Changes in body posture alter plasma nitrite but not nitrate concentration in humans

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Abstract

PURPOSE: This study evaluated the change (Δ) in plasma volume (PV), nitrate [NO_3^-], and nitrite [NO_2^-] concentration following changes in posture in the presence and absence of elevated plasma [NO_3^-] and [NO_2^-]. **METHODS:** Fourteen healthy participants completed two trials that were preceded by either supplementation with NO_3^--rich beetroot juice (BR; total of ~31 mmol NO_3^-) or no supplementation (CON). Both trials comprised 30 min of lying supine followed by 2 min of standing, 2 min of sitting and 5 min of sub-maximal cycling. Measurements of plasma [NO_3^-] and [NO_2^-] were made by gas-phase chemiluminescence and ΔPV was estimated using the Dill and Costill method. **RESULTS:** Plasma [NO_2^-] decreased from baseline (CON: 120 ± 49 nM, BR: 357 ± 129 nM) after lying supine for 30 min (CON 77 ± 30 nM; BR 231 ± 92 nM, both $P<0.01$) before increasing during standing (CON 109 ± 42 nM; BR 297 ± 105 nM, both $P<0.01$) and sitting (CON 131 ± 43 nM; BR 385 ± 125 nM, both $P<0.01$). Plasma [NO_2^-] remained elevated following exercise only in CON (125 ± 61 nM $P=0.02$). Plasma [NO_3^-] was not different between measurement points in either condition ($P>0.05$). PV increased from baseline during the supine phase before decreasing upon standing, sitting, and exercise in both trials (all $P<0.05$). **CONCLUSIONS:** Changing body posture causes rapid and consistent alterations in plasma [NO_2^-]. Researchers should therefore carefully consider the effect of posture when measuring this variable.

Key Words: beetroot juice, exercise, plasma volume

1. Introduction

Nitric oxide (NO) is a ubiquitous signalling molecule which is synthesised endogenously from L-arginine by NO synthases (NOS) in the presence of oxygen (O_2) and regulates a multitude of biological processes (Forstermann and Sessa 2012). For example, NO is transiently released from endothelial cells, diffusing from the production site to smooth muscle cells, resulting in dilation of the vasculature (Ignarro et al. 1987). Nevertheless, the majority of NO does not reach its target cells due to rapid oxidation to nitrite (NO_2^-) and nitrate (NO_3^-) (Kelm 1999).

However, when pO_2 is reduced endogenous NO synthesis is diminished and can result in a reduced blood flow to the periphery (Lundberg et al. 2008). Under these conditions it has been shown that NO metabolites can be utilised as a reservoir for NO production via the NO_3^- - NO_2^- - NO pathway. Firstly, NO_3^- can be reduced to NO_2^- by facultative bacteria on the tongue (Duncan et al. 1995) via the entero-salivary system (Lundberg and Govoni 2004). The resultant NO_2^- is ingested and absorbed into the blood plasma where it may be reduced to NO under certain local physiological conditions (Lundberg et al. 1994; Millar et al. 1998; Modin et al. 2001; Castello et al. 2006). However, due to the short half-life of NO, plasma [NO_2^-] is still considered to provide the best approximation of vascular NO bioavailability (Kelm 1999; Lauer et al. 2001). Increased plasma [NO_2^-] is also associated with improved endothelial function (Rassaf et al. 2006) and superior exercise capacity (Totzeck et al. 2012) and is therefore routinely measured in cardiovascular and exercise science research.

NO_3^- is also readily available within our diet and its consumption has been shown to significantly increase plasma [NO_3^-], and thus [NO_2^-]. There is now an abundance of research which has explored the potential therapeutic and ergogenic benefits of supplementation with dietary NO_3^- (Siervo and Lara 2013; Pawlak-Chaouch et al. 2016; McMahon et al. 2017). This

research typically encapsulates repeated measurements of physiological variables during periods of rest and exercise. During the experimental protocols, blood samples may be routinely collected while participants are either supine (Muggeridge et al. 2015), seated (Sandbakk et al. 2015), or standing (Wylie et al. 2013b). However, it is well-established that alterations in posture lead to marked changes in plasma volume (PV) that can alter the molecular concentrations of common biochemical analytes (Thompson et al. 1928; Fawcett and Wynn 1960; Hagan et al. 1978; Hagan et al. 1980; Lippi et al. 2015). A probable explanation is that postural-induced alterations in hydrostatic pressure force fluid and protein to shift between the blood and interstitial space (Cohn 1966; Stokke et al. 1986). Lippi and colleagues (2015) report that PV was decreased by 3% and 14% when moving from a supine posture to sitting and standing, respectively. Furthermore, short duration high-intensity exercise has been shown to decrease PV between 5-22 % in various exercise modalities (Kargotich et al. 1998).

Previous research has shown considerable variability in the values of basal and supplemented plasma [NO_3^-] and [NO_2^-] (Wylie et al. 2013b; Muggeridge et al. 2015; Sandbakk et al. 2015; McMahon et al. 2017). Given that NO_3^- is measured in the micromolar range and NO_2^- in the nanomolar range, the measured concentration of these variables may be subject to postural-induced changes. Indeed, we have recently shown that plasma [NO_2^-] declined significantly over a 30 min period while participants lay supine (Muggeridge et al. 2015), suggesting that posture and PV shifts may alter plasma [NO_2^-]. However, several factors exist which may account for these observations. For example, prolonged sitting is known to decrease shear stress, whereas standing may increase shear stress and endogenous NO production (Uematsu et al. 1995; Sessa 2004; Hsieh et al. 2014; Restaino et al. 2016; Morishima et al. 2017). This

96 demonstrates that further research is required to determine the impact of posture on NO
97 metabolites.

98

99 No study has explored the effects of postural changes on the measured concentrations of plasma
100 [NO_3^-] and [NO_2^-]. The aim of the present study, therefore, was to determine the magnitude of
101 the postural-induced changes in plasma [NO_3^-] and [NO_2^-] and PV at rest and following short-
102 duration exercise. The experiment was conducted both with and without prior dietary NO_3^-
103 supplementation. We hypothesised that postural changes would alter plasma [NO_3^-] and [NO_2^-
104] which would be inversely associated with the change in PV.

105

106 **2. Methods**

107 2.1. Participants

108 Fourteen healthy and recreationally active participants (9 males and 5 females, age 27 ± 4 years,
109 stature 176 ± 7 cm, and body mass 71 ± 11 kg) volunteered to participate in the study. Written
110 informed consent was obtained from all individual participants included in the study. The study
111 was approved by the School of Science and Sport Ethics Committee at The University of the
112 West of Scotland and all procedures were performed in accordance with the 1964 Declaration
113 of Helsinki and its later amendments.

114

115 2.2. Study design

116 Each participant attended the laboratory on two separate occasions with a minimum of six days
117 between each visit. The experimental conditions were identical in each visit with the exception
118 that the first trial was conducted with no dietary intervention (control; CON). The second was

preceded by ingestion of 3 x 70 ml of NO_3^--rich beetroot juice (Beet it, James White Drinks, UK) the day before and 2 x 70 ml, 2 h before the first blood sample (BR; total of ~31 mmol NO_3^-). Participants recorded their diet 24 h prior to CON and were asked to repeat this as closely as possible prior to BR. All trials were completed before 11 a.m. at the same time of day for each participant and following an overnight fast. Participants were instructed to avoid caffeine, foods high in NO_2^- and NO_3^- (e.g. green leafy vegetables and cured meats), alcohol, mouthwash, and strenuous exercise 24 h prior to the experiment. Participants were provided with one bottle of drinking water (Harrogate, UK) prior to CON trial and given instructions to arrive at the lab well hydrated. Participants recorded the volume of water ingested prior to CON and matched the volume prior to BR.

2.3. Procedures

Following standard anthropometric measurements (stature and body mass), participants lay in a supine posture to allow for the insertion of a cannula into the antecubital vein. Following cannulation, participants stood up for 10 min prior to lying supine to start the experimental trial. Baseline measurements (0 min) of venous blood, blood pressure (BP) and heart rate (HR) were recorded immediately. The measurement of BP was conducted using an automated sphygmomanometer (Omron M10, Kyoto, Japan) in triplicate during supine measures and in duplicate for the standing and seated measures. Mean arterial pressure (MAP) was calculated by the following equation:

$$MAP = (2 \times \text{diastolic BP} + \text{systolic BP}) / 3$$

142 Continuous measurement of HR was conducted using telemetry (Polar electro, Oy, Finland).
143 Participants lay supine for a total of 30 min followed immediately by 2 min of standing, 2 min
144 of sitting, and then 5 min of cycling at 60% of the age-predicted maximal heart rate. The
145 duration of the standing, sitting, and exercise phases was kept brief to minimise
146 pharmacokinetic alterations in plasma [NO_3^-] and [NO_2^-] in the BR trial. Plasma [NO_2^-] and
147 [NO_3^-] are known to peak at ~2.5 h and ~1.5 h, respectively (Webb et al. 2008; Lundberg and
148 Weitzberg 2009; Larsen et al. 2010; Wylie et al. 2013a; McIlvenna et al. 2017). Therefore, the
149 end of the supine phase was designed to coincide with the peak in plasma [NO_2^-]. The
150 experimental protocol reflects many exercise physiology research studies that incorporate both
151 resting and exercise phases (Wylie et al. 2013b; Muggeridge et al. 2015; Sandbakk et al. 2015).

152

153 Collection of venous blood, BP and HR were repeated throughout the experiment as detailed
154 in Figure 1. The measurement of BP was not made during exercise due to difficulties in
155 obtaining a stable measurement. Venous blood was collected in 10 ml aliquots and the cannula
156 flushed with sterile 0.9% saline solution between samples to keep the line patent. Whole blood
157 was initially separated into EDTA vacutainers (BD Vacutainer). One vacutainer was
158 refrigerated at 4°C for the later analysis of haemoglobin concentration and haematocrit. All
159 samples were analysed within 6 h. The other vacutainer was centrifuged at 4000 rpm and 4ºC
160 for 10 min within 3 min of collection (Pelletier et al. 2006; Bailey et al. 2009). The plasma was
161 then separated, frozen at −80 °C, and analysed within 4 months of initial collection for
162 determination of [NO_3^-] and [NO_2^-].

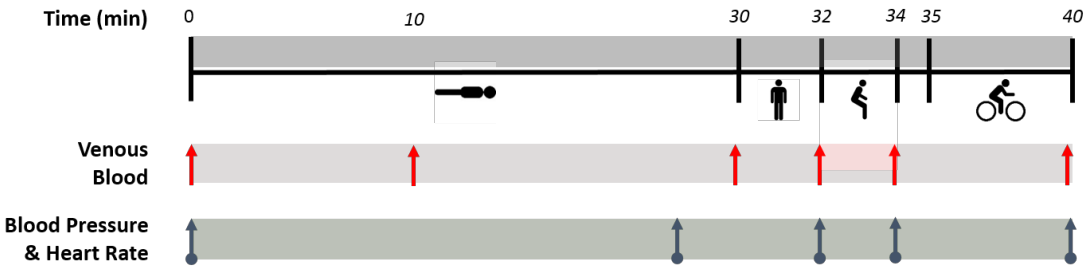


Fig. 1. Schematic of measurement time points for CON and BR trials.

2.4. Plasma Nitrite and Nitrate analysis

Measurements of [NO_3^-] and [NO_2^-] were made using ozone-based chemiluminescence (Rogers et al. 2005). For the measurement of [NO_2^-], tri-iodide reagent (2.5 ml glacial acetic acid, 0.5 ml of 18 Ω deionised water and 25 mg sodium iodide) and 100 µL of anti-foaming agent were placed into a customised glass purge vessel infused with nitrogen and inlet that was heated to 50 °C. This purge vessel was further connected to an NO analyser (Sievers NOA 280i, Analytix, UK). A standard curve was produced by injecting 100 µL of NO_2^- solutions (1000 nM, 500 nM, 250 nM, 125 nM, and 62.5 nM) and control sample containing deionised water. The area under the curve (AUC) for the latter was subtracted from the NO_2^- solutions to account for NO_2^- in the water used for dilutions. Following this, plasma samples were thawed in a water bath at 37 °C for 3 min and 100 µL of the sample was injected into the purge vessel in duplicate. The concentration of NO cleaved during the reaction was then measured by the NO analyser. The AUC was calculated using Origin software (version 7) and divided by the gradient of the slope. The coefficient of variation for the measurement of [NO_2^-] in the current study was 3%.

For the measurement of plasma [NO_3^-], vanadium reagent (32 mg of vanadium tri-chloride, 4 ml of 1M hydrochloric acid and 500 µL of water) and 100 µL of anti-foaming agent were placed into the glass purge vessel and heated to 95 °C. A standard curve was produced by injecting 25-50 µL of NO_3^- solutions (100 µM, 50 µM, 25 µM, 12.5 µM, and 6.25 µM) and a control sample containing deionised water. Plasma samples were thawed and de-proteinised (200 µL of sample, 400 µL of zinc sulphate in deionised water at 10% weight/volume and 400 µL of sodium hydroxide in deionised water at ratio of 1:1). Subsequently, 15-25 µL of the sample was injected into the purge vessel in duplicate and plasma [NO_3^-] calculated as previously described for the NO_2^- assay. The coefficient of variation for the measurement of [NO_3^-] in the current study was 6%.

2.5. Determination of Plasma Volume Change

To determine the haematocrit, a small volume of venous blood was extracted into heparinised capillary tubes that were sealed at the distal end with a wax seal. The capillary tubes were then spun for 8 min at 15,000 revolutions/min in a micro-haematocrit centrifuge before the haematocrit was measured in triplicate using a Hawksey haematocrit reader. The coefficient of variation for the measurement of haematocrit in the current study was 0.4%. Haemoglobin concentration was determined using the Randox colorimetric method (RX Monza, Randox Laboratories, UK). Briefly, 20 µL of whole blood was mixed in a cuvette with 2.5 ml of haemoglobin reagent before being incubated for 3 min at 25 °C. The haemoglobin concentration was determined by measuring absorbance when light at a wavelength of 546 nm was passed through the cuvette. The coefficient of variation for the measurement of haemoglobin in the current study was 2%. Total blood volume (TBV) and total PV (TPV) at baseline were estimated using the Nadler equations (Nadler et al. 1962):

206

207 Males TBV = (0.3669 x height in meters^3) + (0.03219 x body mass in kilograms) + 0.6041

208

209 Females TBV = (0.3561 x height in meters^3) + (0.03308 x body mass in kilograms) + 0.1833

210

211 PV = TBV * (1 – Haematocrit)

212

213 The percentage change (Δ) in PV was estimated using the change in haematocrit and
214 haemoglobin values using the method described by Dill and Costill (1974). The PV values for
215 each time point are expressed as estimated TPV and the percentage Δ from the baseline (0 min)
216 sample (Fig. 4).

217

218 2.6. Statistical Analysis

219 All analyses were carried out using the Statistical Package for Social Sciences, Version 22
220 (SPSS Inc., Chicago, IL, USA). GraphPad Prism version 7 (GraphPad Software Inc., San
221 Diego, USA) was used to create the figures. Data are expressed as the mean ± standard
222 deviation unless otherwise stated. The distribution of the data was tested using the Shapiro-
223 Wilk test. A one-way repeated-measures ANOVA was used to examine the differences
224 between measurement points [NO_3^-], [NO_2^-], PV, HR and BP. *Post-hoc* analysis was used to
225 determine the difference from the baseline and all other time points using a paired samples t-
226 tests with Bonferroni correction for multiple pairwise comparisons. The association between
227 absolute and Δ plasma [NO_3^-], [NO_2^-], and TPV values was determined using Pearson's

correlation coefficient. Statistical significance was declared when $P<0.05$. Probability values are expressed with 95% confidence intervals (95% CI) where appropriate.

3. Results

3.1. Nitrite

Baseline values of $[NO_2^-]$ were significantly elevated in the BR trial compared to the CON trial ($P<0.01$, 95% CI 154-320 nM, Fig. 2) and at all other time points. There was a significant main effect of measurement point on plasma $[NO_2^-]$ in both trials ($P<0.001$). Plasma $[NO_2^-]$ was not different to baseline after 10 min of lying supine in either condition (both $P>0.05$) but decreased significantly after 30 min (CON: $P=0.02$, 95% CI 5-82 nM; BR: $P<0.01$, 95% CI 39-213 nM). In the CON trial, plasma $[NO_2^-]$ increased from the 30 min supine time point upon standing ($P<0.01$, 95% CI 15-51 nM) and sitting ($P<0.01$, 95% CI 32-76 nM) and remained higher following exercise ($P<0.05$, 95% CI 7-89 nM). In the BR trial, plasma $[NO_2^-]$ increased from 30 min supine upon standing ($P<0.01$, 95% CI 35-98 nM) and sitting ($P<0.01$, 95% CI 58-251 nM). Following exercise, plasma $[NO_2^-]$ was significantly reduced compared to sitting ($P=0.02$, 95% CI 13-180 nM) but was not different to the 30 min supine time point ($P=0.14$). Plasma $[NO_2^-]$ was not correlated with TPV either for absolute (CON, R=0.04, $P=0.74$; BR, R=0.03, $P=0.78$) or Δ values (CON, R=-0.12, $P = 0.31$; BR R=-0.11, $P = 0.39$).

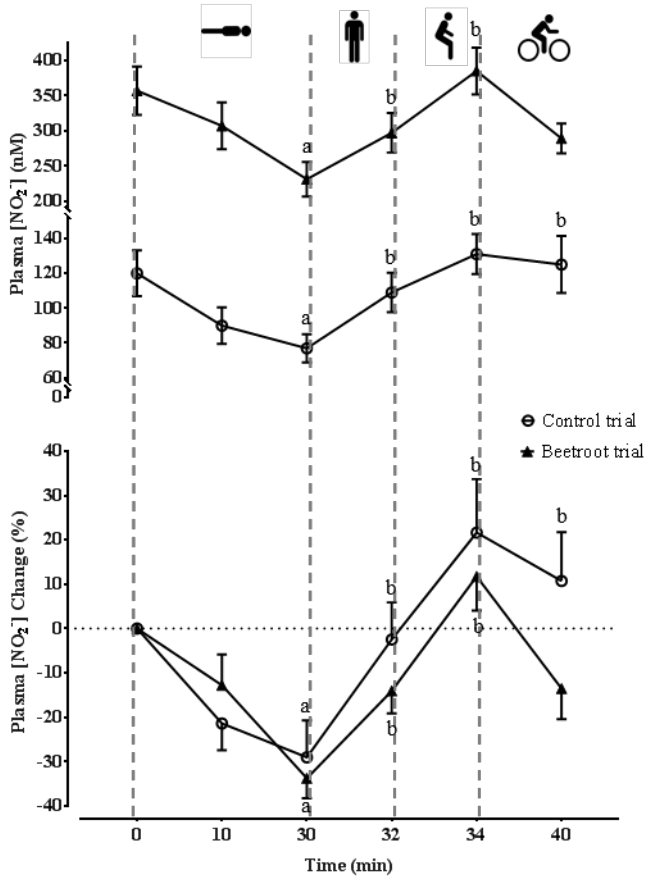


Fig. 2. Changes in mean ± SEM plasma [NO_2^-] expressed as absolute values (top) and percentage change from baseline (bottom). The graphical images at the top of the figure denote the supine, standing, seated, and exercise phases of the trial. a denotes a significant decrease compared to baseline (P<0.05). b denotes a significant increase compared to 30 min time point (P<0.05). All time points in the BR trial were significantly higher than the CON trial (P<0.01).

3.2. Nitrate

Plasma [NO_3^-] at baseline was higher in the BR trial compared to CON (P<0.01, 95% CI 395-561 μM, Fig. 3) and at all other time points. There was a significant main effect of measurement point on plasma [NO_3^-] in the CON trial (P<0.01) but not the BR trial (P=0.20). In the CON trial, plasma [NO_3^-] was higher after exercise compared to 10 min of lying supine (P<0.01,

95% CI 4-24 μM) but was not different between any other measurement points. Plasma [NO_3^-] was not correlated with TPV either for absolute (CON, R=-0.10, *P*=0.37; BR, R=-0.18, *P*=0.10) or Δ values (CON, R=-0.21, *P*=0.08; BR R=-0.02, *P*=0.86).

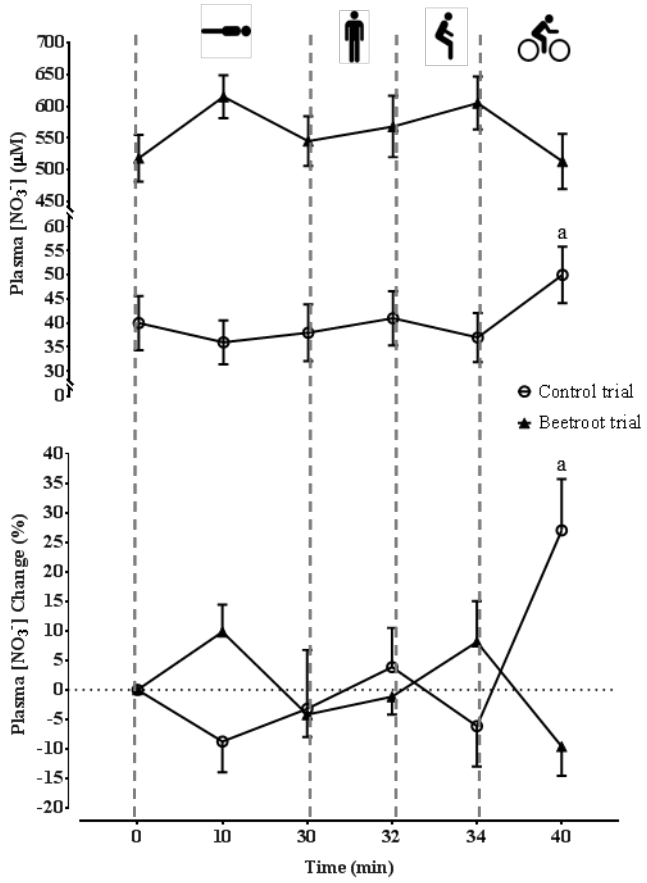


Fig. 3. Changes in mean ± SEM plasma [NO_3^-] expressed as absolute values (top) and percentage change from baseline (bottom). The graphical images at the top of the figure denote the supine, standing, seated, and exercise phases of the trial. a denotes a significant increase compared to 10 min time point (*P*<0.01). All time points in the BR trial were significantly higher than the CON trial (*P*<0.01).

3.3. Plasma Volume

After 10 min of lying supine, PV increased from baseline in both conditions (all $P<0.01$, CON 95% CI 0.09-0.51 L, BR 95% CI 0.12-0.42 L, Fig. 4) and remained elevated at the 30 min measurement point (all $P<0.05$, CON 95% CI 0.04-0.63 L, BR 95% CI 0.15-0.53 L). PV then declined significantly from the 30 min supine measurement upon standing, sitting, and exercise in both conditions (all $P<0.05$).

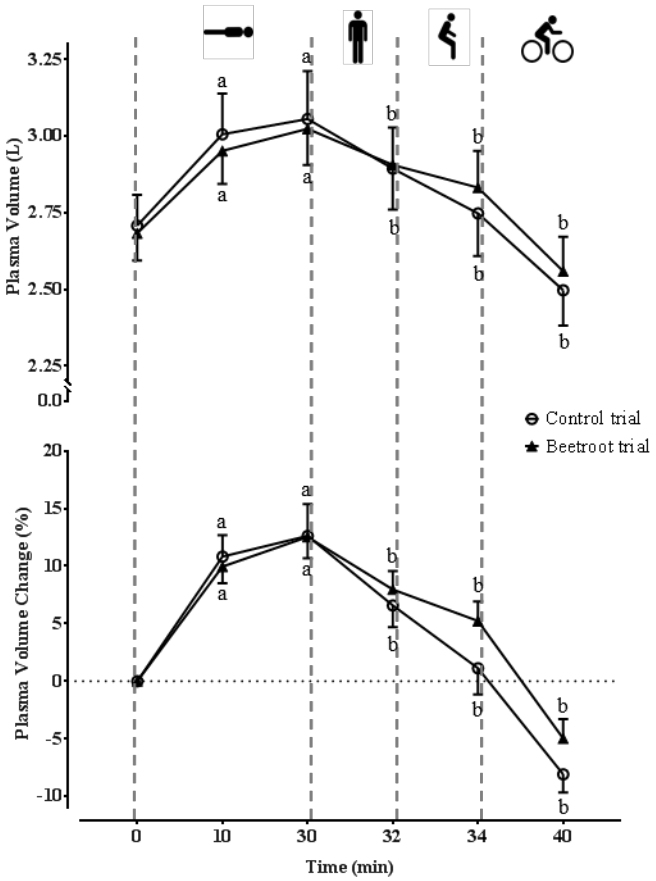


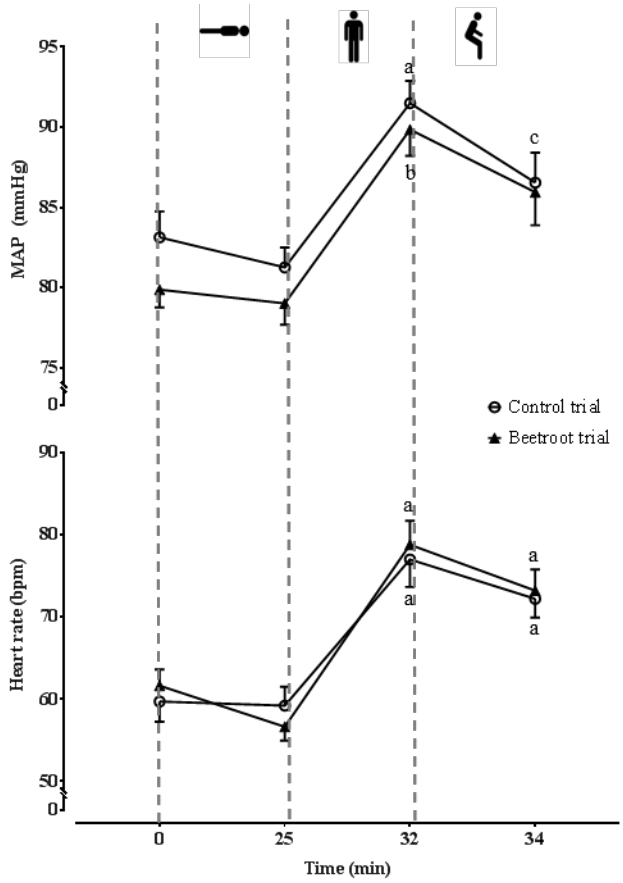
Fig. 4. Changes in mean ± SEM plasma volume expressed as absolute values (top) and percentage change from baseline (bottom). The graphical images at the top of the figure denote the supine, standing, seated, and exercise phases of the trial. a denotes a significant increase compared to baseline ($P<0.01$). b denotes a significant decrease compared to 30 min of laying supine ($P<0.05$).

3.4. Blood Pressure

Baseline measurements of MAP were significantly lower in the BR trial compared to CON (P<0.01, 95% CI 1-5 mmHg, Fig. 5) but was not different at any other time point. There was a significant main effect of time on MAP in both conditions (P<0.01). MAP was higher during standing compared to baseline (CON, P<0.01, 95% CI 4-13 mmHg; BR, P<0.01, 95% CI 4-16 mmHg) and following 25 min lying supine (CON, P<0.01, 95% CI 6-14 mmHg; BR, P<0.01, 95% CI 4-17 mmHg). In the CON trial, MAP was higher during sitting compared to lying supine for 25 min (P=0.04, 95% CI 0-10 mmHg). In the BR trial, MAP was higher during standing compared to sitting (P=0.03, 95% CI 0-7 mmHg).

3.5 Heart Rate

There was a significant main effect of time (P<0.01) on HR in both the CON and BR trials. HR was typically higher during the standing and sitting phases compared to the supine time points (all P<0.01). There was no difference in HR between sitting and standing measurements (both P>0.05). In both conditions, post-exercise HR was higher than all other time points (all P<0.01).



297

298 Fig. 5. Changes in mean ± SEM mean arterial pressure (top) and heart rate (bottom) expressed
299 as absolute values. The graphical images at the top of the figure denote the supine, standing,
300 seated, and exercise phases of the trial. a denotes a significant increase compared to baseline
301 and 25 min time point (P<0.01). b denotes a significant increase compared to all other time
302 points (P<0.05). c denotes significant increase compared to 25 min time point (P<0.05).
303 Baseline measurements of mean arterial pressure were lower in the BR trial compared to the
304 CON trial (P<0.01).

305

306 **4. Discussion**

307 To our knowledge, this study is the first to report that plasma [NO_2^-] is substantially altered by
308 varying body posture while these changes have minimal impact on plasma [NO_3^-]. Here, we

report that plasma [NO_2^-] is increased during sitting and standing compared to lying supine which substantially extends our previous findings that plasma [NO_2^-] declines during a period of lying supine (Muggeridge et al. 2015).

As expected, moving between different body postures resulted in consistent, marked and rapid changes in PV. Following a period of standing upright, PV increased by ~10% after 10 min in the supine posture. There was a further increase in PV at the 30 min time point, although this was of a much smaller magnitude (~13% from baseline). Previous data suggests that PV stabilises approximately 20 min in the supine posture (Hagan et al. 1978) which is pertinent when measuring the concentration of blood metabolites in exercise studies. On the other hand, moving from supine to standing resulted in an almost immediate (~2 min) reduction in PV which reduced further as participants continued to the seated posture. Again, the magnitude of the response was profound, with PV dropping by 6-10% following brief periods of standing and sitting from the end of the supine phase. A short period of exercise caused a further large reduction in PV which corresponded to a decline of ~19% from the end of the supine phase. These data are broadly in line with values reported elsewhere in the literature (Hagan et al. 1978) although others (Hansen 1968; Lippi et al. 2015) have reported larger declines in PV during standing (~14%), likely due to longer period of time in this posture.

These postural-induced alterations in PV are readily explainable and the likely mechanisms have been known for some time (Thompson et al. 1928). Adopting a standing posture increases local hydrostatic pressure, particularly in the lower limbs, which forces fluid and some molecules from the intravascular to the interstitial space (Krogh et al. 1932). The augmented reduction in PV during exercise is most likely caused by an increased intra-capillary pressure

in the contracting muscles (Hansen 1968). These fluid shifts do eventually stabilise due to counter pressure exerted by the tissue and an increase in the intravascular oncotic pressure (Youmans et al. 1934). As evidenced in the present study, the reduction in circulating blood volume and venous return can lead to an increase in HR and BP. It is important to note, however, that standing may initially reduce BP and transient changes in this response may vary between individuals (Eşer et al. 2007).

For the first time, we demonstrate that plasma [NO_2^-] changes substantially between supine, standing and sitting phases. Where PV increases during the supine phase, [NO_2^-] decreases during lying supine and increases on standing and sitting. These data are perhaps not surprising given postural-induced PV shifts have previously been reported to alter the concentration of other constituents in the blood in a similar fashion (Thompson et al. 1928; Fawcett and Wynn 1960; Lippi et al. 2015). Consequently, it might be expected that a considerable proportion of the change in plasma [NO_2^-] can be accounted for by a "dilution effect" where, for example, an increased PV reduces the measured concentration of the number of NO_2^- particles. However, there was no correlation between either absolute or Δ plasma [NO_2^-] and PV values suggesting plasma fluid shifts account for only a small proportion of the variance in [NO_2^-] during postural changes. Furthermore, plasma [NO_3^-] does not change uniformly as posture is altered and was not correlated with PV. Instead, it seems probable that postural-induced alterations in NO metabolism may account for these findings.

In line with previous research, both plasma [NO_2^-] and [NO_3^-] were considerably elevated by ingestion of NO_3^--rich beetroot juice (198% and 1163%, respectively). However, given plasma [NO_2^-] is reported to peak ~2.5 h after acute ingestion of beetroot juice (Webb et al. 2008), it

357 is perhaps surprising that plasma [NO_2^-] in the present study declined during the supine phase
358 of the BR trial to the same extent as CON (i.e. 2 – 2.5 h after ingestion). Nevertheless, we
359 (McIlvenna et al. 2017) and others (James et al. 2015) have previously demonstrated that
360 plasma NO_2^- pharmacokinetics following dietary NO_3^- ingestion appear to vary substantially
361 between individuals. For example, Wylie and colleagues (2013a) reported that time taken for
362 plasma [NO_2^-] to peak following administration of a similar dose of NO_3^--rich beetroot juice
363 ranged between 77 and 213 min. Therefore, it is plausible that while dietary-derived NO_2^- was
364 still increasing in the plasma in some participants, it was declining in others.

365

366 Measurement of plasma [NO_2^-] and [NO_3^-] is further complicated by the fact that changes in
367 posture also alter the rate of endogenous NO production. Shear stress is a frictional force
368 exerted by blood moving across the endothelium and is reported to increase during standing
369 compared to sitting (Morishima et al. 2017). Endothelial cells rapidly respond to shear stress
370 with an acute increase in intracellular calcium that enhances the binding of calmodulin to eNOS
371 and increases eNOS activity and NO production (Boo and Jo 2003; Rassaf et al. 2006). On the
372 contrary, moving from a supine to a seated positon has been demonstrated to reduce shear rate
373 in young but not old participants (Trinity et al. 2015). Given that shear rate was not measured
374 in the current study, we can only speculate as to how this may have impacted endogenous
375 synthesis of NO and related metabolites. Conversion of NO to NO_3^- by heme proteins in the
376 blood and tissues occurs fairly rapidly (Shiva et al. 2006), such that NO_3^- is considered to be
377 the major breakdown product in the presence of sufficient amounts of O_2 (Kelm 1999).
378 Conversely, NO can be oxidised to NO_2^- via various oxidants in plasma and tissues (Shiva et
379 al. 2006). It should also be noted that a considerable portion of NO_2^- and NO_3^- are stored in
380 tissues. In rodents, the liver, blood, and skeletal muscle contain equivalent amounts of NO_2^-
381 (~0.5 – 0.7 nmol/g) whereas NO_3^- is considerably higher in muscle (~200 nmol/g) compared

382 to blood (~80 nmol/g) and the liver (~10 nmol/g) (Piknova et al. 2015). Furthermore,
383 Nyakayiru et al. (2017) have recently shown that ingestion of sodium NO_3^- results in a
384 substantial and sustained increase in muscle [NO_3^-], with reported values exceeding those in
385 plasma. However, muscle [NO_2^-] was below the detection limit both before and after NO_3^-
386 supplementation. Therefore, when considering the impact of postural-induced fluid shifts in
387 the context of measuring [NO_2^-] and [NO_3^-], we must also factor in the change in endogenous
388 NO production, the oxidation of NO to various metabolic endpoints, and the transfer of these
389 metabolites to and from different tissues.

390

391 Data from this study also demonstrates that short-duration sub-maximal cycling exercise leads
392 to a reduction in plasma [NO_2^-] in both conditions but a variable response in plasma [NO_3^-].
393 Although plasma [NO_3^-] did not differ statistically between time points overall, there was a
394 substantial increase from pre- to post-exercise in the CON trial and a reduction in the BR trial.
395 The reduction in plasma [NO_2^-] is consistent with some (Larsen et al. 2007; Kelly et al. 2014),
396 but not all (Larsen et al. 2010) previous studies and potentially results from an increased
397 conversion of NO_2^- to NO during exercise. Differences between study cohorts and the intensity
398 and duration of the exercise protocols may explain the inconsistencies in these data. Cosby and
399 colleagues (2003) suggest that NO_2^- is a major bioavailable pool of NO and present data
400 demonstrating an increased reduction of NO_2^- to NO by deoxyhemoglobin during exercise.
401 Furthermore, in animal studies, the initiation of exercise has been shown to increase the
402 demand for NO and upregulate eNOS activity (Maiorana et al. 2003). Therefore, post-exercise
403 changes in plasma [NO_2^-] and [NO_3^-] in dietary NO_3^- supplementation studies must be
404 interpreted cautiously due to the aforementioned pharmacokinetics of these metabolites and the
405 individual variability in the response (James et al. 2015; McIlvenna et al. 2017).

406

407 Although the precise mechanisms explaining the alterations in plasma [NO_2^-] remain unclear,
408 the magnitude of the change in this outcome during the adoption of different postures highlights
409 the importance of standardising posture in experimental trials where this outcome is important.
410 Indeed, an inconsistent approach to the posture of participants during blood collection may at
411 least partly explain why measurements of plasma [NO_3^-] are comparable between different
412 studies in healthy participants while [NO_2^-] varies considerably. The present study is not
413 without limitations as the phases of sitting, standing, and exercise were very brief and the fate
414 of the ingested NO_3^- is impossible to determine without more advanced measurement methods.
415 Furthermore, the order of the trials was not randomised and nor was there inclusion of a placebo
416 condition that required the ingestion of a matched volume of NO_3^--depleted beetroot juice.
417 However, our data demonstrates the proportional change in PV, [NO_3^-] and [NO_2^-] were
418 consistent between BR and CON conditions suggesting these experimental limitations do not
419 diminish confidence in the findings. Notwithstanding, there are two primary recommendations
420 that emanate from this work. Firstly, if differences in [NO_2^-] are to be compared either within
421 or between participants in an experimental trial, participants should be lying supine for a
422 standardised period of time before blood collection. For baseline measurements the supine
423 period should be a minimum of 20 – 30 min. For post-exercise measurements the supine period
424 should be brief but standardised. Secondly, the posture of participants during blood collection
425 and the duration that this posture was maintained before blood collection should be clearly
426 documented in research manuscripts to allow better comparison of data between studies.

427

428 **5. Conclusion**

The principal finding from this study is that posture has a profound impact on the concentration of plasma NO_2^- regardless of whether [NO_2^-] was normal or elevated by dietary NO_3^- supplementation. The lack of correlation between PV and [NO_2^-] suggests that fluid shifts cannot solely account for this response. While postural alterations in shear stress and endogenous NO production may be contributing factors, we do not have experimental data to support this notion. Nevertheless, researchers should standardise the posture of participants at rest and post exercise when multiple blood samples are to be collected and fully document these procedures during dissemination of their data.

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