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Venous occlusion during blood collection decreases plasma nitrite but not nitrate concentration in humans

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Abstract

Background: To maintain vascular tone and blood flow when tissue oxygenation is reduced, nitrite anions are reduced to nitric oxide (NO). From a practical perspective, it is unclear how the application of a tourniquet during blood collection might influence measurement of NO metabolites. Accordingly, this study evaluated the effect of venous occlusion on plasma nitrite and nitrate during venous blood collection. **Methods**: Fifteen healthy participants completed two trials that were preceded by the ingestion of nitrate-rich beetroot juice (BRJ; total of ~8.4 mmol nitrate) or no supplementation (control). In both trials, blood was collected using a venepuncture needle while a tourniquet was applied to the upper arm and using an indwelling intravenous cannula, from opposing arms. The venepuncture samples were collected at 35 s post occlusion. Changes in the oxygenation of forearm flexor muscles were assessed using near-infrared spectroscopy. Plasma nitrite and nitrate were analysed using gas-phase chemiluminescence. **Results**: In the control trial, plasma nitrite was significantly elevated when collected via the cannula (179 ± 67 nM) compared to venepuncture (112 ± 51 nM, P=0.03). The ingestion of BRJ increased plasma nitrite and values remained higher when sampled from the cannula (473 ± 164 nM) compared to venepuncture (387 ± 136 nM, P<0.001). Plasma nitrate did not differ between collection methods in either trial (all P>0.05). The delta changes in total- , deoxy-, and oxy-haemoglobin were all significantly greater during venepuncture sample compared to the cannula sample at the point of blood collection (all P<0.05). **Conclusions**: Venous occlusion during venepuncture blood collection lowers plasma nitrite concentration, potentially due to localised changes in haemoglobin concentration and/or a suppression of endogenous NO synthesis. Accordingly, the method of blood collection to enable measurements of NO metabolites should be carefully considered and consistently reported by researchers.

Key Words: beetroot juice; cannula; ischemia; blood flow; nitric oxide

Highlights

- Application of a tourniquet during blood collection reduces plasma nitrite
- Use of a tourniquet does not influence plasma nitrate concentration
- Venous occlusion alters tissue oxygenation which may alter local nitric oxide production
- Methods of blood collection should be carefully considered when measuring nitrite

1 Introduction

Endothelial derived nitric oxide (NO) is a universal signalling molecule that plays an important role in vascular homeostasis (Moncada and Higgs, 1993) and is essential for cardiovascular health (Moncada and Higgs, 2006). The maintenance of basal vascular tone and blood pressure is regulated by NO in the vasculature (Vallance, Collier and Moncada, 1989). The two main pathways for NO generation are the L-arginine – endothelial NO synthase pathway (Wood et al., 2013) and the nitrate-nitrite-NO pathway (Lundberg and Govoni, 2004). The NO produced from endothelial NO synthase can be rapidly oxidised to nitrite and nitrate (Ignarro et al., 1993, Joshi et al., 2002). As it is impractical to measure NO directly, plasma [nitrite] is routinely measured as the primary marker of NO synthase activity (Lauer et al., 2001).

The reactive nature of plasma nitrite poses a methodological challenge when attempting to measure these variables in blood samples. We have previously demonstrated that the posture of the participant during blood collection has a significant impact on plasma [nitrite] (Liddle et al., 2018). It is, therefore, reasonable to assume that the methods of collection and sample processing will also influence the measurements of [nitrite] and [nitrate] in blood. For example, a tourniquet is commonly used during venepuncture to make the vein more prominent by impeding venous blood flow (Shaw, 2018). Conversely, blood samples collected from an indwelling venous cannula are collected when blood flow is unrestricted. This is important because venous occlusion (~50 mmHg) of the forearm can increase the concentration of deoxyhaemoglobin and deoxymyoglobin (deoxy[Hb+Mb]) within 1 min of cuff inflation (Hampson and Piantadosi, 1988). This increase in deoxy[Hb+Mb] may potentially augment the conversion of nitrite to NO (Cosby et al., 2003, Nagababu et al., 2003) in the tissue and blood.

To our knowledge, no study has examined the influence of blood collection method on the concentration of plasma NO metabolites. The aim of the current study, therefore, was to compare measurements of plasma [nitrite] and [nitrate] between venous blood samples collected using a venepuncture and cannula. These comparisons were made with basal levels of NO metabolites and when nitrite and nitrate were elevated by prior ingestion of dietary nitrate. A secondary aim was to elucidate whether potential differences in plasma NO metabolites were a direct consequence of tourniquet application during sample collection. We hypothesised that the application of a tourniquet during blood collection would lead to localised increases in deoxy[Hb+Mb] and reduce plasma [nitrite].

2 Methods

2.1 Participants

Fifteen healthy and recreationally active participants (10 males and 5 females, age 27 ± 4 years, stature 176 ± 7 cm, and body mass 71 ± 11 kg) volunteered to participate in the study. Written informed consent was obtained from all participants. The study was approved by the School of Science and Sport Ethics Committee at The University of the West of Scotland and all procedures were performed in accordance with the 1964 Declaration of Helsinki and its later amendments.

2.2 Study design

Participants attended the laboratory on two separate occasions with a minimum of six days between each visit. Each experiment trial was identical (Fig. 1, A) with the exception that one trial was conducted with no dietary intervention (control; CON) and the other was preceded by ingestion of 2 x 70 ml of nitrate-rich beetroot juice (BRJ, Beet it organic shot, James White Drinks, UK; total of ~8.4 mmol nitrate) (Fig. 1). In the BRJ trial, the

experiment commenced 2.5 h following ingestion of the BRJ to coincide with the expected peak in plasma [nitrite] (Webb, Patel, et al., 2008, Lundberg and Weitzberg, 2009, Larsen et al., 2010, Wylie et al., 2013, McIlvenna et al., 2017).

Participants recorded their diet 24 h prior to the first trial and were asked to repeat this as closely as possible prior to the second visit. 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 nitrite and nitrate (e.g. green leafy vegetables and cured meats), alcohol, mouthwash, and strenuous exercise 24 h prior to the experiment. Participants were provided with one 500 ml bottle of drinking water (Harrogate, UK) before each trial and given instructions to arrive at the lab in a euhydrated state. Fluid intake was recorded prior to the first laboratory visit and replicated for the subsequent experimental trial.

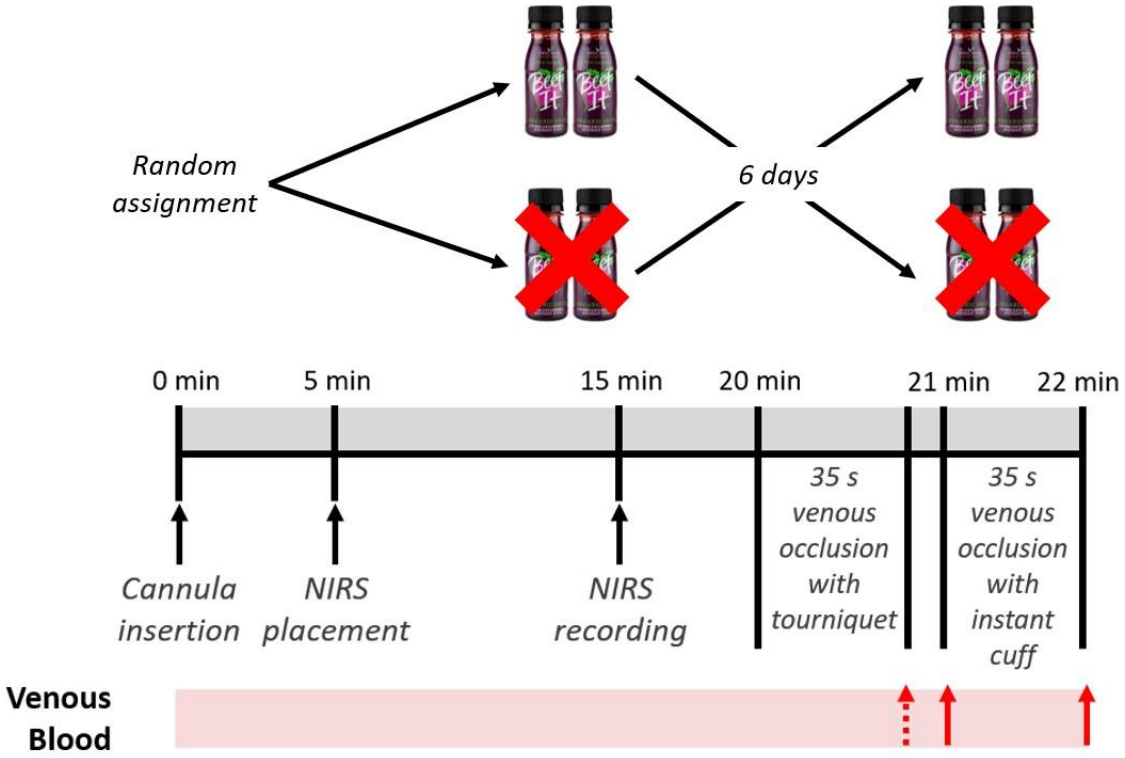


Figure 1 Schematic of study design and procedures. The dashed arrow depicts the collection of venous blood from a venepuncture needle whilst the solid arrows represent the samples collected from the cannula.

2.3 Procedures

Following standard anthropometric measurements (stature and body mass), participants were instructed to lay in a fixed semi-supine position for the duration of each trial (Liddle et al., 2018). Both arms were placed on arm rests in the sagittal and horizontal position to allow for the insertion of an intravenous cannula (20G, 1 x 32 mm, 54 ml/min, Ref 391452, BD Venflon, Helsingborg, Sweden) into an antecubital vein. The placement of the cannula in either the left or right arm was randomised throughout the study. In two separate trials, the cannula was not inserted successfully on the first attempt. On each occasion, the cannula was inserted into the opposing arm and samples of blood were collected at least 15 min

later. The cannula was initially flushed with sterile 0.9% saline solution to keep the line patent.

Two probes were then placed horizontally across the flexor muscles of the upper forearm to enable the measurement of muscle oxygenation using spatially resolved near infrared spectroscopy (NIRS) (NIRO-200NX, Hamamatsu Phototonics, Hamamatsu City, Japan). The same sites were used on both forearms and replicated on the second visit due to known spatial heterogeneity in deoxy[Hb+Mb] kinetics (Koga et al., 2007, 2011). Before probe placement, the hair was removed with a disposable razor (Bic Sensitive, Bic, France) and the site was cleansed with an alcoholic wipe. The probes were placed in a manufacturer-supplied black rubber holder (with a fixed emitter-detector distance of 3 cm and a path length of 17 cm) and attached to a double-sided adhesive pad placed on the participant's skin. The NIRO-200NX has an LED light source that emits infrared light at wavelengths of 735, 810, and 850 nm. It is important to note that the spectral absorbance of the chromophores Hb and Mb are very similar (Chance et al., 1988, Hampson and Piantadosi, 1988, Wilson et al., 1989). However, the contribution of Hb and Mb to the NIRS signal has been reviewed and estimations have been made based on anatomical and experimental data (Davis and Barstow, 2013).

The continuous change (from baseline) in deoxy[Hb+Mb] and oxyhaemoglobin plus oxymyoglobin (oxy[Hb+Mb]) were measured in the area of probe placement. The change in total haemoglobin and myoglobin (t[Hb+Mb]) was then calculated using the equation below:

$$\Delta t[Hb+Mb]\ (\mu M) = \Delta deoxy[Hb+Mb] + \Delta oxy[Hb+Mb]$$

All NIRS data are expressed as the change from baseline and presented as a 10 s average encompassing the 5 s before and 5 s during blood collection.

Prior to blood collection, an easy-release elasticated tourniquet was applied around the triceps and biceps muscles of the opposing arm to which the cannula was inserted. Following 35 s of venous occlusion, a venepuncture needle was inserted into the antecubital vein to draw blood (23G, 0.6 x 19 mm x 178 mm, Ref 367284, BD Vacutainer, Plymouth, UK). The occlusion time of 35 s reduces the risk of haemolysis in the blood samples (Saleem et al., 2009, Makhumula-Nkhoma, Whittaker and Mcsherry, 2015) but allows sufficient filling of the veins to make them prominent. The venous blood was drawn directly into 2 x 4 ml EDTA vacutainers (BD Vacutainer, BD Ltd, Plymouth, UK). All blood samples drawn by venepuncture were successful on the first attempt. Immediately after the venepuncture, a small volume of blood (~2 ml) was drawn through the cannula and discarded to remove any remaining saline within the sample line. A further 8 ml of blood was then drawn from the cannula using a sterile syringe (BD Plastipak, BD Ltd, Plymouth) and separated into 2 x 4 ml EDTA vacutainers. A rapid inflation cuff (E20 Rapid Cuff Inflator, Hokanson, Bellevue, WA) was then placed around the upper portion of the cannulated arm which was immediately inflated to a sub-diastolic pressure (50-60 mmHg) and a blood sample drawn through the cannula 35 s later. The pressure of 50-60 mmHg ensured limited impedance to arterial blood flow (Patterson and Shepherd, 1954). The venous occluded cannula samples were taken to address the secondary aim of the study. The collection of blood commenced immediately at 35 s post-occlusion for both venepuncture and venous occluded cannula samples. There was, however, some unavoidable minor variation in the time for vacutainers to fill when collecting blood through the venepuncture needle.

One vacutainer of blood from each measurement method was centrifuged at 4000 rpm and 4°C for 10 min (Harrier 18/80, Henderson Biomedical. UK) within 3 min of collection (Pelletier et al., 2006, Bailey et al., 2009). The plasma was separated, frozen at −80 °C, and analysed within 4 months of initial collection for determination of [nitrate] and [nitrite]. The other vacutainer was refrigerated at 4°C for the later analysis of Hb concentration and haematocrit. All samples were analysed within 6 h of collection.

2.4 Plasma nitrite and nitrate analysis

Measurements of [nitrate] and [nitrite] were made using ozone-based chemiluminescence (Rogers et al., 2005). For the measurement of plasma [nitrite], 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 that was heated to 50 °C and connected to an NO analyser (Sievers NOA 280i, Analytix, UK). A standard curve was produced by injecting 100 μL of nitrite solutions up to 1000 nM (supplementary data file). 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 and calculated using Origin software (version 7) and divided by the gradient of the slope. The coefficient of variation for the measurement of plasma [nitrite] in the current study was 1.4%.

For the measurement of plasma [nitrate], 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 15-50 μL of nitrate solutions up to 100 μM (supplementary data file). Plasma samples were thawed and de-proteinised (200 μL of sample, 400 μL of zinc sulphate

in deionised water at 10% w/v and 400 µL of 0.5M sodium hydroxide). Subsequently, 15-25 µL of the sample was injected into the purge vessel in duplicate and plasma [nitrate] calculated as previously described for the nitrite assay. The coefficient of variation for the measurement of plasma [nitrate] in the current study was 2.5%.

2.5 Determination of plasma volume change

To measure haematocrit, a small volume of venous blood from all samples 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%. The concentration of Hb 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 Hb reagent before being incubated for 3 min at 25 ^{o}C. The Hb 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 Hb in the current study was 1.5%. Total blood volume and total plasma volume at baseline were estimated using the Nadler equations (Nadler, Hidalgo and Bloch, 1962):

$$\text{Males total blood volume} = (0.3669 \times \text{height in meters}^3) + (0.03219 \times \text{body mass in kilograms}) + 0.6041$$

$$\text{Females total blood volume} = (0.3561 \times \text{height in meters}^3) + (0.03308 \times \text{body mass in kilograms}) + 0.1833$$

240 Total plasma volume = Total blood volume × (1 – Haematocrit)

241

242 **2.6 Statistical analysis**

243 All statistical analyses were carried out using JAMOVI (0.9.1.5) and GraphPad Prism
244 (version 7, GraphPad Software Inc., San Diego, USA) was used to create the figures. Data
245 are expressed as the mean ± standard deviation unless otherwise stated. The distribution of
246 the data were tested using the Shapiro-Wilk test. A two-way repeated measures ANOVA
247 was used to examine the effect of 'collection method' (venepuncture, cannula, and venous
248 occluded cannula), 'condition' (CON and BRJ), and the 'collection method*condition'
249 interaction on measurements of plasma [nitrite], [nitrate], Δ t[Hb+Mb], Δdeoxy[Hb+Mb],
250 Δoxy[Hb+Mb], and total plasma volume. *Post-hoc* tests were conducted using paired
251 samples t-tests with Bonferroni correction for multiple pairwise comparisons when a
252 significant main effect or interaction was found. Statistical significance was declared when
253 $P<0.05$. Probability values are expressed with 95% confidence intervals (95% CI) where
254 appropriate.

255

256 **3 Results**

257 **3.1 Plasma nitrite and nitrate**

258 There was a significant main effect of 'condition' ($P<0.001$) and 'collection method'
259 ($P<0.001$) on plasma [nitrite] but no 'collection method*condition' interaction ($P=0.6$).
260 Plasma [nitrite] was significantly elevated in the BRJ trial (venepuncture 387 ± 136 nM;
261 cannula 473 ± 164 nM; venous occluded cannula 384 ± 124 nM) compared to the CON
262 trial (venepuncture 112 ± 51 nM; cannula 179 ± 67 nM; venous occluded cannula 109 ± 43
263 nM; all $P<0.001$, Fig. 1B and 1C). Plasma [nitrite] was higher in cannula samples compared
264 to venepuncture samples (CON: $P=0.03$, 95% CI 39-94 nM; BRJ: $P<0.001$, 95% CI 25-

165 nM) and venous occluded cannula samples (CON: P=0.02, 95% CI 34-106 nM; BRJ: P<0.001, 95% CI 20-157 nM) in both trials. Plasma [nitrite] was not significantly different between venepuncture and venous occluded cannula samples in either trial (both P=1.0).

There was a significant main effect of 'condition' (P<0.001) on plasma [nitrate] but no effect of 'collection method' (P=0.5) or 'collection method*condition' interaction (P=0.8). Plasma [nitrate] was significantly elevated in the BRJ trial compared to the CON trial (all P<0.001, Fig. 1A).

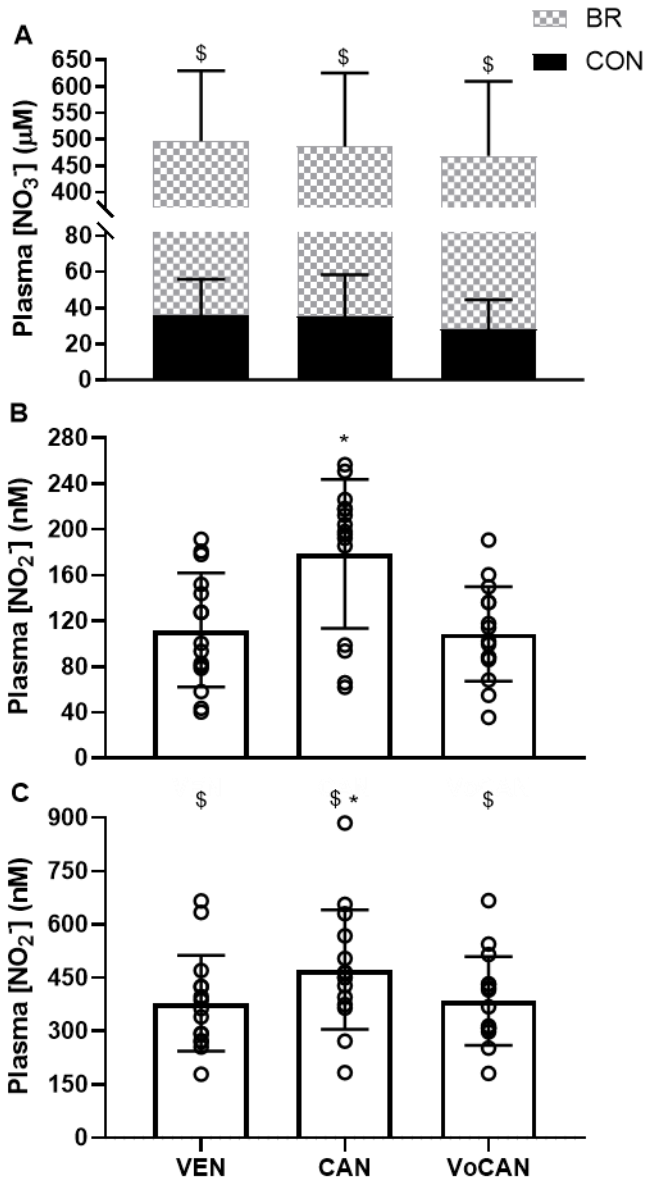


Figure 2 Plasma [nitrate] for CON and BRJ (A), plasma [nitrite] for CON (B), and plasma [nitrite] for BRJ trials for venepuncture, cannula, and venous occluded cannula. * denotes significant difference from VEN and VoCAN within the same condition ($P<0.05$). $ denotes significant difference compared to CON ($P<0.001$). VEN, venepuncture; CAN, non-occluded cannula; VoCAN, venous occluded cannula; NO_2^-, nitrite; NO_3^-, nitrate.

3.2 Tissue Oxygenation

The influence of venous occlusion on Δt[Hb+Mb], Δdeoxy[Hb+Mb], and Δoxy[Hb+Mb] was measured using NIRS. There was a significant main effect of 'collection method' (all $P<0.001$) on all three markers but no effect of 'condition' (all $P=0.5$) or 'collection method*condition' interaction (all $P>0.3$). The Δt[Hb+Mb], Δdeoxy[Hb+Mb], and Δoxy[Hb+Mb] was significantly less in cannula samples compared to venepuncture and venous occluded cannula in both trials (all $P<0.01$, Fig. 2).

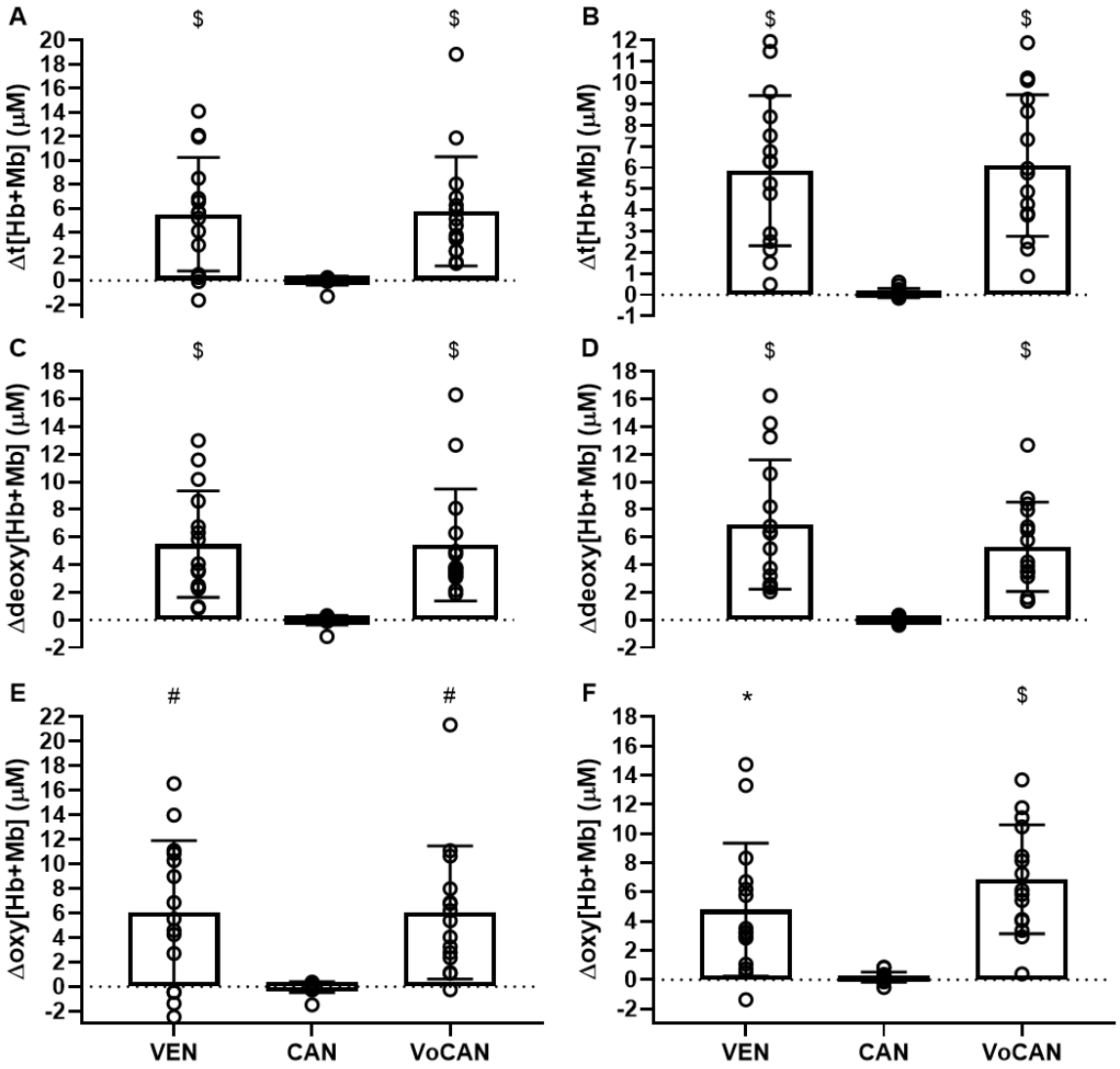
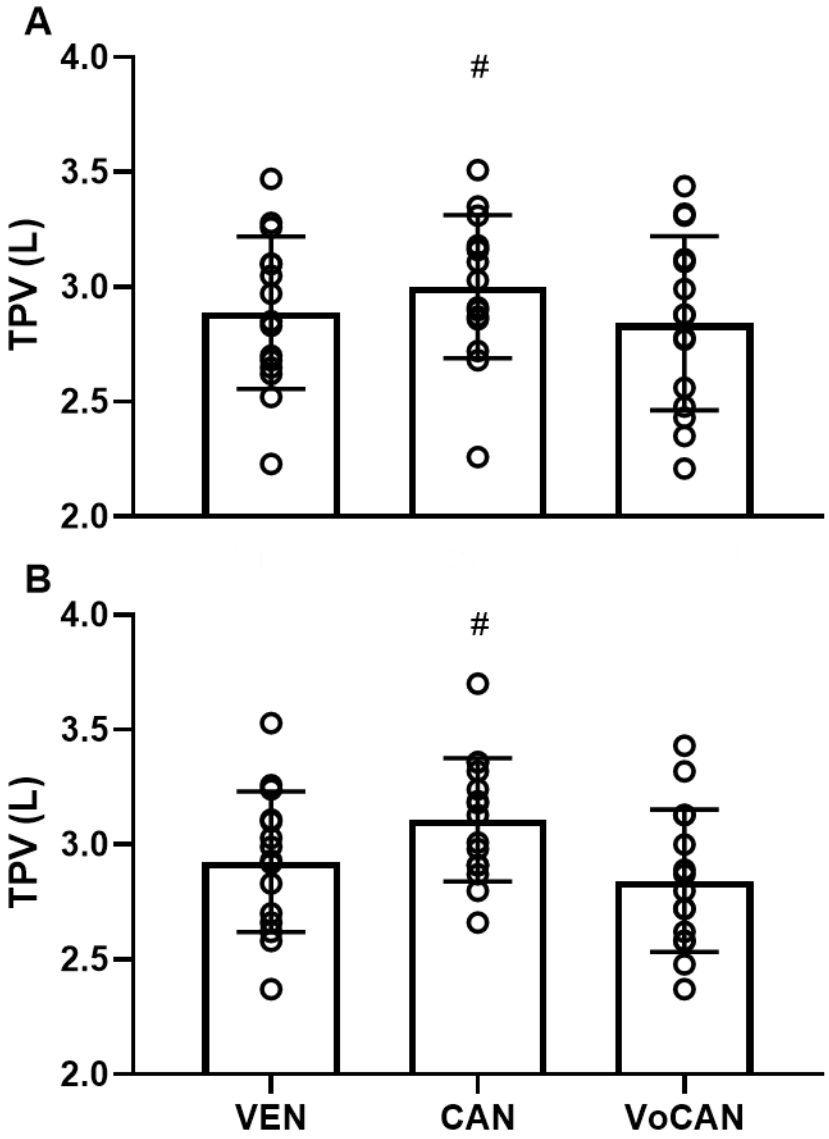


Figure 3 Delta change from baseline in t[Hb+Mb] during CON (A) and BRJ (B) trials. Delta change from baseline in deoxy[Hb+Mb] during CON (C) and BRJ (D) trials. Delta change from baseline in oxy[Hb+Mb] during CON (E) and BRJ (F) trials. Symbols denote a significant difference from the cannula sample: * (P<0.05), # (P<0.01) and $ (P<0.001). VEN, venepuncture; CAN, non-occluded cannula; VoCAN, venous occluded cannula.

3.3. Total plasma volume

There was a significant main effect of 'collection method' (P<0.01) and 'collection method*condition' (P=0.045) on total plasma volume but no effect of 'condition' (P=0.5).

Total plasma volume was significantly elevated in cannula samples when compared to venepuncture (P=0.03, 95% CI 0.05-0.18 L) and venous occluded cannula samples (P<0.001, 95% CI 0.02-0.3 L), in the CON trial. Total plasma volume was significantly elevated in cannula samples when compared to venepuncture (P<0.001, 95% CI 0.12-0.25 L) and venous occluded cannula samples (P<0.001, 95% CI 0.15-0.39 L) in the BRJ trial. In both trials, total plasma volume was not significantly different between venepuncture and venous occluded cannula samples (all P>0.9).



312

313 **Figure 4** Total plasma volume during all three collection methods in the CON (A) and BRJ

314 (B) trials. # denotes significant difference compared to venepuncture and venous occluded

315 cannula within the same trial ($P \leq 0.01$). VEN, venepuncture; CAN, non-occluded cannula;

316 VoCAN, venous occluded cannula; TPV, total plasma volume.

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4. Discussion

The principal finding of the present study was that plasma [nitrite] was substantially lower when blood flow of the upper arm was occluded during sample collection from the antecubital vein using venepuncture in comparison to an unrestricted cannula. These differences were consistent with basal levels of plasma NO metabolites or when elevated by prior ingestion of BRJ. On the other hand, the method of blood collection did not impact the concentration of plasma nitrate. This experiment further demonstrates that the lower [nitrite] in venepuncture samples was a direct consequence of the application of a tourniquet and not due to differences in the blood collection apparatus. It appears that localised changes in deoxy[Hb+Mb] and oxy[Hb+Mb] or other factors emanating from a reduction in blood flow may act to reduce nitrite availability.

The data presented herein quantifies the specific impact of an isolated methodological factor on the measurement of plasma [NO_2^-]. Along with previous data from our laboratory (Liddle et al., 2018, 2019), the results suggest that differences in experimental methods may partly explain the large variation in the reported measurements of basal and elevated concentrations of plasma nitrite between studies (James et al., 2015). Importantly, the differences in plasma [nitrite] between occluded and non-occluded collection methods were substantial and exceed the critical difference (Liddle et al., 2019). This is important in the context of quantifying the effects of factors that may influence the bioavailability of NO, including exercise (Muggeridge et al., 2017), diet (Burleigh et al., 2019), ageing (Goubareva et al., 2007), and disease (Assmann et al., 2016). For individual studies, it is unlikely that the method of blood collection will influence the interpretation of plasma nitrite data with the assumption that methods and procedures are consistent between measurement points. Indeed, the increase in plasma nitrite following the ingestion of BRJ

was not different whether the blood was collected using venepuncture (+275 nM) or a cannula (+294 nM). On the other hand, the percentage increase in plasma nitrite appears higher with venepuncture (+246%) compared to cannula samples (+164%) given the differences in baseline values. We are not aware of any study where different methods of blood collection were utilised between samples, but our findings highlight that this would be problematic. Of note, our observations are limited by the fact that many authors do not fully detail blood collection methods.

The influence of blood collection method may become important when one attempts to combine results from various studies in a meta-analysis or meta-regression. For example, Assmann et al. (2016) conducted a meta-analysis to evaluate the association between plasma NO levels and diabetes mellitus status. McMahon et al. (2017) conducted a meta-regression to assess whether the magnitude of the % change in plasma nitrite influenced the effects on exercise performance. It is likely that these meta-analyses combined studies that used varying combinations of blood collection methods. It is not possible to ascertain how this impacted on the outcomes of these studies, but it is an important point to consider for future work.

In the present study we used NIRS to explore forearm oxygenation when a tourniquet was applied for a brief period. As expected, deoxy[Hb+Mb] increased significantly when venous blood flow was occluded (venepuncture and venous occluded cannula) but remained unchanged when unrestricted. This is consistent with previous research that reported increases in deoxy[Hb+Mb] within 1 min of venous occlusion at 50 mmHg (Hampson and Piantadosi, 1988). It is well-established that the reduction of nitrite to NO in the blood is accelerated during conditions of hypoxia in an attempt to maintain oxygen

delivery (Gladwin et al., 2000, Crawford et al., 2006, Webb, Milsom, et al., 2008). We hypothesised that an increase in deoxy[Hb+Mb] would decrease plasma [nitrite] as this previously been reported in both *in vitro* and *in vivo* studies (Doyle et al., 1981, Cosby et al., 2003, Nagababu et al., 2003, Huang et al., 2005). However, the increase in oxy[Hb+Mb] adds to the complexity of the potential mechanisms involved as an increase in oxy[Hb+Mb] has been reported to increase the oxidation of NO to nitrite and nitrate (Ignarro et al., 1993, Joshi et al., 2002). It must also be recognised that heterogeneity in the tissue depth of flexor muscles may influence deoxy[Hb + Mb] kinetics between participants (Koga et al., 2011).

Alternatively, the decrease in plasma [nitrite] during blood flow restriction may be a consequence of an increase in local blood volume. Venous occlusion can increase hydrostatic pressure and force some plasma into the surrounding tissue as evidenced by the reduced total plasma volume during venous occluded venepuncture and cannula samples. The higher deoxy[Hb+Mb], oxy[Hb+Mb], and t[Hb+Mb] that followed venous occlusion also supports the notion that local blood volume has increased (Kime et al., 2009). This is consistent with some (Ferrari et al., 1992) but not all (Hampson and Piantadosi, 1988) previous research that shows venous occlusion (50-60 mmHg) increases blood volume in the forearm. Conversely, full arterial occlusion decreases oxy[Hb+Mb] (Chance et al., 1988, Hampson and Piantadosi, 1988, Van der Sluijs et al., 1998, Bopp, Townsend and Barstow, 2011) suggesting the extent of the occlusion may influence the magnitude of localised tissue oxygenation. The pooling effect of the blood in this region may decrease the shear rate on the endothelium as previously reported (Padilla et al., 2009). It has also been shown that a retrograde in shear rate attenuates endothelial function in a dose dependent manner (Thijssen et al., 2009). Shear rate stimulates NO production from endothelial cells via an acute increase in calcium that enhances the binding of calmodulin

to endothelial NO synthase and increases endothelial NO synthase activity (Boo and Jo, 2003).

A notable strength of this study is that we isolate the impact of a single methodological factor on the measurement of highly reactive NO metabolites. These data are important given plasma nitrite concentration is routinely measured in human participants to best approximate NO synthase activity (Lauer et al., 2001). This study is, however, not without some unavoidable experimental limitations. It is possible that between-arm differences in blood flow and/or NO metabolism may contribute to the observed effects of venous blood occlusion on plasma nitrite. However, the consistency of the findings between the venepuncture and occluded cannula measurements seem to suggest that this is unlikely. The comparison of NIRS data between different arms is also not ideal as NIRS can only reliably track changes in Hb+Mb within individual detector channels. Finally, local changes in total plasma volume were estimated using the Nadler equations (Nadler, Hidalgo and Bloch, 1962) rather than through direct measurement. While these data must be interpreted cautiously, the Nadler equations have been shown to provide accurate estimations of total blood volume (Sharma and Sharma, 2018).

Based upon our findings, future studies should examine how the duration of tourniquet application influences plasma nitrite. This is important to understand how protracted use of a tourniquet during blood collection from participants with poor venous access may alter plasma nitrite concentration. It is likely that discrepancies in other procedural aspects of blood collection will also influence plasma nitrite measurements, including the posture of the participant (Liddle et al., 2018) and the anticoagulant used in vacutainers (Ricart-Jané, Llobera and López-Tejero, 2002). Furthermore, plasma nitrite is variably measured using

either chemiluminescence (Burleigh et al., 2019, Bescos et al., 2020) or high-pressure liquid chromatography (HPLC) (Kim et al., 2015, Coggan et al., 2018) although it is unclear how the sensitivity of these methods differs. Plasma nitrite may also be subject to circadian or seasonal variation (Liu et al., 2014, Muggeridge et al., 2015, Monaghan et al., 2018) although these factors have not been subject to focused research.

5 Conclusion

Venous occlusion of the upper arm decreases plasma [nitrite] but does not alter plasma [nitrate]. These effects are likely a consequence of an accelerated reduction of nitrite to NO during localised changes in Hb and/or a suppression of endogenous NO production when venous blood flow is restricted. Researchers who use a tourniquet to aid blood collection for the measurement of [nitrite] should carefully consider how short-duration venous occlusion impacts this parameter. The blood collection method should be fully documented in the methods section and, if relevant, the tourniquet application time should be standardised between samples and documented.

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