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1   **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 $\pm$ 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

5

119   preceded by ingestion of 3 x 70 ml of NO_3^--rich beetroot juice (Beet it, James White Drinks,

120   UK) the day before and 2 x 70 ml, 2 h before the first blood sample (BR; total of ~31 mmol

121   NO_3^-). Participants recorded their diet 24 h prior to CON and were asked to repeat this as

122   closely as possible prior to BR. All trials were completed before 11 a.m. at the same time of

123   day for each participant and following an overnight fast. Participants were instructed to avoid

124   caffeine, foods high in NO_2^- and NO_3^- (e.g. green leafy vegetables and cured meats), alcohol,

125   mouthwash, and strenuous exercise 24 h prior to the experiment. Participants were provided

126   with one bottle of drinking water (Harrogate, UK) prior to CON trial and given instructions to

127   arrive at the lab well hydrated. Participants recorded the volume of water ingested prior to CON

128   and matched the volume prior to BR.

129

130   2.3. Procedures

131   Following standard anthropometric measurements (stature and body mass), participants lay in

132   a supine posture to allow for the insertion of a cannula into the antecubital vein. Following

133   cannulation, participants stood up for 10 min prior to lying supine to start the experimental

134   trial. Baseline measurements (0 min) of venous blood, blood pressure (BP) and heart rate (HR)

135   were recorded immediately. The measurement of BP was conducted using an automated

136   sphygmomanometer (Omron M10, Kyoto, Japan) in triplicate during supine measures and in

137   duplicate for the standing and seated measures. Mean arterial pressure (MAP) was calculated

138   by the following equation:

139

140   MAP = (2 x diastolic BP + systolic BP) / 3

141

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 4°C

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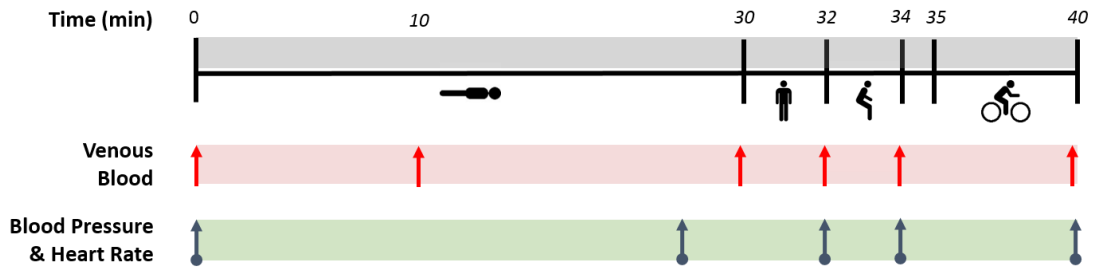
Fig. 1. Schematic of measurement time points for CON and BR trials.

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%.

8

182   For the measurement of plasma [NO_3^-], vanadium reagent (32 mg of vanadium tri-chloride, 4

183   ml of 1M hydrochloric acid and 500 μL of water) and 100 μL of anti-foaming agent were placed

184   into the glass purge vessel and heated to 95 °C. A standard curve was produced by injecting

185   25-50 μL of NO_3^- solutions (100 μM, 50 μM, 25 μM, 12.5 μM, and 6.25 μM) and a control

186   sample containing deionised water. Plasma samples were thawed and de-proteinised (200 μL

187   of sample, 400 μL of zinc sulphate in deionised water at 10% weight/volume and 400 μL of

188   sodium hydroxide in deionised water at ratio of 1:1). Subsequently, 15-25 μL of the sample

189   was injected into the purge vessel in duplicate and plasma [NO_3^-] calculated as previously

190   described for the NO_2^- assay. The coefficient of variation for the measurement of [NO_3^-] in the

191   current study was 6%.

192

193   2.5. Determination of Plasma Volume Change

194   To determine the haematocrit, a small volume of venous blood was extracted into heparinised

195   capillary tubes that were sealed at the distal end with a wax seal. The capillary tubes were then

196   spun for 8 min at 15,000 revolutions/min in a micro-haematocrit centrifuge before the

197   haematocrit was measured in triplicate using a Hawksey haematocrit reader. The coefficient of

198   variation for the measurement of haematocrit in the current study was 0.4%. Haemoglobin

199   concentration was determined using the Randox colorimetric method (RX Monza, Randox

200   Laboratories, UK). Briefly, 20 μL of whole blood was mixed in a cuvette with 2.5 ml of

201   haemoglobin reagent before being incubated for 3 min at 25 °C. The haemoglobin

202   concentration was determined by measuring absorbance when light at a wavelength of 546 nm

203   was passed through the cuvette. The coefficient of variation for the measurement of

204   haemoglobin in the current study was 2%. Total blood volume (TBV) and total PV (TPV) at

205   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

228 correlation coefficient. Statistical significance was declared when $P<0.05$. Probability values

229 are expressed with 95% confidence intervals (95% CI) where appropriate.

230

3. Results

232 3.1. Nitrite

233 Baseline values of $[NO_2^-]$ were significantly elevated in the BR trial compared to the CON trial

234 ($P<0.01$, 95% CI 154-320 nM, Fig. 2) and at all other time points. There was a significant main

235 effect of measurement point on plasma $[NO_2^-]$ in both trials ($P<0.001$). Plasma $[NO_2^-]$ was not

236 different to baseline after 10 min of lying supine in either condition (both $P>0.05$) but

237 decreased significantly after 30 min (CON: $P=0.02$, 95% CI 5-82 nM; BR: $P<0.01$, 95% CI

238 39-213 nM). In the CON trial, plasma $[NO_2^-]$ increased from the 30 min supine time point upon

239 standing ($P<0.01$, 95% CI 15-51 nM) and sitting ($P<0.01$, 95% CI 32-76 nM) and remained

240 higher following exercise ($P<0.05$, 95% CI 7-89 nM). In the BR trial, plasma $[NO_2^-]$ increased

241 from 30 min supine upon standing ($P<0.01$, 95% CI 35-98 nM) and sitting ($P<0.01$, 95% CI

242 58-251 nM). Following exercise, plasma $[NO_2^-]$ was significantly reduced compared to sitting

243 ($P=0.02$, 95% CI 13-180 nM) but was not different to the 30 min supine time point ($P=0.14$).

244 Plasma $[NO_2^-]$ was not correlated with TPV either for absolute (CON, R=0.04, $P=0.74$; BR,

245 R=0.03, $P=0.78$) or Δ values (CON, R=-0.12, $P = 0.31$; BR R=-0.11, $P = 0.39$).
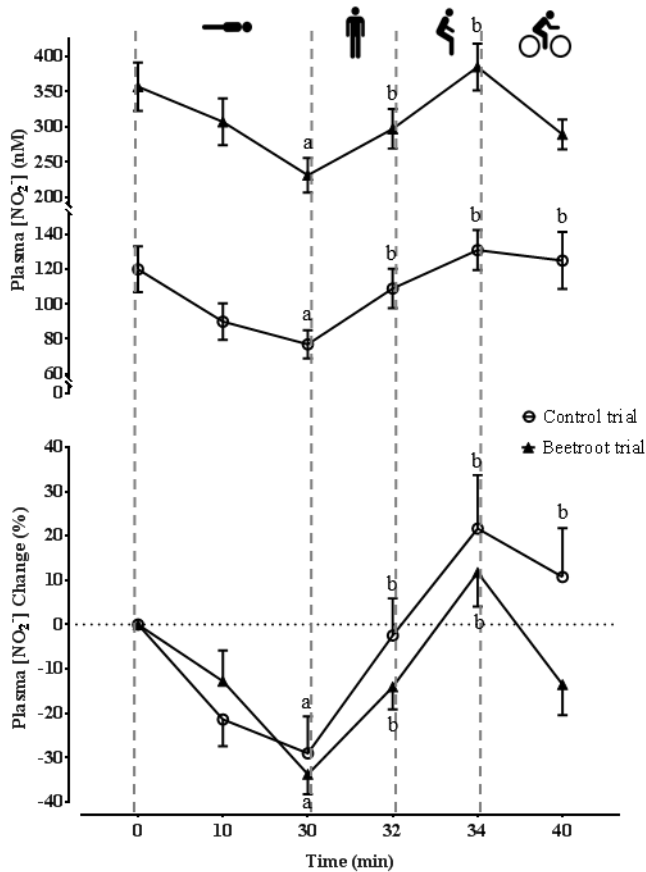
Fig. 2. Changes in mean ± SEM plasma [NO_2^-] expressed as absolute values (top) and percentage change from baseline (bottom). The graphical images at the top of the figure denote the supine, standing, seated, and exercise phases of the trial. a denotes a significant decrease compared to baseline ($P<0.05$). 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 ($P<0.01$,

258   95% CI 4-24 µM) but was not different between any other measurement points. Plasma [NO$_3^-$

259   ] was not correlated with TPV either for absolute (CON, R=-0.10, *P*=0.37; BR, R=-0.18,

260   *P*=0.10) or Δ values (CON, R=-0.21, *P*=0.08; BR R=-0.02, *P*=0.86).
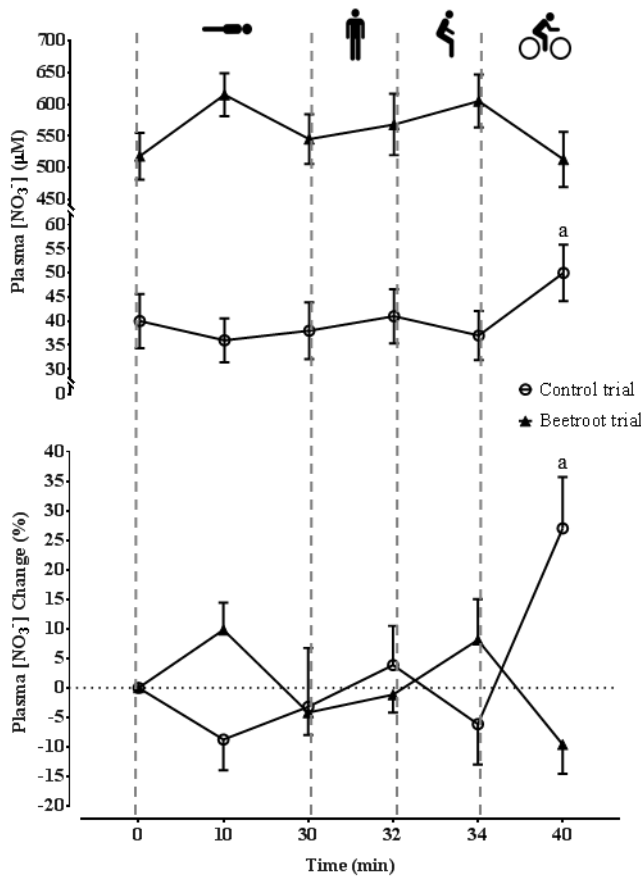
261

262   Fig. 3.   Changes in mean ± SEM plasma [NO$_3^-$] expressed as absolute values (top) and

263   percentage change from baseline (bottom). The graphical images at the top of the figure denote

264   the supine, standing, seated, and exercise phases of the trial. a denotes a significant increase

265   compared to 10 min time point (*P*<0.01). All time points in the BR trial were significantly

266   higher than the CON trial (*P*<0.01).

267

268   3.3. Plasma Volume

269   After 10 min of lying supine, PV increased from baseline in both conditions (all $P<0.01$, CON

270   95% CI 0.09-0.51 L, BR 95% CI 0.12-0.42 L, Fig. 4) and remained elevated at the 30 min

271   measurement point (all $P<0.05$, CON 95% CI 0.04-0.63 L, BR 95% CI 0.15-0.53 L). PV then

272   declined significantly from the 30 min supine measurement upon standing, sitting, and exercise
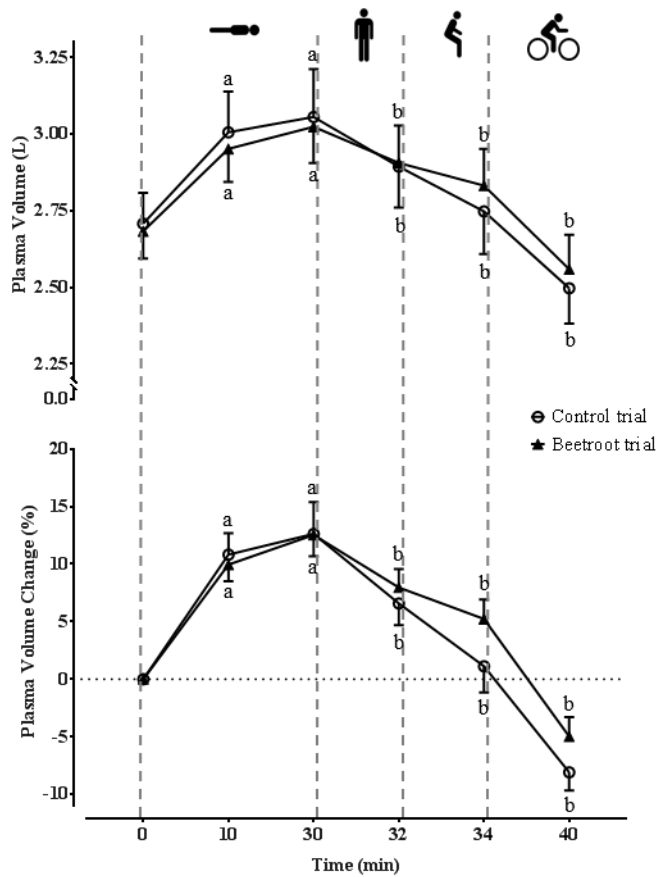
273   in both conditions (all $P<0.05$).

274

275   Fig. 4.   Changes in mean ± SEM plasma volume expressed as absolute values (top) and

276   percentage change from baseline (bottom). The graphical images at the top of the figure denote

277   the supine, standing, seated, and exercise phases of the trial. a denotes a significant increase

278   compared to baseline ($P<0.01$). b denotes a significant decrease compared to 30 min of laying

279   supine ($P<0.05$).

280

281   3.4. Blood Pressure

282   Baseline measurements of MAP were significantly lower in the BR trial compared to CON

283   ($P<0.01$, 95% CI 1-5 mmHg, Fig. 5) but was not different at any other time point. There was a

284   significant main effect of time on MAP in both conditions ($P<0.01$). MAP was higher during

285   standing compared to baseline (CON, $P<0.01$, 95% CI 4-13 mmHg; BR, $P<0.01$, 95% CI 4-16

286   mmHg) and following 25 min lying supine (CON, $P<0.01$, 95% CI 6-14 mmHg; BR, $P<0.01$,

287   95% CI 4-17 mmHg). In the CON trial, MAP was higher during sitting compared to lying

288   supine for 25 min ($P=0.04$, 95% CI 0-10 mmHg). In the BR trial, MAP was higher during

289   standing compared to sitting ($P=0.03$, 95% CI 0-7 mmHg).

290

291   3.5 Heart Rate

292   There was a significant main effect of time ($P<0.01$) on HR in both the CON and BR trials.

293   HR was typically higher during the standing and sitting phases compared to the supine time

294   points (all $P<0.01$). There was no difference in HR between sitting and standing measurements

295   (both $P>0.05$). In both conditions, post-exercise HR was higher than all other time points (all

296   $P<0.01$).

297
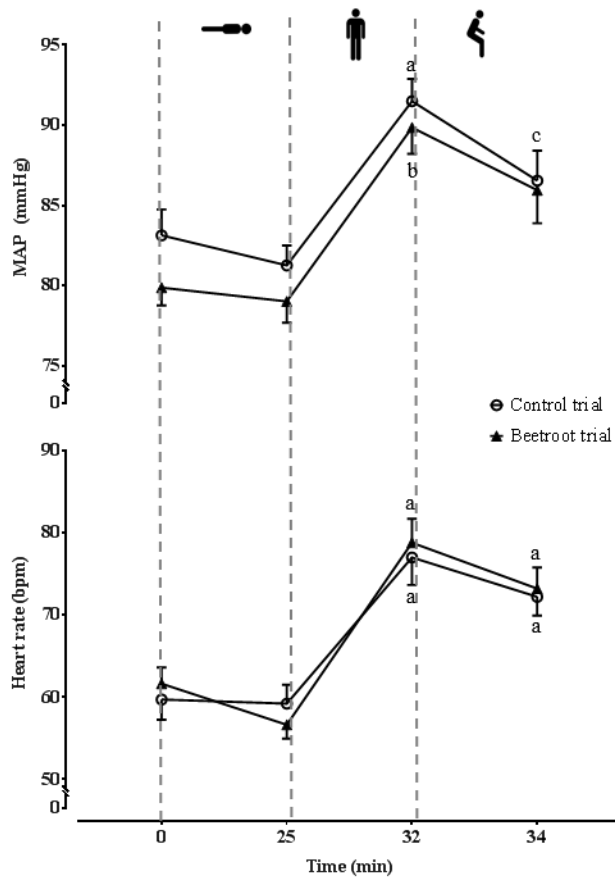
Fig. 5. Changes in mean ± SEM mean arterial pressure (top) and heart rate (bottom) expressed

as absolute values. 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

and 25 min time point ($P<0.01$). b denotes a significant increase compared to all other time

points ($P<0.05$). c denotes significant increase compared to 25 min time point ($P<0.05$).

Baseline measurements of mean arterial pressure were lower in the BR trial compared to the

CON trial ($P<0.01$).

305

4. Discussion

To our knowledge, this study is the first to report that plasma [NO_2^-] is substantially altered by

varying body posture while these changes have minimal impact on plasma [NO_3^-]. Here, we

16

309 report that plasma $[NO_2^-]$ is increased during sitting and standing compared to lying supine

310 which substantially extends our previous findings that plasma $[NO_2^-]$ declines during a period

311 of lying supine (Muggeridge et al. 2015).

312

313 As expected, moving between different body postures resulted in consistent, marked and rapid

314 changes in PV. Following a period of standing upright, PV increased by ~10% after 10 min in

315 the supine posture. There was a further increase in PV at the 30 min time point, although this

316 was of a much smaller magnitude (~13% from baseline). Previous data suggests that PV

317 stabilises approximately 20 min in the supine posture (Hagan et al. 1978) which is pertinent

318 when measuring the concentration of blood metabolites in exercise studies. On the other hand,

319 moving from supine to standing resulted in an almost immediate (~2 min) reduction in PV

320 which reduced further as participants continued to the seated posture. Again, the magnitude of

321 the response was profound, with PV dropping by 6-10% following brief periods of standing

322 and sitting from the end of the supine phase. A short period of exercise caused a further large

323 reduction in PV which corresponded to a decline of ~19% from the end of the supine phase.

324 These data are broadly in line with values reported elsewhere in the literature (Hagan et al.

325 1978) although others (Hansen 1968; Lippi et al. 2015) have reported larger declines in PV

326 during standing (~14%), likely due to longer period of time in this posture.

327

328 These postural-induced alterations in PV are readily explainable and the likely mechanisms

329 have been known for some time (Thompson et al. 1928). Adopting a standing posture increases

330 local hydrostatic pressure, particularly in the lower limbs, which forces fluid and some

331 molecules from the intravascular to the interstitial space (Krogh et al. 1932). The augmented

332 reduction in PV during exercise is most likely caused by an increased intra-capillary pressure

333   in the contracting muscles (Hansen 1968). These fluid shifts do eventually stabilise due to

334   counter pressure exerted by the tissue and an increase in the intravascular oncotic pressure

335   (Youmans et al. 1934). As evidenced in the present study, the reduction in circulating blood

336   volume and venous return can lead to an increase in HR and BP. It is important to note,

337   however, that standing may initially reduce BP and transient changes in this response may vary

338   between individuals (Eşer et al. 2007).

339

340   For the first time, we demonstrate that plasma $[NO_2^-]$ changes substantially between supine,

341   standing and sitting phases. Where PV increases during the supine phase, $[NO_2^-]$ decreases

342   during lying supine and increases on standing and sitting. These data are perhaps not surprising

343   given postural-induced PV shifts have previously been reported to alter the concentration of

344   other constituents in the blood in a similar fashion (Thompson et al. 1928; Fawcett and Wynn

345   1960; Lippi et al. 2015). Consequently, it might be expected that a considerable proportion of

346   the change in plasma $[NO_2^-]$ can be accounted for by a "dilution effect" where, for example,

347   an increased PV reduces the measured concentration of the number of NO_2^- particles. However,

348   there was no correlation between either absolute or Δ plasma $[NO_2^-]$ and PV values suggesting

349   plasma fluid shifts account for only a small proportion of the variance in $[NO_2^-]$ during postural

350   changes. Furthermore, plasma $[NO_3^-]$ does not change uniformly as posture is altered and was

351   not correlated with PV. Instead, it seems probable that postural-induced alterations in NO

352   metabolism may account for these findings.

353

354   In line with previous research, both plasma $[NO_2^-]$ and $[NO_3^-]$ were considerably elevated by

355   ingestion of NO_3^--rich beetroot juice (198% and 1163%, respectively). However, given plasma

356    $[NO_2^-]$ 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**

429   The principal finding from this study is that posture has a profound impact on the concentration

430   of plasma NO_2^- regardless of whether $[NO_2^-]$ was normal or elevated by dietary NO_3^-

431   supplementation. The lack of correlation between PV and $[NO_2^-]$ suggests that fluid shifts

432   cannot solely account for this response. While postural alterations in shear stress and

433   endogenous NO production may be contributing factors, we do not have experimental data to

434   support this notion. Nevertheless, researchers should standardise the posture of participants at

435   rest and post exercise when multiple blood samples are to be collected and fully document

436   these procedures during dissemination of their data.

437

442

443   **7. Conflict of interest:**   The authors declare no conflict of interests.

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