Pharmacokinetics of Nitrate and Nitrite Following Beetroot Juice Drink Consumption

Emily Margaret Jakubcik 1, Kay Rutherford-Markwick 2,3, Marsanne Chabert 4, Marie Wong 5 and Ajmol Ali 1,3,*

1 School of Sport, Exercise and Nutrition, Massey University, Auckland 0745, New Zealand; emilyjakubcik@outlook.com
2 School of Health Sciences, Massey University, Auckland 0745, New Zealand; K.J.Rutherford@massey.ac.nz
3 Centre for Metabolic Health Research, Massey University, Auckland 0745, New Zealand
4 Institut Polytechnique de Bordeaux, 33300 Bordeaux, France; marsannechabert@orange.fr
5 School of Food and Advanced Technology, Massey University, Auckland 0745, New Zealand; M.Wong@massey.ac.nz
* Correspondence: a.ali@massey.ac.nz

Abstract: Background: Nitrate (NO\textsubscript{3}\textsuperscript{−})-rich beetroot (BR) juice supplementation has been shown to improve cardiovascular function via reduction to nitrite (NO\textsubscript{2}−) and then to the bioactive molecule nitric oxide (NO). However, limited research exists for the role of inorganic NO\textsubscript{2}− that is contained naturally within BR. Objective: As BR juice can naturally contain both NO\textsubscript{3}− and NO\textsubscript{2}− the objective of this study was to evaluate the individual effects of NO\textsubscript{3}− and NO\textsubscript{2}− consumed from BR on plasma [NO\textsubscript{3}−]/[NO\textsubscript{2}−] and their subsequent effects on various cardiovascular measures. Design: In four separate treatments, 11 healthy adults consumed 250 mL of BR containing one of the following: (i) high NO\textsubscript{3}−, low NO\textsubscript{2}− (HL; 572 mg NO\textsubscript{3}−, 32 mg NO\textsubscript{2}−); (ii) medium NO\textsubscript{3}−, medium NO\textsubscript{2}− (MM; 280 mg NO\textsubscript{3}−, 237 mg NO\textsubscript{2}−); (iii) low NO\textsubscript{3}−, medium NO\textsubscript{2}− (LM; 43 mg NO\textsubscript{3}−, 262 mg NO\textsubscript{2}−); (iv) placebo (PL; low NO\textsubscript{3}−, low NO\textsubscript{2}−: 8 mg NO\textsubscript{3}−, 5.8 mg NO\textsubscript{2}−). Plasma [NO\textsubscript{3}−]/[NO\textsubscript{2}−], blood pressure, heart rate, mean arterial pressure (MAP), cardiac output and stroke volume were measured at baseline and every hour or second hour for 6 h post-BR consumption. Outcomes: Ingestion of the HL and MM BR increased plasma [NO\textsubscript{3}−] and [NO\textsubscript{2}−] after 2 h, with both remaining elevated after 6 h (p < 0.05). LM increased plasma [NO\textsubscript{3}−] (p < 0.05) but did not increase plasma [NO\textsubscript{2}−] compared to PL (p = 0.177). MAP was lower following the consumption of HL at 4 h and LM at 6 h (p < 0.05). Conclusion: Inorganic NO\textsubscript{2}− consumption is the critical factor in elevating plasma [NO\textsubscript{3}−] and [NO\textsubscript{2}−]; however, both NO\textsubscript{2}− and NO\textsubscript{3}− show potential to reduce MAP. The known reduction of systolic blood pressure (SBP)/diastolic blood pressure (DBP) following NO\textsubscript{3}− supplementation was not observed, making it unclear if NO\textsubscript{2}− contributes to a reduction in SBP/DBP alongside NO\textsubscript{3}−.

Keywords: nitric oxide; cardiovascular disease; cardioprotective; blood pressure

1. Introduction

Cardiovascular disease (CVD) places a significant burden on the health system, accounting for approximately one-third of all deaths [1]. A major risk factor for the development of CVD is increased blood pressure (BP) or hypertension [2,3] which is often the target for CVD-based interventions. Epidemiological evidence suggests a diet rich in fruit and vegetables reduces BP and the subsequent risk of CVD [4–7]. This effect has been previously attributed to the abundance of antioxidants and vitamins which are present in fruit and vegetables [4,7]. However, other studies failed to see any cardioprotective effect of antioxidants and vitamins [8–10], thus prompting researchers to investigate potential cardioprotective effects of other compounds present in fruits and vegetables. While it is likely that the cardioprotective effect observed with fruit and vegetable intake is multifactorial, the consumption of high nitrate (NO\textsubscript{3}−) vegetables [11,12] has been shown to lead
to the greatest reduction in BP compared to other vegetables [13,14]. As a result, there has been increased interest in the potential of NO$_3^-$ derived from fruit and vegetables to lead to a reduction in BP [13].

Beetroot juice (BR) is used as a supplement because of its high inorganic nitrate (NO$_3^-$) content, a compound found naturally in vegetables and in processed meats, where it is used as a preservative [15]. Beetroot juice contains varying amounts of both inorganic NO$_3^-$ and inorganic nitrite [16–18] which in the human body can reduce to the bioactive molecule nitric oxide (NO) through an exogenous pathway [19]. This exogenous pathway begins with the absorption of NO$_3^-$ in the gastrointestinal tract, which is then distributed to various locations, including the mouth [16]. In the mouth NO$_3^-$ is reduced to NO$_2^-$ by anaerobic bacteria [20]. NO$_2^-$ is then swallowed and re-enters the gastrointestinal tract; when it reaches the stomach, the acidic environment reduces NO$_2^-$ to NO [21]. NO enters the circulation and is then able to lower BP by relaxing and dilating the endothelium [22–24].

Consistent with the exogenous pathway, multiple studies have reported a reduction of systolic BP (SBP; 5–22 mmHg) and diastolic BP (DBP; 2.4–18.3 mmHg) following supplementation with BR [20,25–29]. This effect has been shown to occur in a dose-dependent manner, with a larger dose of NO$_3^-$ reducing BP to a greater extent than a smaller dose [25,29,30]. However, some studies have shown no change in either SBP or DBP following BR consumption [31,32]. Although the exact reason for the contradictory data is unclear, it may relate to dosage level—one of these studies [31] used a low NO$_3^-$ dose (119 mg; 1.4 mmol/day) which was likely insufficient to result in a reduction in BP.

Interestingly, several studies support NO$_2^-$ having a cardioprotective effect [33–35]. Specifically, in humans the oral consumption of NO$_2^-$ via NaNO$_2^-$ elicits an elevated plasma NO$_2^-$ [36], which correlates with a reduction in BP [37–39]. When specifically investigating the cardioprotective effect of NaNO$_2^-$ capsules (80 mg NO$_2^-$ or 160 mg NO$_3^-$), an improvement in brachial artery flow was seen but, interestingly, no change in BP [40]. Studies in mice have shown that NO$_2^-$ infusion led to a reduction in mean arterial pressure [41,42] and reversed endothelial dysfunction [43]. The cardioprotective effects of NO$_2^-$ can be explained via the exogenous NO pathway, and the ability for NO$_2^-$ to also produce the bioactive molecule NO [21,40]. While there is potential for NO$_2^-$ to reduce BP via the exogenous NO pathway, to date there has been no consideration as to whether the NO$_2^-$ in BR contributes to the observed reductions in BP.

Therefore, we determined the impact of different doses of NO$_3^-$ and NO$_2^-$ from BR on plasma levels of NO$_3^-$ and NO$_2^-$ and examined the effect of these different doses on cardiovascular measures. In addition, correlations between the plasma NO$_3^-$ and NO$_2^-$ levels and physiological outcomes were investigated.

2. Methods and Materials

2.1. Participants

Eleven healthy adults (18–50 years old) volunteered for this study (5 M; 6 F). All participants completed a health-screening questionnaire to determine eligibility. Inclusion criteria included healthy men and pre-menopausal women aged 18–50 years who were proficient in English and could attend the laboratory on multiple occasions. Those who regularly consumed NO$_3^-$-based dietary supplements, or were unable to participate in blood collection, or had a beetroot allergy, or were pregnant or had known health issues, e.g., hypertension or cardiovascular disease, were excluded. Prior to their involvement, the study was explained to all participants and each was provided with an information sheet. Participants were fully informed of any risks associated with the experiments before giving their informed written consent to participate in the investigation. The study was approved by the Massey University Human Ethics Committee (SOA 18/35).

2.2. Study Design and Procedures

Participants were required to visit the laboratory (18–20 °C, 40–60% relative humidity) on five occasions. During the first visit they were familiarised with the testing procedures
and equipment, including an ultrasonic cardiac output monitor (USCOM1A; Uscom Ltd.; Sydney, Australia) and automated sphygmomanometer (deluxe HEM-7310; OMRON Healthcare CO. Ltd.; Kyoto, Japan). Height was measured using a stadiometer (Seca portable stadiometer, Amtech, New Zealand) and body mass using scales accurate to 0.1 kg (A & D Weighing, HV-200KGL, Adelaide, Australia).

For 24 h prior to visit 2, participants recorded their food and fluid intake and were asked to replicate these diets for each 24-h period prior to the remaining visits (3 to 5). Participants were instructed to arrive at the laboratory in a fasted state, and to have refrained from caffeine ingestion, strenuous exercise and alcohol for 24 h pre-trial. Each trial commenced in the morning at approximately the same time of day (9 a.m. +/− 1 h).

Each participant was randomly allocated the order which the supplemented beverages would be consumed in a double-blinded, randomized crossover design. Visits 2 to 5 were the experimental trials and each trial was separated by a one-week washout period to allow for complete normalization of nitrate levels in the body [44–46]. The drink was consumed within a 10-min period alongside a standardised, isocaloric breakfast with a macronutrient distribution of 15 g protein, 30 g carbohydrate and 10 g fat. Three hours post-supplement intake the participants received a standardised, isocaloric lunch with a macronutrient distribution of 25 g protein, 45 g carbohydrate and 16 g fat.

On experimental trial days, baseline measures of blood pressure and a resting blood sample were taken on arrival and prior to supplementation. The allocated drink and breakfast were consumed and the timer started immediately after the drink was finished. Blood samples, BP and heart rate (HR) were measured each hour and other hemodynamic measurements (stroke volume (SV), mean arterial pressure (MAP) and cardiac output (CO)) were carried out via the USCOM every second hour (0 h, +2 h, +4 h, +6 h). Participants remained in the laboratory for the duration of the trial and were able to complete office work between sampling periods. The study protocol is illustrated in Figure 1.

2.3. Intervention

For each of the experimental trials, participants consumed 250 mL of one of the four juices containing different levels of nitrate and nitrite (Table 1). Each drink was similar in both appearance and taste and was not perceived as significantly different (p > 0.05) when presented to a consumer sensory panel [47]. The high and medium doses of NO$_3^-$ (572 mg and 280 mg, respectively) were chosen as these levels have been shown to be sufficient to elicit cardioprotective effects [29]. The chosen nitrite dose was the variable being tested—due to the low concentration of naturally occurring nitrite we were unable to produce a beverage containing >500 mg nitrite.

### Table 1. Nitrate and nitrite content of the drinks (per 250 mL) (as determined by high-performance liquid chromatography (HPLC)).

| Drink                        | Nitrate mg | Nitrite mg | Nitrate mmol | Nitrite mmol |
|------------------------------|------------|------------|---------------|--------------|
| High NO$_3^-$, low NO$_2^-$ (HL) | 572        | 32         | 6.72          | 0.46         |
| Medium NO$_3^-$, medium NO$_2^-$ (MM) | 280        | 237        | 3.29          | 3.43         |
| Low NO$_3^-$, medium NO$_2^-$ (LM) | 43         | 262        | 0.51          | 3.79         |
| Low NO$_3^-$, low NO$_2^-$ (PL) | 8          | 5.8        | 0.09          | 0.08         |

Nitrate (NO$_3^-$); Nitrite (NO$_2^-$).

Freshly prepared BR was blended with apple juice and water, and the flavour adjusted with a natural beetroot powder (low in NO$_3^-$) to produce two standardized drinks of a constant soluble solids concentration (11 °Brix). These drinks were each used as they were and further as the base drink for one other formulation. The drinks were pasteurized and poured into 250 mL plastic bottles, stored frozen and then thawed immediately prior to use. The placebo (PL) was made to a standardized low nitrate (8 mg/250 mL) and low nitrite (5.8 mg/250 mL) concentration which also formed the base of the high NO$_3^-$, low NO$_2^-$ (HL) beverage. The low nitrate, medium nitrite (LM) beverage was standardized to
contain 43 mg nitrate and 262 mg nitrite per 250 mL. This drink was also used as the base formulation for the medium nitrate and medium nitrite beverage (MM).

Figure 1. Protocol of the experimental trials. BP, blood pressure; HR, heart rate; SV, stroke volume; MAP, mean arterial pressure; CO, cardiac output.

The medium nitrate and medium nitrite beverage (MM) was prepared immediately prior to consumption by replacing 30 mL of the LM beverage with 30 mL of a commercial BR with known NO$_3^-$ and NO$_2^-$ content (determined by high-performance liquid chromatography (HPLC)). This produced a 250 mL beverage of 11° Brix containing standardized medium nitrate (280 mg/250 mL) and medium nitrite (237 mg/250 mL). Similarly, to prepare the HL solution, 70 mL of the placebo drink was replaced with 70 mL of the commercial BR immediately prior to consumption to produce a 250 mL beverage of 11° Brix containing standardized high nitrate (572 mg/250 mL) and low nitrite (32 mg/250 mL). The final concentrations of both nitrate and nitrite within the drinks were determined using high-performance liquid chromatography.
2.4. Blood Measurements

Venous blood samples (6 mL) were taken via a cannula (where possible) or by venepuncture within the antecubital area and collected into heparinized tubes. Following cannula blood sampling, 5 mL of a noncoagulant saline solution was then injected through the cannula to prevent clotting and blockage of the line. Samples were mixed and immediately centrifuged (MF-50 Hanil Science Industrial, Gimposi, Korea) at 1300 \( \times \) g for 10 min, and the collected plasma was aliquoted and stored at −80°C for later HPLC analysis of \( \text{NO}_3^- \) and \( \text{NO}_2^- \).

2.5. HPLC Analysis

The quantification of \( \text{NO}_3^- \) and \( \text{NO}_2^- \) in beetroot juice was based on previously described methods \[48,49\]. Briefly, samples were centrifuged, filtered (0.45 µm), diluted and separated on a Gracesmart C-18 column (4.6 × 250 mm; 5 µm), using an isocratic mobile phase of 0.01 M Octylammonium orthophosphate (pH 3–3.5) at a flow rate of 0.8 mL/min (20 °C). Detection occurred at UV wavelengths of 193 nm for \( \text{NO}_2^- \) and 213 nm for \( \text{NO}_3^- \). Quantification was achieved with external standard Na \( \text{NO}_3^- \) and Na \( \text{NO}_2^- \) solutions.

The quantification of \( \text{NO}_2^- \) in plasma was based on the method described by Li et al., \[50\]. The plasma samples were filtered using 10 kD cut-off filters (Amicon Ultra 2 10 KDa 2 mL Thermo LPN 00288739 UFC201024) which had each been washed four times with water. The determination of \( \text{NO}_2^- \) occurs through its derivatisation with 2,3-diaminonaphthalene (DAN) to yield the highly fluorescent 2,3-naphthotriazole (NAT). Briefly, NAT was separated at a flow rate of 1.3 mL/min (20 °C) on a Waters Spherisorb S5C8 column (4.6 × 250 mm; 5 µm) using a mobile phase of 15 mM sodium phosphate (pH 7.5) containing 50% methanol (0–5 min), sequentially followed by 100% Milli-Q water (5.1–7 min), 100% methanol (7.1–10 min) and 100% Milli-Q water (10.1–12 min) before returning to the initial buffer (12.1–17 min). Fluorescence was monitored with excitation at 375 nm and emission at 415 nm.

This same filtration process was utilized for plasma \( \text{NO}_3^- \) analysis. Following the filtration, the method previously described to quantify the \( \text{NO}_3^- \) within juice was used to quantify the \( \text{NO}_3^- \) within plasma following a suitable dilution.

2.6. Cardiovascular Measurements

Blood pressure and HR were measured via an automated sphygmomanometer (mean value of three measurements taken from the left arm \[51\]. The USCOM was used to measure velocity time integral (VIT), HR, SBP and DBP. Utilizing these measurements and various algorithms, the mean arterial pressure, stroke volume and cardiac output were obtained.

2.7. Statistical Analysis

Data were checked for normality using the Shapiro–Wilk test and variance using Levene’s test. Data were expressed as mean ± SD and all analyses were completed on raw data and delta-changed data to eliminate the effect of baseline differences. A p-value < 0.05 was considered to be statistically significant. For groups showing statistical differences, an effect size was calculated, with 0.2 being considered a “small” effect size, 0.5 being considered a “medium” effect size and 0.8 a “large” effect size \[52\]. All statistical analyses were completed using IBM SPSS package version 22 (IBM Corporation, Chicago, IL, USA).

Any differences between individual baseline values for all variables for both different drinks and different trial numbers were determined using one-way ANOVA. The main effect of drink, the main effect of time and the interaction effect of drink and time were determined for each variable using a repeated measures two-way ANOVA. To ensure accuracy, both the measured values (absolute value) and the change from baseline (delta-change) were analyzed. Additionally, to exclude the effect of lunch, analysis of each variable was completed taking into account pre-lunch time points (0 h to 3 h) along with the analysis on all time points (0 h to 6 h) for both forms of data. To limit confounders, this
was repeated for the main effect of trial, the main effect of time and the interaction effect of trial and time. When a significant interaction was found, Holm–Bonferroni post-hoc tests were used to find out where differences lay.

Pearson’s correlation was used to investigate relationships between plasma [NO$_2^-$] and [NO$_3^-$] and cardiovascular measures; where 0.2 inferred a weak correlation, 0.5 inferred a moderate correlation and 0.8 inferred a strong correlation [53].

3. Results

3.1. Participants

All eleven participants completed the BP, HR and USCOM measures. Of the eleven, only eight participants completed all blood samples due to difficulties during the blood collection process. Participant characteristics are reported in Table 2.

| Participant Characteristics | Total (n = 11) |
|----------------------------|--------------|
| Age (y)                    | 24 ± 5.7     |
| Height (cm)                | 173 ± 8.9    |
| Body mass (kg)             | 67.6 ± 13.3  |
| Plasma NO$_2^-$ (µM)       | 0.77 ± 0.086 * |
| Plasma NO$_3^-$ (µM)       | 36.74 ± 1.89 * |
| SBP (mmHg)                 | 111.36 ± 1.73 * |
| DBP (mmHg)                 | 70.83 ± 1.22 * |

Systolic Blood Pressure (SBP); Diastolic Blood Pressure (DBP). * Values are taken as a mean of eligible participants measured at 0 h for each trial.

3.1.1. Plasma NO$_2^-$

There was an interaction of drink and time for plasma [NO$_2^-$] ($p < 0.001$). Specifically, plasma [NO$_2^-$] was elevated in the high NO$_3^-$ trial relative to the high NO$_2^-$ (3 h and 6 h post-consumption) and PL (2 h and 6 h post-consumption; $p < 0.05$; Figure 2). PL and high NO$_2^-$ consumption showed no increases in plasma [NO$_2^-$] at any time point ($p > 0.05$). The delta change values also showed an elevation in plasma [NO$_2^-$] following consumption of HL relative to PL and LM (3 h and 6 h; $p < 0.05$). Plasma [NO$_2^-$] had a higher increase in the MM trial relative to PL (3 h and 6 h) and LM (3 h; $p < 0.05$); however, it was still lower than HL at 6 h ($p < 0.05$) but not at 3 h ($p = 0.16$).

3.1.2. Plasma NO$_3^-$

There was an interaction of drink and time for plasma [NO$_3^-$] ($p < 0.001$). Specifically, plasma [NO$_3^-$] was elevated in the HL, MM and LM trials relative to PL (2 h and 6 h post-consumption; $p < 0.01$; Figure 3). In all trials, time was shown to increase plasma [NO$_3^-$], rising from 1 h ($p < 0.001$) and plateauing at 5 h (5 h vs 6 h $p = 0.80$). The delta change values showed that the highest increase in plasma [NO$_3^-$] occurred following the consumption of HL ($p < 0.001$), with the second highest increase occurring following the consumption of MM ($p < 0.001$).

3.2. Blood Pressure (BP)

There was no interaction of drink and time for SBP ($p = 0.325$). SBP was reduced in all trials (2 h, 3 h, 5 h and 6 h post-consumption; $p < 0.001$), with no difference occurring between trials ($p = 0.783$). These findings were consistent when analysing delta change and pre-lunch data.

There was a trend for an interaction of drink and time for absolute data and change in diastolic blood pressure ($p = 0.059$; $p = 0.058$); this trend was not present pre-lunch ($p > 0.05$). There was no difference in DBP between trials ($p = 0.692$) and no effect of time on DBP ($p = 0.124$).
3.3. Heart Rate

There was no interaction of drink and time for HR in absolute or delta change data ($p > 0.05$). However, there was an interaction of drink and time for pre-lunch HR. Specifically, HR was reduced in the PL trials relative to MM (3 h post-consumption, $p < 0.05$).

There was a main effect of time for HR ($p < 0.05$). Specifically, in all trials HR was reduced, 1 h, 4 h, 5 h and 6 h post-consumption ($p < 0.05$), with the greatest decrease in HR occurring at 3 h ($p < 0.001$).
3.4. USCOM Measures

There was no main effect of time or treatment or interaction of drink and time for CO or SV (all $p > 0.05$). These findings were consistent when analysing delta change and pre-lunch data.

There was an interaction of drink and time for mean arterial pressure ($p = 0.013$). MAP was reduced in the HL trial relative to LM (4 h post-consumption; $p < 0.05$; Figure 4). The delta change values also showed an interaction of drink and time for MAP, which was reduced in the HL and MM trials relative to PL (4 h post-consumption; $p < 0.05$; Figure 5) and in the LM group relative to HL and PL (6 h post-consumption; $p < 0.05$; Figure 5).

![Figure 4](image1.png)

**Figure 4.** Mean arterial pressure (mmHg) over a 6-h period following the ingestion of placebo (PL); high nitrate, low nitrite (HL); low nitrate, medium nitrite (LM); and medium nitrate and medium nitrite (MM) beverages. * Significant difference between HL and LM $p < 0.05$.

![Figure 5](image2.png)

**Figure 5.** Change in mean arterial pressure (mmHg) over a 6-h period following the ingestion of placebo (PL); high nitrate, low nitrite (HL); low nitrate, medium nitrite (LM); and medium nitrate and medium nitrite (MM) beverages. * Significant difference between HL and PL ($p < 0.05$). b Significant difference between MM and PL ($p < 0.05$). c Significant difference between HL and LM ($p < 0.05$). d Significant difference between LM and PL ($p < 0.05$).
3.5. Correlations

Plasma $\left[ NO_3^- \right]$ showed a weak correlation with SBP at 6 h ($r = 0.428$; $p < 0.006$). Additionally, a weak negative correlation was also seen with the delta change data, where plasma $\left[ NO_3^- \right]$ correlated with DBP at 4 h ($r = -0.364$; $p = 0.021$).

4. Discussion

This study examined the effect of acute supplementation with beetroot juice rich in either $NO_3^-$ or $NO_2^-$, or a combination of both, on plasma $\left[ NO_3^- \right]$, plasma $\left[ NO_2^- \right]$ and cardiovascular responses in normotensive adults. The main findings of this study demonstrate that: (1) $NO_3^-$ consumption is the critical factor in elevating both plasma $\left[ NO_3^- \right]$ and $\left[ NO_2^- \right]$; (2) $NO_3^-$ ingestion reduces MAP 4 h post-consumption while $NO_2^-$ reduces MAP 6 h post-consumption; (3) an increase in plasma $\left[ NO_3^- \right]$ leads to decreased DBP after 4 h.

4.1. Plasma $\left[ NO_2^- \right]$ and $\left[ NO_3^- \right]$

This is the first study to directly evaluate the effects of $NO_2^-$ present in BR on plasma $\left[ NO_2^- \right]$ and $\left[ NO_3^- \right]$. Both HL and MM increased plasma $\left[ NO_3^- \right]$ and $\left[ NO_2^- \right]$ to a greater extent than LM and PL, implying that $NO_3^-$ consumption is the critical factor for the rise in plasma $\left[ NO_3^- \right]$ and $\left[ NO_2^- \right]$. Furthermore, HL caused a higher rise in both plasma $\left[ NO_3^- \right]$ and $\left[ NO_2^- \right]$ than MM, a change which is proportional to the $\left[ NO_3^- \right]$ within the drink. Both HL and MM drinks contained similar combined quantities of $NO_3^-$ and $NO_2^-$ (604 mg and 517 mg respectively), but different quantities of $NO_3^-$ (572 mg; 280 mg respectively) and $NO_2^-$ (32 mg; 237 mg respectively), leading to the conclusion that there is little effect of $NO_2^-$ on plasma $\left[ NO_3^- \right]$ or $\left[ NO_2^- \right]$.

Following the consumption of HL, peak plasma $\left[ NO_3^- \right]$ occurred after 1 h, and peak plasma $\left[ NO_2^- \right]$ occurred after 2 h. These findings are consistent with studies in the literature which showed $NO_3^-$-rich BR led to peak plasma $\left[ NO_3^- \right]$ 1–1.5 h post-ingestion [54] and peak plasma $\left[ NO_2^- \right]$ 2.5–3 h post-ingestion [55]. Furthermore, the current study showed that following the consumption of LM, peak plasma $\left[ NO_3^- \right]$ occurred 1 h post-ingestion but with no increase in plasma $\left[ NO_2^- \right]$. This result is inconsistent with previous studies, where the ingestion of NaNO$_2^-$ capsules increased both plasma $\left[ NO_3^- \right]$ and $\left[ NO_2^- \right]$, leading to the conclusion that $NO_2^-$ was 95–98% bioavailable (compared to the same IV dose) [36]. Although the source of supplement was different between our study and Hunault et al.’s [36], there were comparable doses of $NO_2^-$ (193–253 mg vs. 262 mg). Although specific research comparing the effect of $NO_2^-$ supplementation between BR and capsules does not exist, it has been suggested that synthetic nutrients delivered in capsules may not be used by the body in the same way as their natural counterparts [7].

Previous literature suggests that a reduction in BP following BR consumption (400–800 mg NO$_3^-$) is reliant on a rise in plasma $\left[ NO_2^- \right]$ (0.5–0.9 µmol/L) [37–39]. Our findings show for the first time that BR high in $NO_3^-$ but low in $NO_2^-$ (HL) led to the greatest rise in plasma $\left[ NO_2^- \right]$, and therefore $NO_3^-$ consumption may be the critical factor in producing a reduction in BP following BR supplementation. Furthermore, when BR is stored at room temperature, $NO_3^-$ begins to convert to $NO_2^-$ [17]; hence when BR is left at room temperature for a period of time, it may not lead to the same increase in plasma $\left[ NO_2^- \right]$ and therefore may have a reduced ability to lower BP. The current study did not show a correlation between BP and plasma $\left[ NO_2^- \right]$ despite similar $NO_3^-$ ingestion to past studies [26,29].

4.2. Blood Pressure

4.2.1. Systolic and Diastolic Blood Pressure

SBP and DBP decreased following the consumption of all drinks, including PL, suggesting that participants were becoming more relaxed over time. Interestingly, the reduction of SBP was not different between trials, leading to the conclusion that neither $NO_3^-$ or $NO_2^-$ had an influence on SBP. Similarly, participants’ DBP did not differ after consuming the different drinks; however, there was a trend for HL to reduce DBP more than the other
drinks. This may be secondary to the young, healthy participants who volunteered for this study.

Very few studies show no reduction in either SBP or DBP following NO$_3^-$ supplementation [31,32]. More commonly, it is concluded that NO$_3^-$ supplementation (comparable doses to the present study) elicits a reduction in SBP [20,25–30,44,56] and DBP [25,27–30,57]. In studies where an effect was not observed, the presence of confounding factors preventing NO$_3^-$ from reducing BP has been suggested. For example, Floyd et al. [32] suggested that an elevation in plasma [glucose] and [insulin] can prevent a reduction in BP in a younger population (27 ± 6.5 years) following potassium NO$_3^-$ supplementation (2.4 g; 24 mmol NO$_3^-$). In the current study, 15 g of carbohydrate was consumed alongside the supplement, with a further 30 g consumed at lunch (+4 h) which may have prevented a reduction in BP due to the rise in [glucose] and [insulin]. However, the low baseline SBP of 113 mmHg in the participants in Floyd et al. [32] and in the current study (111 mmHg) may better explain the lack of effect. A metaregression, including 19 eligible randomised clinical trials investigating the effects of BR supplementation (316–860 mg NO$_3^-$/day) over 2–56 d, showed that a larger decrease in SBP occurred in participants with a higher baseline SBP measure [58]. Most previous studies which have shown BR consumption to have an effect on SBP had participants with baseline SBP measures ranging between 127–149 mmHg [20,27–31].

4.2.2. Cardiovascular Functions

The current study showed no change in HR, SV or CO following NO$_3^-$-rich BR consumption, which is consistent with previous literature [27,59]. The present study has further shown that consumption of NO$_2^-$-rich BR also had no effect on these variables. The cardioprotective effects observed in previous literature are likely mediated by the relaxation and dilation of the endothelium associated with NO production [21,60]. Additionally, the endogenous formation of NO, via L-arginine supplementation, has shown to limit noradrenaline production, depressing sympathetic activity and increasing the parasympathetic tone [61]. A main characteristic of the sympathetic drive is increased HR, CO and SV [62], implying that endogenous NO has the ability to reduce HR, CO and SV [53]. Exogenous NO$_3^-$ has not been investigated, but the current study, along with previous literature, suggest that this does not have the same effect on noradrenaline and hence does not have the same effect on HR, CO and SV that endogenous NO$_3^-$ does.

It has been concluded that NO$_3^-$-rich supplementation reduces BP, more significantly in those with a higher baseline BP. As the current study failed to show any BP-lowering effect associated with NO$_3^-$, it is unlikely that any effect of NO$_2^-$ on BP would be shown. Further research into the potential effects of NO$_2^-$ on SBP and DBP is required in a population with a higher baseline SBP.

4.2.3. Mean Arterial Pressure

NO$_3^-$, independent of dose (HL and MM), reduced MAP after 4 h, and NO$_2^-$ (LM) reduced MAP after 6 h. Due to the direct conversion of NO$_2^-$ to NO in the body, high NO$_2^-$ was proposed to provide an early onset of cardioprotective effects compared to high NO$_3^-$ [21,64]. Previous literature has shown a decrease in MAP (−8.2 ± 7.6 mmHg; −7 ± 1 mm Hg) following NO$_3^-$-rich BR consumption (300 mg; 450 mg) [65,66], specifically occurring 4 h post-supplementation [66], which is consistent with the current study. However, the delayed effect of NO$_2^-$ on MAP in comparison to NO$_3^-$ cannot be explained by the current literature and is not consistent with the exogenous NO pathway. Further studies are required to ensure that this is an accurate and replicable outcome.

Further research investigating the pharmacokinetics of the NO$_2^-$ present in BR, and the subsequent correlations of both plasma [NO$_3^-$] and [NO$_2^-$], is warranted. The inconsistent findings of SBP and DBP associated with NO$_3^-$-rich BR in the current study indicate a need for further research to confirm the effect of NO$_2^-$ present in BR. Future
studies should include participants with a higher baseline SBP as this has been associated with a larger reduction in SBP.

5. Conclusions

Plasma [NO\textsubscript{3}\textsuperscript{−}] increased in a dose-dependent manner following the ingestion of combined NO\textsubscript{2}\textsuperscript{−} and NO\textsubscript{3}\textsuperscript{−}, while plasma [NO\textsubscript{2}\textsuperscript{−}] increased following the ingestion of NO\textsubscript{3}\textsuperscript{−} only. There was a significantly higher increase of plasma [NO\textsubscript{3}\textsuperscript{−}] following HL supplementation compared to MM supplementation. There was no effect of NO\textsubscript{3}\textsuperscript{−} or NO\textsubscript{2}\textsuperscript{−} ingestion on SBP, DBP, SV or CO compared to PL. MAP decreased following the consumption of HL, MM and LM; however, in comparison LM showed its effect 2 h later. Collectively, these results indicate that consumption of NO\textsubscript{3}\textsuperscript{−} is the critical factor in elevating plasma [NO\textsubscript{3}\textsuperscript{−}] and [NO\textsubscript{2}\textsuperscript{−}], although the consumption of either NO\textsubscript{3}\textsuperscript{−} or NO\textsubscript{2}\textsuperscript{−} can reduce MAP. Further research is required in a population with a higher baseline BP so any effects of NO\textsubscript{2}\textsuperscript{−} consumption on SBP/DBP can be observed.

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