that the peak increase (as determined for each participant) was significantly correlated with decreased resting SBP, DBP and MAP at the corresponding time-point. Neither WB [RSNOs] nor plasma [RSNOs] were correlated with changes in BP. Although the correlation between the change in RBC [RSNOs] and the change in SBP ($r = -0.68$) was stronger than the correlation between the change in RBC [NO_2^-] and the change in SBP ($r = -0.50$), Fisher's Z transformation did not indicate that this difference was significant.

Our novel finding that, in humans, increased RBC [RSNOs] is significantly correlated with decreased BP is consistent, in part, with the findings of Pinheiro et al. [33] who experimentally dissociated the effects of oral administration of NO_2^- (and NO_3^-) on BP from the effects on plasma [NO_2^-]. These authors manipulated gastric pH and the concentration of thiols and demonstrated, in hypertensive rats, that RSNOs play an important role in the BP lowering effects of dietary NO_3^-. Specifically, they reported that, when gastric pH was increased, there was a decreased plasma [RSNOs] and an attenuated antihypertensive effect of oral NO_2^- treatment in the absence of changes in plasma [NO_3^-] or [NO_2^-]. Moreover, lowering the concentration of circulating thiols also resulted in blunted plasma [RSNOs] and BP responses to NO_2^- ingestion whereas increasing thiol levels proportionally augmented the plasma [RSNOs] and BP responses [33]. The importance of the acidic gastric environment to the efficacy of the $NO_3^- \rightarrow NO_2^- \rightarrow NO$ pathway is underlined by the study of Montenegro et al. [76] which demonstrated, in humans, that the reduction in SBP after oral administration of $NaNO_2$ was attenuated following pre-treatment with a proton pump inhibitor (esomeprazole, which is used to lower gastric acidity) despite a similar elevation of plasma [NO_3^-] and [NO_2^-]. Moreover, when participants received $NaNO_2$ intravenously, such that contact between the swallowed NO_2^- and the low pH in the stomach was avoided, no reduction in BP was found despite a significant increase of plasma [NO_2^-] [76]. Because RSNOs are formed in the acidic stomach, these results indicate that RSNOs play an important role in the BP lowering effects of dietary NO_3^- supplementation, and that elevated plasma [NO_2^-] may serve simply as a proxy biomarker for increased NO availability. It should be noted that although the [mercury resistant signal], which will include, for example, nitrosamines (RNNO), was significantly increased following NO_3^- ingestion in all blood compartments, there were no significant correlations with changes in resting BP variables. This strengthens our interpretation that RSNOs play a key role in the BP lowering effects of dietary NO_3^- supplementation.

The RBC has been considered an important site for the generation and transport of RSNOs in the human circulation due to the presence of haemoglobin, which acts as a RSNOs synthase and promotes allosteric NO delivery to tissue [68,77]. However, Pinheiro et al. [33] only measured plasma [RSNOs] in hypertensive rats, while Montenegro et al. [76] only measured plasma nitroso species concentration (which includes RSNOs and other nitroso species, such as N-nitrosamines and Fe-nitrosyl species) in humans, and so possible changes in [RSNOs] in the RBC were not addressed. To our knowledge, this study is the first to investigate correlations between changes in plasma, WB and RBC [RSNOs] and changes in resting BP variables following BR ingestion. Our results suggest that RBC [RSNOs], along with RBC [NO_2^-], may be more appropriate than plasma [NO_2^-] for quantifying increases in NO bioactivity in studies investigating the physiological effects of dietary NO_3^- supplementation.

4.4. Experimental considerations

Numerous studies have demonstrated that acute NO_3^- ingestion reduces resting BP compared to placebo ingestion [2,18–20,42,78]. The present study was designed to investigate the relationships between changes in [NO_3^-], [NO_2^-] and [RSNOs] in plasma, WB, and RBC and changes in resting BP variables (SBP, DBP, and MAP) in healthy adults following acute BR ingestion. In this design a placebo condition was not necessary. Although BP is influenced by the circadian rhythm, Wylie et al. [20] did not find significant changes in BP variables over the same time frame as that used in the present study when participants ingested a NO_3^--depleted placebo beverage. In the present study, concentrated NO_3^--rich BR was used as the dietary NO_3^- source. It should be noted that the antioxidant content of this juice has the potential to enhance NO bioavailability [79,80] and the effects of ingestion of a nitrate salt on the relationships between RBC and plasma [NO_2^-] and [RSNOs] with resting BP requires further investigation. The participants in the present study were relatively young (mean age of 37 years), lean (mean body mass index of 24.0 kg/m^2) and normotensive (mean SBP/DBP of 111/65 mmHg). Future studies should explore the relationships between RBC and plasma [NO_2^-] and [RSNOs] with resting BP in hypertensive individuals, in whom a greater fall in BP would be anticipated following dietary NO_3^- ingestion [2,34]. Finally, although the increase in RBC [RSNOs] was most strongly correlated with the decrease in BP following dietary NO_3^- ingestion in the present study, we note that measuring RSNOs is technically challenging. For example, it has been shown that the dinitrosyl iron complexes (DNICs) are degraded by $HgCl_2$ [81], potentially contributing to [RSNOs] measured in our samples. Therefore, plasma [NO_2^-] remains a practical and convenient general indicator of NO bioavailability.

5. Conclusion

This is the first study to compare the relationships between changes in NO biomarkers (NO_3^-, NO_2^- and RSNOs) in different blood compartments (plasma, WB, and RBC) and changes in resting BP following acute NO_3^- ingestion in healthy adults. We found that SBP was reduced at 3 h, DBP was reduced at 1, 2, and 3 h, and MAP was reduced at 1 and 3 h, following BR ingestion. The peak increase in plasma [NO_2^-], which is the most widely employed biomarker of NO bioavailability in dietary NO_3^- studies, was not correlated with decreases in BP at the corresponding time-point, although the peak increase in RBC [NO_2^-] was correlated with the decrease in SBP. However, significant correlations were found between the peak increase in RBC [RSNOs] and decreases in SBP, DBP, and MAP at the corresponding time-point, implying that RBC [RSNOs] may be an important mediator of the BP lowering effects of dietary NO_3^-. Further studies are required to investigate the influence of dietary NO_3^- supplementation strategy (i.e., dose and duration) on RBC [RSNOs] and RBC [NO_2^-] and their relationship to BP variables. Further studies are also required to determine whether RBC or tissue [RSNOs] are important in other putative physiological benefits of dietary NO_3^- supplementation such as improvements in cognition and exercise performance [82]. We conclude that RSNOs is a highly relevant biomarker of NO bioactivity and that the RBC plays an important role in mediating the effects of dietary NO_3^- on BP.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2023.05.008.

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