Beetroot Juice versus Chard Gel: A Pharmacokinetic and Pharmacodynamic Comparison of Nitrate Bioavailability

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Highlights

- When matched for nitrate content both beetroot juice and chard gels, known to be rich in nitrate, increased plasma nitrate and nitrite concentrations and reduced blood pressure to a similar extent.
- Inter-individual variability to reach maximal plasma nitrite levels was considerable and should be taken into account when utilizing acute dietary nitrate supplementation.
- Plasma concentrations of total nitrosated products were higher with beetroot juice than with chard gel despite comparable nitrate content.

Abstract

Dietary supplementation with inorganic nitrate (NO_3^-) has been shown to induce a multitude of advantageous cardiovascular and metabolic responses during rest and exercise. While there is some suggestion that pharmacokinetics may differ depending on the NO_3^- source ingested, to the best of our knowledge this has yet to be determined experimentally. Here, we compare the plasma pharmacokinetics of NO_3^-, nitrite (NO_2^-), and total nitroso species (RXNO) following oral ingestion of either NO_3^- rich beetroot juice (BR) or chard gels (GEL) with the associated changes in blood pressure (BP). Repeated samples of venous blood and measurements of BP were collected from nine healthy human volunteers before and after ingestion of the supplements using a cross-over design. Plasma concentrations of RXNO and NO_2^- were quantified using reductive gas-phase chemiluminescence and NO_3^- using high pressure liquid ion chromatography. We report that, [NO_3^-] and [NO_2^-] were increased and systolic BP reduced to a similar extent in each experimental arm, with considerable inter-individual variation. Intriguingly, there was a greater increase in [RXNO] following ingestion of BR in

comparison to GEL, which may be a consequence of its higher polyphenol content. In conclusion, our data suggests that while differences in circulating NO_2^- and NO_3^- concentrations after oral administration of distinct NO_3^--rich supplementation sources are moderate, concentrations of metabolic by-products may show greater-than-expected variability; the significance of the latter observation for the biological effects under study remains to be investigated.

Key Words: nitrite, nitric oxide, dietary supplementation, blood pressure

1. Introduction

Dietary nitrate (NO_3^-) supplementation has been demonstrated to positively influence parameters of exercise performance (2, 25, 36) and vascular health (26, 27, 50, 54). These effects have been achieved utilizing a variety of different vehicles for NO_3^- delivery, including simple sodium (28) or potassium salts (23), NO_3^--rich foods (44), concentrated beetroot juice (BR) (58), and chard gel (GEL) (37, 38). These studies have consistently shown that circulating plasma [NO_3^-] and nitrite ([NO_2^-]) concentrations are increased following ingestion of NO_3^- supplements. Whilst the biological consequences of dietary NO_3^- administration are not fully understood at present, it is known that NO_3^- can be reduced to NO_2^-, which is believed to be subsequently further converted to bioactive nitric oxide (NO) (1, 31). The entero-salivary circulation plays a vital role in NO homeostasis with ~25% of all circulating NO_3^- taken up by the salivary glands and concentrated in the saliva (51). The reduction of NO_3^- to NO_2^- takes place in the oral cavity where commensal facultative anaerobic bacteria on the surface of the tongue reduce NO_3^- to NO_2^- via NO_3^- reductase enzymes (12, 29). Once

78 swallowed, NO_2^- reaches the stomach where a proportion is then converted to NO, with
79 the remainder being absorbed into circulation via the intestinal tract (3, 32, 33).

80 It is well-established that increases in plasma [NO_3^-] and [NO_2^-] following dietary NO_3^-
81 supplementation occur in a dose-dependent manner (4, 19, 21, 23, 58, 59), however the
82 influence of the vehicle, if any, is less certain. Several studies have reported that plasma
83 [NO_3^-] and [NO_2^-] $^-$] reaches maximal quantities at ~ 1–1.5 h and 2.5–3h, respectively,
84 after ingestion of BR (23, 35, 54, 58). Recent work from our laboratory has shown that
85 consuming GEL results in similar plasma NO_3^- pharmacokinetics but plasma [NO_2^-]
86 reaches maximal levels more quickly (~1.5 h) after ingestion (37). It is currently unclear
87 whether the variance in NO_2^- pharmacokinetics between BR and GEL is simply due to
88 the vehicle of administration or profoundly influenced by inter-cohort differences in
89 the response to NO_3^- supplementation. Understanding if the vehicle of NO_3^-
90 supplementation affects the fate of NO-related metabolites may allow for the
91 optimization of dosing strategies for sports performance and other contexts. Therefore,
92 the purpose of this study was to compare the effects of ingesting BR and GEL on plasma
93 NO metabolite pharmacokinetics and blood pressure (BP) pharmacodynamics in
94 healthy individuals.

95

96 **2. Methods**

97 **2.1 Participants**

98 Nine healthy adult males (age 28 ± 4 years, stature: 181 ± 8 cm, body mass: 83.4 ± 10.4
99 kg) volunteered to take part in the study, which was approved by the School of Science
100 and Sport Ethics Committee of the University of the West of Scotland. All participants
101 provided written informed consent and a medical questionnaire before the study began.

Healthy males between the ages of 18 and 45 who were physically active (taking part in recreational activity a minimum of 3 times per week) were eligible to participate in the study. Participants were excluded if they were currently taking dietary supplements or any medication, regularly used mouthwash, were smokers, had a current illness or virus within the previous month, had a known disorder or history of disorders of the hematopoietic system, were hypertensive (≥140/90 mmHg) or had a family history of premature cardiovascular disease. All procedures were conducted in accordance with the Declaration of Helsinki.

2.2 Experimental Design

Our study had a simple randomized cross-over design. Participants visited the laboratory on two separate occasions with a minimum 7-day washout period and a maximum of 14 days between visits. Participants consumed either concentrated BR (Beet It Organic Shot, James White Drinks, Ipswich, UK) or GEL (Science in Sport, GO+ Nitrates, Lancashire, UK) during each trial.

Participants were asked to refrain from the consumption of alcohol, caffeine, NO_3^- rich foods as outlined by Hord and colleagues (22), and to avoid any strenuous exercise for 24 h before each trial. Participants were also asked to refrain from the use of anti-bacterial mouthwash and chewing gum for the duration of the study as they have been shown to disturb the oral bacterial flora required for the conversion of NO_3^- to NO_2^- in the saliva (17, 41). Compliance to these factors was determined at the start of each visit.

Following a 12 h overnight fast, participants reported to the lab in the morning where they were asked to void the contents of their bladder and lie supine on a medical bed. After 15 min, BP was determined using an automated sphygmomanometer (Omron M10, Kyoto, Japan) three times, at 1 min intervals. A cannula was then inserted into the antecubital vein of the arm or a superficial vein on the dorsal surface of the hand and the line was kept patent by regular flushing with intravenous 0.9% saline solution. A sample of venous blood was then collected in a vacutainer containing EDTA and immediately centrifuged at 4000 rpm at 4^{o}C for 10 min (Harrier 18/80, MSE, UK). The plasma was extracted carefully ensuring the cell layer was not disturbed and immediately frozen at -80^{o}C for later analysis of plasma [NO_3^-], [NO_2^-], and total nitrosospecies [RXNO]. Participants then ingested either the BR or GEL supplements within 1 min of pre supplementation blood sampling. The GEL supplement comprised 120 ml of peach flavored sports gel containing 500 mg of NO_3^- from natural chard and rhubarb sources. In the BR trial, participants ingested 117 ml of concentrated BR that also contained 500 mg of NO_3^-. The NO_3^- content of the supplements was later verified using high-pressure liquid ion chromatography (section 2.3).

As outlined in Fig. 1 venous blood samples were collected simultaneously with measurements of BP pre-supplementation then at 1, 1.5, 2, 2.5, 3, 3.5 and 6 h post-ingestion of each supplement. The measurement of BP was carried out in triplicate, with the measurement being performed as close as possible to blood draw. The BP Cuff was placed on the opposite arm to the cannula. Participants remained supine from the first blood sample until the 3.5 h sample, after which they were allowed to sit at a desk, returning 30 min before the final sample. During the experimental trials, participants were provided with standardized meals, which had a low NO_3^- content. Specifically,

participants consumed a cereal bar after 1.5 h and a cheese sandwich 3.5 h after ingestion of BR or GEL. Participants were provided with *ad libitum* access to tap water. The volume consumed in trial 1 was recorded and kept consistent for trial 2.

2.3 Additional Experimental Arm

The aforementioned procedures were conducted to address the primary objective of this experiment whereby doses of GEL and BR matched for NO_3^- content were compared. Whereas the dose of GEL used in this experiment comprised two full gels as provided by the manufacturer (2 x 60g), 23 ml of BR was removed from one 70 ml bottle to ensure a matched NO_3^- content. Given that both researchers and end-users are more likely to utilize the full 140 ml (e.g. (21, 58) the dose of BR used in this experiment was considered to be lacking in ecological validity. To this end, eight of the participants completed an additional experimental trial where they received 140 ml of BR (600 mg of NO_3^-, H-BR) with the procedures repeated as previously described.

2.4 Analysis of Plasma NO Metabolites

High-pressure liquid ion chromatography was used to determine plasma [NO_3^-] and [NO_2^-]. Due to high variability in the NO_2^- measurements, which may relate to lack of specific sample processing without addition of N-ethylmaleimide prior to centrifugation, the NO_2^- data were re-analyzed using chemiluminescence and the latter was used in all calculations. Gas-phase chemiluminescence was used to determine plasma [RXNO]. Samples were thawed at room temperature in the presence of 5 mM N-ethylmaleimide and subsequently analyzed using an automated NOx detection system (Eicom, ENO-20, Kyoto, Japan, combined with a Gilson auto-sampler for [NO_3^-

174])(46) and a NO analyzer (Sievers NOA 280i, Analytix, UK for [NO_2^-] and CLD 77AM
175 sp, ECOphysicis, Durnten, Switzerland for [RXNO]) in conjunction with a custom-
176 designed reaction chamber. NO_2^- levels were determined using 1% potassium iodide in
177 5ml glacial acetic acid at room temperature for reduction of NO_2^- to NO (42); RXNO
178 levels were determined using the triiodide method (13). All samples were analyzed
179 within 3 months of sample collection in order to minimize degradation of NO
180 metabolites.

181

182 **2.5 Data Analysis**

183 All analyses were carried out using the Statistical Package for the Social Sciences,
184 Version 22 (SPSS Inc., Chicago, IL, USA) or GraphPad Prism version 6 (GraphPad
185 Software Inc., San Diego, USA) for kinetic analyses. For brevity, data from the
186 additional H-BR trial are not displayed in figures. The sample size was determined *a*
187 *priori* using a power calculation which revealed that a minimum of eight participants
188 was required to detect differences in the time taken for NO_2^- to peak between GEL and
189 BR conditions. To establish the time to reach maximal [NO_2^-] and [NO_3^-] a log
190 (Gaussian) non-linear regression model was applied to the data using the following
191 equation:

192 *Y=Amplitude*exp(-0.5*(ln(X/Center)/Width)^2).*

193 Data are expressed as the change in the mean (Δ) ± standard error of the mean (S.E.M)
194 as compared to baseline or the mean and 95% confidence interval (CI) for time to reach
195 maximal values. The distribution of the data was tested using the Shapiro-Wilk test. A
196 two-way repeated-measures ANOVA was used to examine the differences between
197 condition and over time for plasma NO_3^-, NO_2^-, RXNO, and BP. *Post-hoc* analysis to

198 determine the difference from the baseline was conducted using a paired samples t-tests
199 with Bonferroni correction. Statistical significance was declared when $P < 0.05$.

200

201 **3. Results and Discussion**

202 Plasma [NO_3^-] and [NO_2^-] at baseline amounted to 26 ± 5.7 μM $NO_3^=$, 95 ± 31.9 nM
203 NO_2^- for BR and 33 ± 3.4 μM NO_3^- and 25 ± 6.7 nM NO_2^- for GEL. As expected, oral
204 NO_3^- supplementation significantly increased plasma [NO_3^-] and [NO_2^-] in each
205 experimental arm ($P < 0.001$) (Δ [NO_3^-] with BR: 319.4 ± 32.1 μM, with GEL: 383.9
206 ± 35.7 μM, Fig. 2; Δ [NO_2^-] with BR: 205.4 ± 51.9 nM, with GEL: 207.4 ± 58.1 nM,
207 Fig. 3). The magnitude of the increase, however, was not different between BR and
208 GEL ($P > 0.10$). In the H-BR arm, [NO_2^-] and [NO_3^-] increased to a greater extent than
209 BR and GEL (Δ [NO_2^-] 277 ± 161 nM, Δ [NO_3^-] 457 ± 22 μM, both $P < 0.01$).
210 Following ingestion of BR, [NO_2^-] reached maximal values at 3 h (95%CI 2.1 – 3.9 h),
211 which was not different to GEL (2.8 h, 95%CI 2.3 – 3.2 h, $P = 0.739$). Likewise, the
212 time taken for plasma [NO_3^-] to reach maximal concentrations was not different
213 between BR and GEL (BR: 1.4 h 95%CI 0.8 – 1.9 h, GEL: 1.4 h 95%CI 0.7 – 2.1 h, P
214 = 0.737). In the H-BR arm, [NO_2^-] and [NO_3^-] reached maximal concentration in the
215 plasma after 3.2 h (95%CI 2.1 – 4.2 h) and 1.5 h (95%CI 0.9 – 2.1 h), respectively.
216 These data collectively suggest that the vehicle of delivery, be it liquid or gel, does not
217 impact the kinetics of the reduction of NO_3^- to NO_2^- or the maximal plasma
218 concentrations of these metabolites. Nevertheless, it remains to be established whether
219 NO_3^- supplementation in solid forms, such as whole vegetables or concentrated BR
220 flapjacks, results in different NO_x pharmacokinetics.

221

In the present study, plasma [NO_2^-] and [NO_3^-] reached maximal quantities within a similar timeframe to previous research with BR (19, 29, 40, 43). However, on this occasion [NO_2^-] took substantially longer after GEL (2.8 h) compared with our own previous work (1.5 h) (37). Given that descriptive and anthropometric variables were similar between the two study cohorts, it seems likely that physiological variations between individuals may account for these differences in time. Although plasma [NO_2^-] is likely to be substantially elevated in most individuals 2.5 h after ingestion of either BR or GEL, the peak may reasonably occur anywhere between 2.1 and 3.9 h. To further highlight this Figure 4 displays the individual variability in the plasma NO_2^- response to both vehicles of supplementation. Another important factor to acknowledge when comparing different studies is the methods of analysis for NO metabolites. The sensitivity of chemiluminescence and HPLC has been highlighted with factors such as sample preparation, type of analyzer used, and duration of sample storage, all potentially influencing the result acquired (8, 42). Whilst the precise mechanisms explaining the disparity in plasma [NO_2^-] pharmacokinetics between these studies are unclear, we speculate that this may at least be partially explained by variances in the gut microbiota (14), pH of oral cavity and stomach (18, 43), and differences in the composition of the oral bacterial flora required for NO_3^- reduction (11, 18). The importance of the oral microbiome for NO_3^- reduction has been clearly established, with the oral reductase capacity substantially interrupted when using anti-bacterial mouthwash (5, 41, 55) or spitting of saliva following NO_3^- supplementation (30, 54). Equally, physical fitness has been suggested to affect the individual response to NO_3^- supplementation (18). In contrast to the direct association between endothelial NO production (as measured by plasma NO_2^-) and exercise performance (47, 53). Porcelli and colleagues (45) demonstrated that there was a negative association between aerobic

247 capacity (VO_{2peak}) and the increase in plasma [NO_2^-] following ingestion of a NO_3^-
248 supplement. Although not measured in either the present study or our previous work on
249 NO_3^- pharmacokinetics (37), it is conceivable that individual differences in physical
250 fitness, diet, or other lifestyle habits may contribute to the between-group variation
251 reported here and elsewhere within the literature (18). Although it has not been
252 thoroughly investigated, it is also conceivable that oral (and gut) microbial flora
253 changes as a result of frequent NO_3^- supplementation. It has been recently demonstrated
254 following 2 weeks of NO_3^- supplementation via BR there is an increase in salivary pH
255 suggesting a role of NO_3^- supplementation in altering composition of the oral
256 microbiome (20).

257

258 Whilst the NO_3^- and NO_2^- responses were similar between experimental arms, an
259 unexpected finding was that ingestion of BR tended to increase plasma [RXNO] to a
260 greater extent in comparison to GEL (Δ in BR: 408.1 ± 127.9 nM vs. Δ in GEL: 148.1
261 ± 35.1 nM, $P = 0.08$, Fig. 5.). Plasma [RXNO] at baseline amounted to 79.5 ± 13.1 nM
262 for BR and 71.9 ± 10.9 nM for GEL. There was, however, a high degree of variability
263 in the change in [RXNO] between individuals and the small sample size likely explains
264 why this finding was not statistically significant. The increase in [RXNO] was even
265 greater in the H-BR trial (Δ563.8 ± 116.7 nM) at 2 h post ingestion than in GEL ($P =$
266 0.004) and BR (P=0.03). Although plasma [RXNO] is not measured routinely in NO_3^-
267 supplementation studies, the magnitude by which [RXNO] increased following BR in
268 the present study is greater than what has been previously reported [6]. Equally
269 surprising was that the rise in [RXNO] exceeded that of [NO_2^-] following ingestion of
270 BR. The explanation for this is presently uncertain and while differences in
271 supplementation regimen, NO_3^- dose, and study participants may explain the disparity

with previous research, further work is required to explore the changes in [RXNO] and [NO_2^-] following ingestion of BR.

What is also unclear is why ingestion of BR increases [RXNO] to a greater extent (at least in the H-BR trial) compared to GEL. Although care was taken to match the supplements for total NO_3^- content, differences in the polyphenol content between beetroot and chard may account for this outcome (24, 57). Furthermore, alongside the primary sources of NO_3^- the BR supplement contained additional ingredients including lemon juice and the GEL contained rhubarb juice, gelling agents, preservatives, and flavorings. While the total antioxidant and polyphenol content of BR has been defined (56, 57) there is no comparable data on GEL. The total polyphenol content of each supplement may be important for overall NO bioavailability. Ingestion of flavonoid rich apples, for example, has been shown to increase [RXNO] in healthy adults (6), and nitrated polyphenols are formed from acidified NO_2^- under simulated stomach conditions (40). Moreover, it has been shown that polyphenols augment the reduction of NO_2^- to NO in the gut (48, 49). Given that S-nitrosothiols (RSNO), a component of RXNO, act as a carrier and store of NO in the blood, a polyphenol-induced increase in the bioavailability of NO may reasonably be exhibited by an increase in total nitroso products following BR. The importance of the polyphenol content of NO_3^- supplements and the role of RXNO in the translation to consequent physiological outcomes has yet to be established. However, the high polyphenol content of BR (56, 57), may explain the greater reduction in oxygen consumption following BR compared to sodium NO_3^- (15). RXNOs are protected from direct NO scavenging by reactive oxygen species allowing NO to be transported by e.g. serum albumin and red blood cells (7, 52). This establishes an NO reservoir for the sustained release of NO from these biological

storage forms (9, 16, 34). Potentially allowing for the targeted delivery of NO to where it is required such as sites of ischemia during exercise.

Systolic (SBP), diastolic (DBP), and mean arterial pressure (MAP) at baseline were as follows SBP: 123 ± 2 mmHg, DBP: 70 ± 1 mmHg, MAP: 88 ± 1 mmHg for BR and SBP: 124 ± 2 mmHg, DBP: 73 ± 2 mmHg, MAP: 90 ± 2 mmHg for GEL. In the present study, both BR and GEL reduced SBP and MAP (Δ SBP with BR: -10 ± 2 mmHg, $P <$ 0.001, vs. Baseline; with GEL: -12 ± 2 mmHg, $P < 0.001$; Δ MAP with BR: -5 ± 2 mmHg, $P = 0.012$ vs Baseline; with GEL: -7 ± 2 mmHg, $P = 0.010$, Fig. 6). The magnitude of the reductions in SBP and MAP were not different between BR and GEL ($P \geq 0.12$). Neither GEL nor BR significantly altered DBP ($P = 0.18$) nor was there any difference between experimental arms ($P = 0.197$). Likewise, SBP (Δ -11 ± 2 mmHg, $P < 0.001$) and MAP (Δ -8 ± 3 mmHg, $P < 0.001$) were reduced and DBP remained unchanged from baseline in the H-BR arm. It must be acknowledged that maintenance of the supine position for a prolonged period of time also likely contributed to a reduction in BP. Without a control condition, however, it is impossible to determine the extent of this effect. Nevertheless, these findings are consistent with previous literature demonstrating that ingestion of either BR or GEL reduces SBP and MAP among healthy individuals (23, 37, 54, 58). The response in DBP appears to be more variable, however, although several previous studies have reported comparable data (2, 10, 23). Given the data presented here, it appears that the plasma [NO_3^-] and [NO_2^-] mirrors acute hemodynamic response to dietary NO_3^- closely. Of notable interest, however, is that the changes in [RXNO] did not appear to be associated with the magnitude of the reduction in BP. This is in contrast to work by Oplander and colleagues (39) who demonstrated that reductions in BP were associated with an

increased plasma availability of RXNO but not NO_2^- following exposure of the skin to ultraviolet radiation. It is conceivable, therefore, that the method by which NO bioavailability is augmented will alter the mechanisms by which BP is reduced.

4. Conclusion

Our data suggests that dietary NO_3^- supplementation via BR and GEL elicits similar plasma [NO_2^-] and [NO_3^-] pharmacokinetics when examined within the same participant cohort. Likewise, both BR and GEL are capable of reducing SBP and MAP with little difference in the magnitude of these effects. Nevertheless, we here present data demonstrating that the time course of ingesting the NO_3^- supplements to maximal [NO_2^-] in blood plasma is profoundly variable between individuals. This is of major relevance for researchers wishing to determine the same. We also report, for the first time, that ingesting BR leads to a greater availability of RXNO compared to GEL, which we speculate may be attributed to the higher polyphenol content of the BR supplement.

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Figure Captions

Figure 1: Study overview: time-points for beetroot juice/chard gel administration, venous blood sampling, blood pressure measurements and food intake.

Figure 2: Changes in plasma nitrate concentrations following supplementation with BR and GEL (Δ Mean ± S.E.M). * Significant difference from baseline (pre-supplementation) ($P < 0.001$).

Figure 3: Changes in plasma nitrite concentrations following supplementation with BR and GEL (Δ Mean ± S.E.M). * Significant difference from baseline (pre-supplementation)

Figure 4: Individual plasma nitrite pharmacokinetics and Systolic BP for BR and GEL. Each participant is represented by the same different colour in each figure.

Figure 5: Changes in total nitroso species concentrations following supplementation with BR and GEL (Δ Mean ± S.E.M). * Significant difference from baseline (pre-supplementation)

Figure 6: Systolic (A), diastolic (B) and mean arterial pressure (C) changes following supplementation with BR and GEL (Δ Mean ± S.E.M). * Significant difference from baseline (pre-supplementation)