FACTORS INFLUENCING THE EFFECTS OF DIETARY NITRATE SUPPLEMENTATION ON NITRIC OXIDE BIOMARKERS AND BLOOD PRESSURE

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Abstract

Ingestion of nitrate (NO_3^-) from natural sources can improve indices of cardiovascular health and exercise tolerance. The aim of this thesis was to determine the impact of dietary NO_3^- as a therapeutic aid when consumed amongst factors that might affect its efficacy such as antibacterial mouthwash, blood donation, different food forms and co-ingestion with alcoholic beverages. Young, healthy, normotensive individuals volunteered to participate in each experiment and undergo an array of physiological assessments. **Chapter 4:** Mouth rinsing with chlorhexidine and non-chlorhexidine mouthwash prior to consumption of concentrated NO_3^--rich beetroot juice (BR), over 6 days, blunted the rise in plasma nitrite concentration ($[NO_2^-]$) by 53 % and 29 % respectively, compared with control. Chlorhexidine mouthwash also elevated systolic (SBP) and mean arterial (MAP) blood pressure (BP) during treadmill walking. **Chapter 5:** Short-term BR ingestion lowered the oxygen (O_2) cost of moderate-intensity exercise (by ~ 4 %), better preserved muscle oxygenation and attenuated the decline in incremental exercise tolerance (by 5 %) following whole blood donation. **Chapter 6:** An array of different NO_3^--rich vehicles, including BR, beetroot flapjack (BF), non-concentrated beetroot juice (BL) and beetroot crystals (BC), elevated salivary, plasma and urinary NO_3^- concentration ($[NO_3^-]$) and $[NO_2^-]$ when compared with baseline and control, with the largest increases in plasma $[NO_2^-]$ occurring in BF and BR. BR also reduced SBP (~5 mmHg) and MAP (~ 3-4 mmHg), and BF reduced diastolic BP (DBP; ~ 4 mmHg). **Chapter 7:** A high NO_3^- salad, accompanied by polyphenol-rich (NIT-RW) and -low (NIT-A) alcoholic beverages and a water control (NIT-CON) elevated salivary, plasma and urinary $[NO_3^-]$ and $[NO_2^-]$ compared with control (CON). SBP was reduced 2 h post consumption of NIT-RW (-4 mmHg), NIT-A (-3 mmHg) and NIT-CON (-2 mmHg) compared with CON. DBP and MAP were also lower in NIT-A, and more so in NIT-RW, compared with NIT-CON.

Overall, the findings in this thesis demonstrate the efficacy of naturally derived NO_3^- on NO metabolites, BP and exercise tolerance. The potential for such benefits to arise may be maximised if antibacterial mouthwash is avoided during supplementation and if NO_3^- is consumed as BR, BF or as a green leafy salad with or without an alcoholic beverage. It may also be suggested that NO_3^- ingestion can offset decrements in exercise tolerance following blood donation.

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Chapter 8: General Discussion

Symbols and Abbreviations

[ ]	concentration
Δ	difference, change, delta
ADI	acceptable daily intake
ANOVA	analysis of variance
ANT	adenine nucleotide translocator
Asc	Ascorbic acid
ATP	adenosine triphosphate
BC	beetroot crystals
BF	beetroot flapjack
BL	non-concentrated beetroot juice
BP	blood pressure
BR	concentrated beetroot juice
Ca^{2+}	calcium
cGMP	cyclic guanosine monophosphate
CO_2	carbon dioxide
CON	control
COPD	chronic obstructive pulmonary disease
COX	cytochrome-*c* oxidase
CWR	constant work rate
DBP	diastolic blood pressure
eNOS	endothelial nitric oxide synthase
GET	gas exchange threshold
h	hour
H^+	hydrogen ion, proton
Hb	haemoglobin
HbO_2	oxygenated haemoglobin
HCl	hydrochloric acid

HHb	deoxygenated haemoglobin
HNO_2	nitrous acid
H_2O	water
HR	heart rate
iNOS	inducible nitric oxide synthase
KNO_3	potassium nitrate
MAP	mean arterial pressure
NaI	sodium iodide
$NaNO_3$	sodium nitrate
$NaNO_2$	sodium nitrite
NaOH	sodium hydroxide
NIRS	near-infrared spectroscopy
NIT-A	nitrate and alcohol
NIT-RW	nitrate and red wine
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
N_2O_3	dinitrogen trioxide
NOS	nitric oxide synthase
O_2	oxygen
O_2^-	superoxide
PAD	peripheral arterial disease
PPO	peak power output
Ph	phenol
PL	placebo
P/O	oxygen cost of ATP resynthesis
RER	respiratory exchange ratio

RNI	reactive nitrogen intermediates
RPM	revolutions per minute
SBP	systolic blood pressure
SD	standard deviation
SE	standard error
SPSS	Statistical Package for the Social Sciences
TOI	tissue oxygenation index
TTE	time to exhaustion
TTF	time to task failure
UCP	uncouling protein
VCl_3	vanadium chloride
$\dot{V}CO_2$	pulmonary carbon dioxide output
$\dot{V}E$	pulmonary ventilation (expired)
$\dot{V}O_2$	pulmonary oxygen uptake
$\dot{V}O_{2peak}$	peak oxygen uptake
W	Watt
WHO	World Health Organisation

Declaration

The material contained within this thesis is original work conducted and written by the author. The following publications and communications are a direct consequence of the work.

Refereed Journal Articles

McDonagh STJ, Wylie LJ, Vanhatalo A, Jones AM. (2015). The effects of chronic nitrate supplementation and the use of strong and weak antibacterial agents on plasma nitrite and exercise blood pressure. *International Journal of Sports Medicine, 36*(14), 1177-1185. DOI: 10.1055/s-0035-1554700.

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McDonagh STJ, Wylie LJ, Webster JMA, Vanhatalo A, Jones AM. (2018). Influence of dietary nitrate food forms on nitrate metabolism and blood pressure in healthy normotensive adults. *Nitric Oxide, 72*, 66-74. DOI: 10.1016/j.niox.2017.12.001.

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Black MI, Jones AM, Blackwell JR, Bailey SJ, Wylie LJ, **McDonagh STJ**, Thompson C, Kelly J, Sumners P, Mileva KJ, Bowtell JL, Vanhatalo A. (2017). Muscle metabolic and neuromuscular determinants of fatigue during cycling in different exercise intensity domains. *Journal of Applied Physiology, 122*(3), 446-459. DOI: 10.1152/japplphysiol.00942.2016.

Thompson C, Wylie LJ, Blackwell JR, Fulford J, Black MI, Kelly J, **McDonagh STJ**, Carter J, Bailey SJ, Vanhatalo A, Jones AM. (2017) Influence of dietary nitrate supplementation on physiological and muscle metabolic adaptations to sprint interval training. *Journal of Applied Physiology, 122*(3), 642-652. DOI: 10.1152/japplphysiol.00909.2016.

Thompson C, Wylie LJ, Fulford J, Kelly J, Black MI, **McDonagh STJ**, Jeukendrup AE, Vanhatalo A, Jones AM (2015). Dietary nitrate improves sprint performance and cognitive function during prolonged intermittent exercise. *European Journal of Applied Physiology, 115*(9), 1825-1834. DOI: 10.1007/s00421-015-3166-0.

Other Publications

Jones AM, Kelly J, **McDonagh STJ,** Wylie LJ. *Dietary nitrate and exercise. Professionals in Nutrition for Exercise and Sports Newsletter, 2013.*

Works in Review/Progress

McDonagh STJ, Mejzner N, Clark, CE. Prevalence of postural hypotension in primary, community and institutional care: A systematic review and meta-analysis.

Clark CE, Smith LFP, Cloutier L, Konya J, Todkar SK, **McDonagh STJ**, Clark OM, Glynn LG, Taylor RS, Campbell JL. Allied health professional-led interventions for improving control of blood pressure in patients with hypertension. *Cochrane Review*.

Clark CE, **McDonagh STJ**, McManus RJ. Accuracy of automated blood pressure measurements in the presence of atrial fibrillation: systematic review and meta-analysis.

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Conference Activity

Poster Presentation: Interventions to improve control of hypertension; what works (and what doesn't)? British and Irish Hypertension Society Annual Scientific Meeting, Robinson College, Cambridge, England, September 2018.

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Dedication

I dedicate this thesis to my family and friends - thank you for your never-ending support.

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Chapter 1: Introduction

Recently, there has been a surge in the use of dietary nitrate (NO_3^-) as an ergogenic and therapeutic aid. The scientific and medical communities have led many investigations into the physiological effects of dietary NO_3^- ingestion in sport and exercise and on indices of cardiovascular health and well-being. The emerging evidence base (see Chapter 2) has resulted in the promotion of NO_3^-, in many different forms, as a tool for improving athletic performance and the vasculature.

One of the first reports of NO_3^- supplementation on human physiological functioning was by Larsen et al. (2006) who showed that ingestion of sodium NO_3^- ($NaNO_3$) for 3 days reduced resting blood pressure (BP) in healthy, normotensive persons. This finding has since been replicated, with different doses of NO_3^- salt (Bahra et al., 2012; Kapil et al., 2010). The same group later reported that NO_3^- salts were also capable of lowering the oxygen (O_2) cost of cycling exercise (Larsen et al., 2007), a phenomenon previously considered to be unaffected by exercise training or breathing hyperoxic gas. These remarkable findings provoked many others to explore the impact of NO_3^-, particularly in its more natural forms, such as beetroot juice and vegetables, on BP, health and performance. In 2009, Bailey and colleagues found that 500 mL of beetroot juice, consumed every day, for 6 days, reduced systolic BP (SBP) and the O_2 cost of submaximal cycle exercise and improved tolerance to high intensity exercise. Further research reported that NO_3^- ingestion reduced systemic BP (Webb et al., 2008) and the O_2 cost of exercise in a number of other exercise modalities (Bailey et al., 2010; Lansley et al., 2011; Muggeridge et al., 2013) and improved time-trial (Cermak, Gibala & van Loon, 2012; Lansley 2011a), intermittent exercise (Thompson et al., 2016) and cognitive performance (Thompson et al., 2015).

However, NO_3^- ingestion has not always resulted in reductions in BP (Gilchrist et al., 2013; Wilkerson et al., 2012) or improvements in exercise performance and cognitive parameters (Kelly et al., 2013), and this may be due to a number of factors influencing the effectiveness of the supplement, such as the population under investigation or the type and dose of NO_3^- administered (James et al., 2015).

Whilst most research studies carefully control experimental conditions when investigating the efficacy of NO_3^- supplementation on physiological parameters, the influence of certain factors, particularly those which relate to 'normal' lifestyle routines, in combination with NO_3^- ingestion, remain unknown. It is evident that some choices, such as the source of NO_3^- ingested (Jonvik et al., 2016), training status (Wilkerson et al., 2012) and smoking (Bailey et al., 2016) can influence the efficacy of the physiological response to dietary NO_3^-. It has also been reported that chlorhexidine-containing mouthwash can attenuate the rise in NO_3^- derivatives, like nitrite (NO_2^-) and the vasodilator, nitric oxide (NO; Govoni et al., 2008) and the BP reducing effect (Kapil et al., 2010; Petersson et al., 2009) of NO_3^- salt ingestion. However, further research is required to determine the impact of the prolonged use of chlorhexidine and weaker antiseptic agents on exercise BP when a NO_3^--rich vegetable source is ingested.

Although many studies now support a therapeutic and ergogenic role for NO_3^-, particularly in hypoxic (Kelly et al., 2014; Masschelein et al., 2012) and ischaemic conditions (Hendgen-Cotta et al., 2012), which may occur during exercise and in disease states (Kenjale et al., 2011), it is not known whether NO_3^- supplementation can counteract the reduction in O_2-carrying capacity and subsequent decrements in exercise performance which occur after voluntary blood donation and this is an avenue for future work.

Chapter 1: Introduction

The acute and chronic physiological effects of beetroot juice have been compared (Vanhatalo et al., 2010; Wylie et al., 2016). Whilst the pharmacokinetic and dose-response relationship to NO_3^--rich salts and beetroot juice have been determined (Kapil et al., 2010; Wylie et al., 2013), the optimal NO_3^- vehicle (from the many commercially available products that exist) needed to maximise supplementation regimens remains unknown.

Mediterranean and Japanese diets have often been endorsed for their positive influence on cardiovascular health, mainly due to the high NO_3^- content of the vegetables (Hu, 2003; Sobko et al., 2010). In addition, polyphenols, particularly those found in red wine, have been reported to promote NO formation in the stomach after a $NaNO_3$ bolus (Gago et al., 2007). Similarly, the combined ingestion of NO_3^- and alcohol has also been shown to increase ethyl NO_2^-, a vasodilator (Rocha et al., 2015). However, the pharmacokinetic response to the combination of a typical vegetable based NO_3^--rich meal and accompanying alcoholic beverage, whether high or low in polyphenols, is not known and warrants further investigation.

The purpose of this thesis was to determine the impact of a number of factors, some of which are typical daily choices, on the physiological and therapeutic response to dietary NO_3^- supplementation. Specifically, the following literature review develops a rationale for the investigation of the physiological response to NO_3^- ingestion in different forms, alongside chronic antibacterial mouthwash use, after blood donation, and in conjunction with polyphenol-rich and -low alcoholic beverages.

Chapter 2: Literature Review

Nitric Oxide

NO, a clear, odourless gas, was discovered in the late 1700’s by Joseph Priestley and was predominantly known as an atmospheric pollutant. In the 1980’s, Furchgott, Ignarro and Murad identified that the molecule produced in the body that was responsible for vasodilation, known at the time as endothelium derived relaxing factor (EDRF), was, in fact, NO. Nowadays, NO is known as a key and widespread mammalian signalling molecule that can regulate both physiological and pathophysiological processes (Nathan, 1992). It is one of the most researched molecules in physiology and medicine today due to its influence on BP (Rees, Palmer & Moncada, 1989; Webb et al., 2008), blood flow (Shen et al., 1994), immunity (Coleman, 2001; Kilbourn, 1991; Tripathi, 2007), blood clotting (Radomski, Palmer & Moncada, 1990), skeletal muscle glucose uptake (Merry, Lynch & McConell, 2010), calcium handling and skeletal muscle contractility (Hart & Dulhunty, 2000; Viner et al., 2000), skeletal muscle fatigue (Percival et al., 2010), mitochondrial efficiency (Larsen et al., 2011) and neurotransmission (Garthwaite, 2008). NO has a short half-life in *vivo* (~ 0.1 s; Kelm & Schrader, 1990) and a reduction in its bioavailability has been associated with both cardiovascular (Förstermann, 2010) and metabolic (Huang, 2009) disease. Therefore, it may be suggested that maintaining continual NO production, via either of the two known pathways, the NO synthase (NOS)-dependent pathway and the NOS-independent NO_3^--NO_2^--NO pathway, is critical for normal biological functioning.

Endogenous, NOS-dependent NO production

It is well established that NO can be produced at a number of sites in the body by a family of NOS enzymes, including the endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) isoforms (Moncada et al., 1989; Stamler & Meissner, 2001; Villanueva & Giulivi, 2010). These enzymes catalyse the complex oxidation of one of the guanidine nitrogen groups on the amino acid, L-arginine, to yield NO and L-citrulline (Stamler & Meissener, 2001; Stuehr et al., 1991). However, this reaction is only possible in the presence of molecular O_2 and several essential co-factors, such as nicotinamide adenine dinucleotide phosphate (NADPH, acting as an electron donor), tetrahydrobiopterin (BH_4), haem, calmodulin, calcium, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD; Alderton et al., 2001; Machha & Schechter; Stuehr et al., 2004; White & Marletta, 1992). A reduction in the bioavailability of one or more of these co-factors can hinder the production of NO via the NOS pathway (Crabtree et al., 2009). In fact, NOS can become uncoupled under particular conditions and cannot catalyse the conversion of L-Arginine to L- citrulline, yielding NO, instead, superoxide (O_2^-) is formed via the donation of an electron from NADPH to molecular O_2 (Roe and Ren, 2012). The causes of uncoupling are not fully understood at present but the oxidation of BH_4 by O_2^- and alterations in NOS phosphorylation or destabilisation of the functional NOS dimer have all been considered to play a role (Alp and Channon, 2004).

Increasing age (Lyons et al., 1997) and cardiovascular (Försterman, 2010) and metabolic morbidities (Huang, 2009; Wu et al., 2009) have been associated with a decline in endogenously formed NO and can also result in impaired exercise capacity (Lauer et al., 2008). It is therefore evident that NO availability plays a crucial role in the preservation of normal endothelial function and tolerance to exercise.

It is important to note that NO formation is very sensitive to alterations in the physiological milieu, and conditions such as hypoxia or acidosis can limit NO production via the NOS pathway (Griffith and Stuehr, 1995). This may be due to the reduced availability of O_2 per se, decreased expression of eNOS (Ho et al., 2012; McQuillan et al., 1994; Torporsian et al., 2000) or even the favouring of NO production via an alternative route (i.e. by the reduction of NO_2^- through the NOS independent pathway, which suggests an existence of a crosstalk between the two pathways; Carlström et al., 2015). Recently, an additional O_2 independent pathway for NO generation has been identified, in which the products of the reaction, NO_3^- and NO_2^-, are progressively reduced to create NO (Benjamin et al., 1994). This means of NO production is a way of maintaining NO availability when the oxidation of L-arginine to form NO is compromised, by, for example, areas of low O_2 concentration (Bryan et al., 2008; Carlström et al., 2010; Lundberg et al., 2008) or in individuals with NOS dysfunction (Lauer et al., 2008).

NO_3^--NO_2^--NO pathway

NO_3^- and NO_2^- were originally viewed as biologically inert oxidation products of endogenously derived NO (Moncada & Higgs, 1993). Specifically, NO_3^- can be produced via the reaction of NO_2^- or NO with oxyhaemoglobin (Cooper, 1999), and NO_2^- can be formed by the reaction of NO with O_2 (Ignarro et al., 1993), ceruloplasmin (Shiva et al., 2006) or with the active copper site in cytochrome *c* oxidase (Cooper et al., 1997).

However, nowadays, there is a wealth of evidence to suggest that NO_3^- and NO_2^- can be serially reduced *in vivo* to form NO, particularly in hypoxic and acidic environments, which may manifest during exercise and disease (Van Faassen et al., 2009). NO can also be stimulated by the consumption and serial reduction of NO_3^- from dietary sources (Bryan & Hord, 2010) and this is a focus of the current thesis (See Figure 1.1).

NO_3^- can be ingested as a salt or as part of a natural nutritional regimen. Typically, 60-80 % of daily NO_3^- intake in a Western diet is derived from vegetables (Ysart et al., 1999). It is important to note, however, that ingested NO_3^- and NO_2^- results in a similar amount of NO production compared to that derived from endogenous vascular (~ 70 % of systemic NO is derived via eNOS in the endothelium) or gastrointestinal (e.g. through the reduction of recycled NO_2^- in the acidic environment of the stomach, in the absence of dietary NO_3^- ingestion; Weitzberg and Lundberg, 1998) sources, assuming most of the endogenously derived NO is oxidised to NO_3^- and NO_2^- (Hord, Tang & Bryan, 2009).

Once ingested, 100 % of dietary NO_3^- is rapidly absorbed from the upper gastrointestinal tract and enters the systemic circulation within ~ 60 min (Lundberg & Weitzberg, 2009), where it merges with endogenously derived NO_3^- (Florin et al., 1990; van Velzen et al., 2008). Plasma NO_3^- concentration ($[NO_3^-]$) remains elevated for up to ~ 5 h (Wagner et al., 1983), although more recent research by Wylie et al. (2013) suggests that NO_3^- may remain above baseline values until 24 h post ingestion of the NO_3^- source. Although ~ 60-70 % of NO_3^- is ultimately excreted in the urine (Bartholemew & Hill, 1984; Lundberg & Weitzber, 2009), approximately 25 % is taken up by the salivary glands, with the aid of the transporter protein sialin (Qin et al., 2012), and concentrated in the saliva by ~ 20 fold (Lundberg & Govoni, 2004; McKnight et al., 1997).

A number of species of facultative and strict anaerobic bacteria (including Veillonella, Rothia and Neisseria; Burleigh et al., 2018; Doel et al., 2005; Hyde et al., 2014; Vanhatalo et al., 2018), located on the dorsal surface of the tongue, facilitate the reduction of ~ 20 % of salivary NO_3^- (~ 5 % overall dietary NO_3^- intake) to NO_2^- (Duncan et al., 1995).

After ingestion of a NO_3^- source, salivary NO_2^- concentration ($[NO_2^-]$) has been reported to rise up to 2 mM, and, once swallowed, some of this NO_2^- is reduced to NO in the acidic environment of the stomach (e.g. $NO_2^- + H^+ \rightarrow HNO_2$, $2HNO_2 \rightarrow 2N_2O_3 + H_2O$, $N_2O_3 \rightarrow NO + NO_2$; Benjamin et al., 1994; Lundberg & Govoni, 2004). The reduction of NO_2^- to NO can be enhanced by the presence of vitamin C (e.g. $2HNO_2 + Asc \rightarrow 2NO + dehydrogAsc + 2H_2O$) and polyphenols (e.g. $Ph\text{-}OH + HNO_2 \rightarrow Ph\text{-}O\bullet + \bullet NO + H_2O$; Weitzberg and Lundberg 1998; Gago et al., 2007). Gastric NO, produced enzymatically by activated leukocytes (Malawista et al., 1992; Salvemini et al., 1989), or when acting as an effector molecule for macrophages (Brunet, 2001; Cenci et al., 1993), can play a role in host defence by destroying or inhibiting swallowed pathogens (Benjamin et al., 1994; McKnight et al., 1997). Despite this, some NO_2^- enters the systemic circulation, increasing plasma $[NO_2^-]$ (Dejam, Hunter, Schechter & Gladwin, 2004; Lundberg & Govoni, 2004). The time to peak plasma $[NO_3^-]$ and $[NO_2^-]$ has typically been reported to occur at ~ 1-2 and 2-3 h, after an acute dose of NO_3^-, respectively (Webb et al., 2008; Wylie et al., 2013). It is noteworthy that antibacterial mouthwash (Govoni et al., 2008) and failure to swallow saliva (Webb et al., 2008) have been shown to blunt the increase in plasma $[NO_2^-]$ following NO_3^- ingestion. This suggests that the reduction of NO_3^- to NO_2^- is highly dependent on the presence of the NO_3^- reducing bacteria found in the oral cavity.

NO synthesis from NO_2^-

The final step in the NO_3^--NO_2^--NO pathway is the one electron reduction of NO_2^- to NO via both enzymatic and non-enzymatic means (Lundberg et al., 2008; van Faassen et al., 2009). This pathway is facilitated by several catalysts including deoxyhaemoglobin (Cosby et al., 2003), deoxymyoglobin (Shiva et al., 2007), cytochrome P-450 (Kozlov et al., 2003) aldehyde oxidase (Li et al., 2008), xanthine oxidase (Zhang et al., 1998), NOS (Vanin et al., 2007), components of the mitochondrial electron transport chain (Kozlov, Staniek & Nohl, 1999), ascorbate (Carlsson et al., 2001) and polyphenols (Gago et al., 2007; Peri et al., 2005). Unlike the O_2-dependent NOS route, acidic (Modin et al., 2001) and hypoxic (Castello et al., 2006) physiological environments can enhance the production of NO via the NO_3^--NO_2^--NO pathway (van Faassen et al., 2009). In fact, Ferguson and colleagues (2016) recently found that in rats, NO_2^- infusion, alongside NO blockade, restores skeletal muscle vascular control during exercise to levels reported in healthy rats with normal NOS function. Overall, it may be suggested that dietary NO_3^- supplementation can provide an alternative means for elevating NO_2^- and NO concentrations when the NOS pathway of generating NO is comprised, by, for example, hypoxic or acidic conditions which may occur during exercise (Richardson et al., 1999), oxidative stress and disease (Van Faassen et al., 2009; Williams et al., 2002).

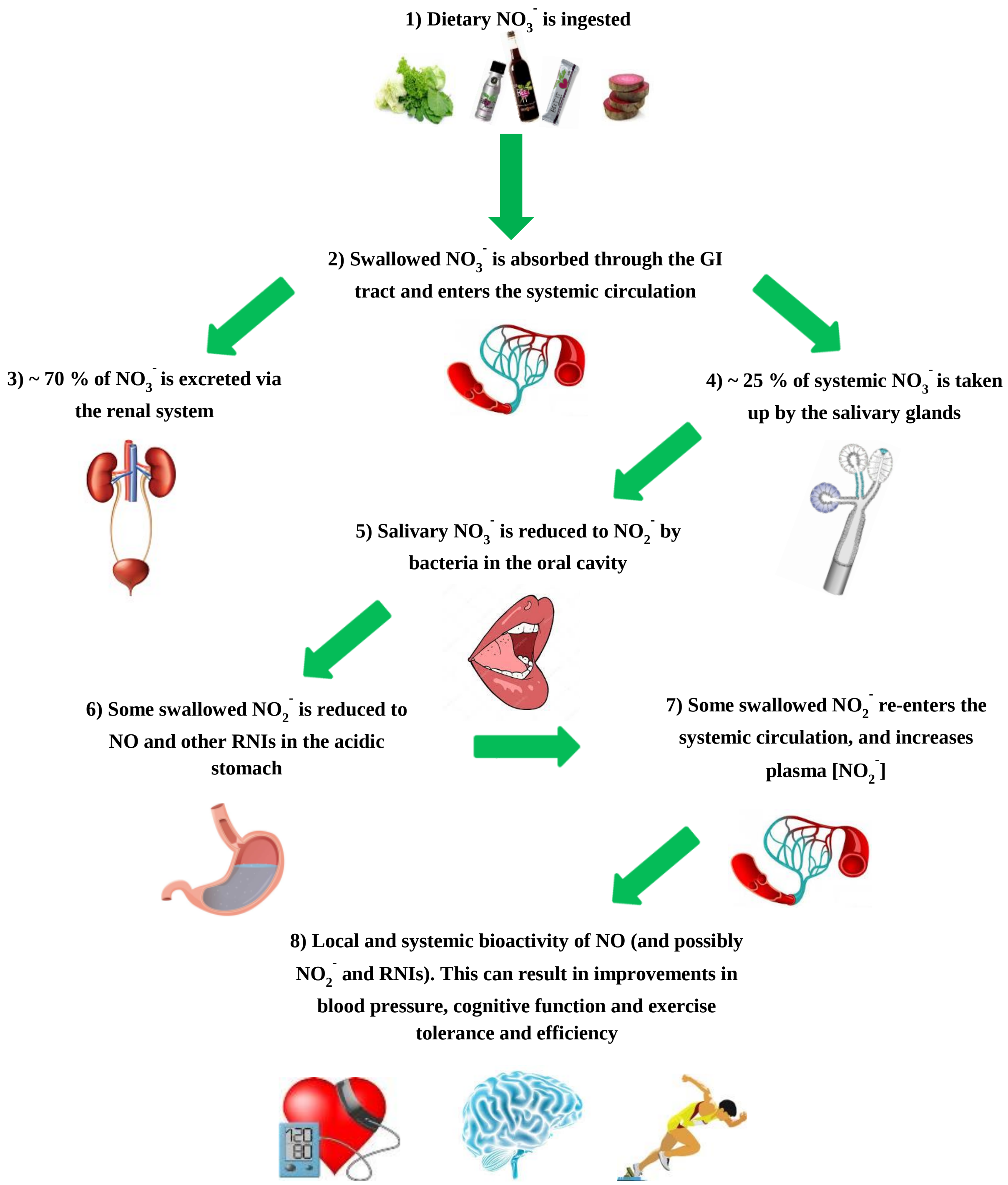

Figure 2.1 The nitrate (NO_3^-) -nitrite (NO_2^-) -nitric oxide (NO) pathway in the human body

Typical dietary NO_3^- and NO_2^- intake

NO_3^- is usually ingested as part of a natural diet, with the main source being vegetables, which can contain up to ~ 400 mg of NO_3^- per 100 g of fresh produce (Wang, Wei & Li, 2000). Sixty to 80 % of daily NO_3^- intake in a Western diet is derived from leafy greens, such as spinach, rocket, lettuce and red beetroot (*Beta vulgaris rubra*; Ysart et al., 1999). However, NO_3^- is also present in drinking water and cured meats where it is used as a preservative (Hord et al., 2009). It has been estimated that NO_3^- intake in humans can range from 31-350 mg per day (Hord et al., 2009; Pennington, 1998). This variation in NO_3^- consumption may be due to a number of factors, including the type and portion size of vegetable ingested, fertiliser use and the growth, storage and transport conditions of the NO_3^- source (Hord et al., 2009; Pennington, 1998).

The average daily intake of NO_2^- by humans ranges from 0 to 20 mg per day in U.K (Knight et al., 1987; Walters, 1980) and U.S (Fassett, 1973) diets, with the main source in the diet being from processed meats, where it is used to enhance taste and prevent bacterial growth (Pennington, 1998). NO_2^- is also found in vegetables but at much lower concentrations than NO_3^-, ranging from 10-100 mg per kg (Hord et al., 2009; Pennington, 1998; Santamaria, 2006; Sušin et al., 2006).

Health concerns relating to NO_3^- consumption

The consumption of NO_3^- has traditionally been considered harmful to health due to links with infantile methaemoglobinaemia (Comly et al., 1945), increased nitrosamine production (Mirvish, 1975) and carcinogenesis (Newberne et al., 1979). The NO_3^- anion is relatively inert, with any harmful effects being related to its conversion to the more reactive ion, NO_2^-. As a result, the World Health Organisation (WHO) have declared the acceptable daily intake (ADI) of NO_3^- as 3.7 mg per kg of body mass (e.g. ~ 4.5 mmol of NO_3^- for a 75 kg person; WHO, 2002). Despite these guidelines, vegetarians and those who follow initiatives such as the Dietary Approaches to Stop Hypertension (DASH) diet tend to exceed the ADI considerably, often by consuming up to 20 mmol of NO_3^- per day (Appel et al., 1997; Sacks et al., 1995).

Methaemoglobin is formed when one of the iron atoms in the haem group of oxyhaemoglobin is oxidised (by NO_2^-), converting ferrous (Fe^{2+}) iron into the ferric state (Fe^{3+}; Stryer, 1988). This prevents the haemoglobin molecule from binding with O_2 and therefore can result in cell hypoxia (Fan & Steinberg, 1996; McKnight et al., 1999). The initial concern with regard to NO_3^- and methaemoglobinaemia ('blue baby syndrome') arose in 1945 and was associated with infants that had been ingesting NO_3^- containing well water (Comly et al., 1945). This remains the basis for the regulation of NO_3^- in drinking water today, despite suggestions that the development of methaemoglobinaemia is unlikely to occur in the absence of bacterial contamination (Avery, 1999) and the lack of subsequent reports of methaemoglobinaemia following both NO_3^- and NO_2^- consumption across a number of countries (Cornblath & Hartmann, 1948; Dejam et al., 2007; Kortboyer et al., 1997). However, infant NO_3^- poisoning still remains a problem in the U.S., particularly in rural areas where milk formula may be prepared with contaminated well water (Johnson & Kross, 1990; Knobeloch & Proctor, 2001; Kross, Ayebo & Fuortes, 1992).

It has been reported in rodents that nitrosamines (a potential carcinogen) can be formed endogenously after the ingestion of NO_2^- (Mirvish, 1975). In fact, a few incidences of lymphoma were reported in rats after chronic NO_2^- exposure. However, it must be noted that the dose administered in this study was supra-physiological and unlikely to occur in humans (Newberne et al., 1979). NO_3^- and NO_2^- are often used as preservatives in processed meats and a high consumption of such foods has been associated with an increased risk of gastric cancer due to the formation of carcinogenic N-nitroso compounds, by, for example, the nitrosation of amines or amides by NO_2^- in the stomach (Larsson, Orsini & Wolk, 2006; Song, Wu & Guan, 2015). A recent meta-analysis has revealed, however, that NO_3^- intake is linked with a reduced risk of gastric cancer (Song et al., 2015) and many other studies have also failed to provide evidence that dietary NO_3^- consumption is linked to an increased risk of cancer in humans (Beresford, 1985; Forman et al., 1985; Gangolli et al., 1994; WHO, 2010). Actually, high NO_3^- foods, particularly vegetables and beetroot products, are a rich source of antioxidants and polyphenols (Kavalcovά et al., 2015; Khanam et al., 2012; Shepherd et al., 2015), which are known to inhibit the formation of carcinogenic *N*-nitroso compounds (Mirvish et al., 1998; Wootton-Beard & Ryan, 2012).

There is an expanding body of evidence to suggest that dietary NO_3^- consumption may be both cardio-protective and ergogenic. These findings have resulted in an increase in research exploring the use of NO_3^- as a therapeutic and performance enhancing aid, but the influence of a number of factors, particularly daily choices, and their role in the effectiveness of NO_3^- ingestion has yet to be determined.

Salivary, plasma and urinary [NO_3^-] and [NO_2^-]

NO can be produced endogenously or formed from the serial reduction of ingested dietary NO_3^-. However, due to its high reactivity and short half-life *in vivo* it is extremely difficult to measure in biological fluids (Kelm & Schrader, 1990). NO reacts rapidly with, for example, O_2 or haemoglobin, and therefore, it is likely that its free transport is limited, particularly in the blood (Hakim et al., 1996). Many NO storage and transport forms exist, including S-nitrosothiols, S-nitrosohaemoglobin (Doctor & Stamler, 2011; Pinheiro et al., 2015), NO_3^-, and NO_2^-, with the latter two forms being the predominant, stable and easily detectable pools found in the human body (Cosby et al., 2003; Silver, 2011). Measuring the concentration of NO_3^- and NO_2^- in the saliva, plasma and urine will allow further insight into the metabolism of NO *in vivo*, particularly after dietary NO_3^- supplementation. In addition, determination of the area under the curve, peak concentrations and the time taken to achieve peak and return to baseline concentrations of the stated NO metabolites, across a range of biological fluids, will provide crucial information for supplementation regimes where the aim is to improve indices of cardiovascular health. More specifically, knowledge of the kinetic pattern, including the magnitude and duration of exposure to these NO metabolites, will allow individuals to tailor supplementation of NO_3^- to maximise and prolong the expected physiological responses, such as improvements in BP or exercise performance.

Resting [NO_3^-] and [NO_2^-] in plasma (15-60 µM; 50-1000 nM), saliva (200-600 µM; 30-210 µM) and urine (250-2000 µM; < 1 µM) are susceptible to variation, depending on the individual’s age, nutritional intake and health status (Bescós et al., 2012; Green et al., 1982; Lundberg & Govoni, 2004; Pannala et al., 2002). The measurement techniques employed, such as ozone based chemiluminescence, high performance liquid chromatography, electrophoresis and colorimetric assays, may also impact the [NO_3^-] and [NO_2^-] reported in biological fluids (Moorcroft, Davis & Compton, 2001; Tsikas, 2005). It is important to note here that chemiluminescence is the gold standard technique for determining [NO_3^-] and [NO_2^-] due to its high sensitivity and therefore ability to detect concentrations in the nM range (Tsikas, 2005).

Typically, endothelial dysfunction results in lower levels of systemic NO_3^- and NO_2^- (Kleinbongard et al., 2006), whilst exercise (Green et al., 2004) and inflammatory disease (Crawford et al., 2004) can increase baseline circulating concentrations of NO_3^-. Exercise has also been found to increase plasma [NO_2^-] (Allen et al., 2010; Gladwin et al., 2000), but more frequently it has been shown, particularly at high intensities, to reduce plasma [NO_2^-], suggesting that circulating NO_2^-, after NO_3^- intake, may be reduced to NO during exercise in both normoxia and hypoxia (Bescós et al., 2011; Dreissigacker et al., 2010; Kelly et al., 2014; Larsen et al., 2010; Wylie et al., 2013a). Dietary NO_3^-, in the form of salts and vegetables, has consistently been shown to increase plasma (Kapil et al., 2010; Webb et al., 2008), salivary (Kapil et al., 2015; Lundberg & Govoni, 2004; Woessner et al., 2016) and urinary (Pannala et al., 2002) [NO_3^-] and [NO_2^-].

Time to peak salivary [NO_3^-] and [NO_2^-] usually occurs between 1-3 h post ingestion of NO_3^- (Pannala et al., 2002; Woessner et al., 2016). Although a direct relationship has been noted between NO_3^- intake and salivary [NO_3^-] and [NO_2^-], a number of factors, such as salivary flow-rate, oral pH and the activity of the NO_3^- reducing bacteria can be altered by daily choices (like using mouthwash or the type and dose of the NO_3^- source ingested) and subsequently influence the concentration of NO metabolites in the mouth (Djekoun-Bensoltane et a., 2007).

A dose-dependent increase in markers of NO bioavailability, such as plasma [NO_3^-] and [NO_2^-], has been reported after beetroot juice and potassium NO_3^- (KNO_3) ingestion (Kapil et al., 2010), with baseline levels evident by 24 h after ingestion of lower NO_3^-

doses (Wylie et al., 2013). Increases in plasma [NO_3^-] and [NO_2^-] after KNO_3 have been associated with reductions in SBP and diastolic BP (DBP), albeit a little later (6 and 3 h, respectively) than those detected following beetroot juice ingestion (Kapil et al., 2010). Time to peak plasma [NO_3^-] and [NO_2^-] typically occur within 2-4 h (Wylie et al., 2013). However, it seems that the kinetic response and time to maximum elevation in NO bioavailability, namely plasma [NO_3^-] and [NO_2^-], may differ depending on the NO_3^- vehicle ingested (Fleuck et al., 2016; Jonvik et al., 2016; Muggeridge et al., 2014; van Velzen et al., 2008). For example, Muggeridge et al. (2014) reported a faster time to peak plasma [NO_3^-] and [NO_2^-] (60 and 90 min) following ingestion of Swiss chard and rhubarb extract gels, respectively.

Approximately 60-75 % of both endogenously and exogenously derived NO_3^- is excreted in the urine (Hobbs et al., 2013; Pannala et al., 2002; Wagner et al., 1983) and this can be shown by increases in urinary [NO_3^-] and [NO_2^-] or total NO_3^- and NO_2^- output (Oldreive et al., 2001), which peak approximately 4-6 h post ingestion (Bartholomew et al., 1984; Cortas et al., 1991; Hobbs et al., 2012; Pannala et al., 2002).

Many NO_3^--rich products are commercially available, but the pharmacokinetic response to different food forms (e.g. solid or liquid) has not been determined and warrants investigation. This information would help to optimise supplementation regimens where the aim is to increase plasma [NO_2^-] and lower BP after ingestion of various types of NO_3^- containing foodstuffs or products.

Beneficial effects of dietary NO_3^- ingestion

The remainder of this review will summarise the established physiological effects of dietary NO_3^- supplementation and highlight particular factors that may alter these responses.

Blood pressure

The beneficial effect of a diet rich in fruit and vegetables on cardiovascular health (Gilchrist et al., 2010) and life expectancy (Visioli et al., 2005) is well established. The consumption of plant foods, such as green leafy vegetables and beetroot, has been estimated to lower the risk of cardiovascular morbidity and mortality (Hord, Tang & Bryan, 2009). These effects have been partly attributed to the high NO_3^- content of such vegetables (Santamaria, 2006) and the vasodilatory effects of its derivatives, NO_2^- (Classen, Stein-Hammer & Thöni, 1990) and NO (Ignarro et al., 1987).

Vascular changes and risk for cardiovascular events and disease can be monitored and predicted using a variety of different techniques, such as ankle-brachial index, pulse wave velocity, electrocardiograms, laser-Doppler, pulse pressure and standard BP measurements, amongst many others. Typically, BP measurements performed using a single arm are utilised in everyday general practice and in research studies as it is a simple, non-invasive and effective method of predicting cardiovascular events and total mortality (Lewington et al., 2002; Psaty et al., 2001) and therefore this technique has been employed throughout this thesis.

There is now a plethora of evidence to suggest that the consumption of NO_3^-, in the form of beetroot juice, leafy vegetables or NO_3^- salts, can reduce resting BP in healthy individuals (Ashworth et al., 2015; Bailey et al., 2010; Jonvik et al., 2016; Larsen et al., 2006; Webb et al., 2008) and in those with chronic obstructive pulmonary disease

(COPD; Berry et al., 2015; Curtis et al., 2015) and peripheral arterial disease (PAD; Kenjale et al., 2011). Indeed, short term (Kapil et al., 2010; Webb et al., 2008; Wylie et al., 2013) NO_3^- supplementation has been found to positively influence BP and these effects have been known to last up to four weeks in healthy (Thompson et al., 2016a; Wylie et al., 2016) and hypertensive (Kapil et al., 2015) persons. More specifically, Webb et al., (2008) reported a reduction in SBP (~ 10 mmHg), DBP (~ 8 mmHg) and mean arterial pressure (MAP; ~ 8 mmHg) approximately 2.5-3 h post 500 mL of beetroot juice (~ 22.5 mmol of NO_3^-) consumption. These reductions are similar to those observed after anti-hypertensive medication and are likely important as it has been suggested that a decrease of just 5 mmHg in SBP could lower the risk of stroke- and heart disease- related mortality by 14 and 9 %, respectively (Stamler et al., 1991). Even a 2 mmHg reduction in SBP, if sustained, has been suggested to lower stroke mortality by 10 % and cardiovascular disease mortality by 7 % (Lewington et al., 2002).

Both Wylie et al. (2013) and Kapil et al. (2010) reported a dose-dependent increase in plasma [NO_2^-] and reduction in BP after different quantities of NO_3^- were ingested. Alterations in BP have typically been reported to have returned to pre-supplementation baseline values by 24 h (Webb et al., 2008; Kapil et al., 2010), but this has not always been the case (Wylie et al., 2013).

A meta-regression by Siervo and colleagues (2013) indicated that greater reductions in SBP were associated with a higher daily dose of dietary NO_3^- (in the form of both salts and beetroot juice), but not increased study duration or plasma [NO_2^-]. However, a more recent systematic review reported reductions in SBP and DBP following beetroot juice ingestion, with larger changes in SBP noted following interventions with higher doses of NO_3^- and when supplementation was continued for more than 14 days (Bahadoran et al., 2017). Another review by Jackson et al. (2018) showed that KNO_3, beetroot juice and adoption of a high NO_3^- diet (e.g. ingestion of green leafy vegetables) was associated with reductions in BP, with more pronounced effects noted in healthy individuals compared with patient participants. In addition, BP reduction was greater following acute supplementation (< 24 h) compared with longer interventions (> 24 h) and higher doses of NO_3^- were associated with larger reductions in SBP but not DBP. Overall, further work is required to confirm the effects of inorganic NO_3^- on BP.

The reduction of NO_3^- to NO is suggested to be accountable for the reduction in BP following NO_3^- supplementation (Ignarro et al., 1987), although it must be noted that NO_2^- itself may also produce direct vasodilatory effects on the vasculature (Alzawahra et al., 2008; Demoncheaux et al., 2002; Pinder et al., 2009), but such findings remain equivocal and further work is required to confirm the role of NO_2^- as a direct vasodilator in humans (Lauer et al., 2001). An elevated level of intracellular NO is known to encourage the binding of NO with guanylate cyclase (Ignarro et al., 1986), which, in turn, stimulates the release of cyclic guanosine monophosphate (cGMP). An increase in cGMP activates a number of protein kinases to phosphorylate substrate proteins, which, subsequently, reduces intracellular calcium concentration ($[Ca^{2+}]$) and leads to smooth muscle relaxation, lowering BP (Lohmann et al., 1997). Therefore, it may be suggested that dietary NO_3^- ingestion has the potential to treat and/or prevent hypertension and associated vascular diseases.

However, it must be stated that NO_3^- supplementation does not always lower BP (Beijers et al., 2017; Cermak, Gibala & van Loon, 2012; Larsen et al., 2010; Wilkerson et al., 2012). A number of factors other than health status, and in particular, lifestyle choices, such as the type of food and drink one might ingest during a typical day, may influence the BP response to dietary NO_3^- consumption. Kapil et al. (2010) reported a negative correlation between baseline BP and the BP response to NO_3^- supplementation i.e. the lower the baseline BP, the smaller the peak BP reduction achieved. It has also been reported that the frequent use of a chlorhexidine-containing mouthwash can abolish the beneficial effects on BP afforded by NO_3^- ingestion (Kapil et al., 2013; Petersson et al., 2009; Woessner et al., 2016). The presence of dietary components, such as polyphenols and vitamin C, which are found in fruit and vegetables, are likely to promote the production of NO from NO_2^- in the stomach (Gago et al., 2007; Lundberg et al., 2010) and the consumption of alcohol may also increase NO storage pools (Rocha et al., 2015) and subsequently lead to vasodilation (Gago et al., 2008). Nowadays, a number of different NO_3^--rich products are on the market but the impact of such products on resting BP has not been determined. Future studies that characterise the BP response and metabolism of NO_3^- and NO_2^- following dietary NO_3^- ingestion alongside factors (for example, mouthwash use, alcohol consumption and the type of NO_3^- food form ingested) that might impact its effectiveness on these parameters, will provide important information for the regular use of NO_3^- as a potentially therapeutic aid amongst habitual practices.

Oxygen cost of submaximal exercise

In recent years, dietary NO_3^- supplementation has been found to positively affect the physiological response to exercise (Bailey et al., 2009; Lansley et al., 2011; Larsen et al., 2007). Larsen and colleagues (2007) were the first to report a reduction (~ 5 %) in the O_2 cost of submaximal exercise in well trained athletes after 3 days of $NaNO_3$ ingestion (0.1 $mmol \cdot kg^{-1} \cdot d^{-1}$). This decrease in O_2 consumption ($\dot{V}O_2$) was evident in the presence of an increase in plasma [NO_2^-] (by ~ 82 %) and in the absence of detectable changes in ventilation, blood lactate concentration and respiratory exchange ratio (RER), suggesting an improvement in muscle oxidative metabolic efficiency rather than alterations in the O_2 cost of cardiopulmonary processes, substrate utilization or increases in energy supply from non-oxidative sources (Larsen et al., 2007).

In 2009, Bailey et al. also found a reduction in $\dot{V}O_2$ (~ 5 %) during moderate-intensity constant work rate (CWR) cycling after 4-6 days of supplementation with a natural NO_3^- source, beetroot juice (0.5 $L \cdot d^{-1}$, containing 5.5 $mmol \cdot d^{-1}$ of NO_3^-). Many studies have since reported similar reductions in the O_2 cost of exercise in various modalities following up to six days of NO_3^- supplementation (Bailey et al., 2010; Cermak et al., 2012; Lansley et al., 2011; Muggeridge et al., 2013; Whitfield et al., 2016; Wylie et al., 2016). Decreases in $\dot{V}O_2$ during moderate-intensity exercise have been reported to be dose-dependent (Wylie et al., 2013) and can occur just 1-3 h after acute bolus consumption of NO_3^- salts (~ 0.033 $mmol \cdot kg^{-1}$ of body mass; Larsen et al., 2010) and beetroot juice (~5-8 mmol; Vanhatalo et al., 2010; Muggeridge et al., 2013; 2014; Thompson et al., 2014) and these effects may still be evident if daily supplementation with concentrated beetroot juice (BR) is continued for four weeks (~13 $mmol \cdot d^{-1}$; Thompson et al., 2016a). Acute (Masschelein et al., 2012) and chronic (Kelly et al., 2014; Muggeridge et al., 2014) beetroot juice ingestion has also been found to decrease steady state $\dot{V}O_2$ in hypoxia. However, it is unknown whether similar effects are present when O_2-carrying capacity is limited, such as after whole blood withdrawal.

Lowering the O_2 cost of exercise may improve functional capacity and the quality of life of older persons and those with cardiovascular, respiratory or metabolic disease. Vanhatalo and colleagues (2016) recently reported that 10 days of beetroot juice ingestion reduced the O_2 cost of treadmill walking in healthy older persons (70-80 years of age). In addition, a lowered $\dot{V}O_2$ during submaximal cycling has also been found in individuals with COPD (Curtis et al., 2015) and during a cardiopulmonary treadmill test in patients with PAD (Kenjale et al., 2011). A number of studies, however, have not found any influence of beetroot juice ingestion on the O_2 cost of exercise (Breese et al., 2013; Kelly et al., 2013; Thompson et al., 2015), particularly in highly trained cohorts (Bescós et al., 2011; Christensen et al., 2013; Peacock et al., 2012; Boorsma et al., 2014; Sandbakk et al., 2015) and in persons with type 2 diabetes (Shepherd et al., 2015), COPD (Berry et al., 2015; Shepherd et al., 2015) and in those with heart failure with reduced (Coggan et al., 2018; Hirai et al., 2017) or preserved (Zamani et al., 2015) ejection fraction. A recent systematic review reported that dietary NO_3^- can indeed reduce the O_2 cost of moderate- and even heavy- intensity exercise in healthy subjects, but such effects were not evident in individuals with chronic disease (Pawlak-Chaouch et al., 2016). The reason for such disparity in findings is not known but the timing of NO_3^- ingestion, type of supplement used and the training/health status and habitual actions or daily lifestyle choices (e.g. mouthwash use and diet) of the subjects under investigation may account for these discrepancies.

The mechanistic bases for a lower O_2 cost of submaximal exercise following NO_3^- supplementation may include a reduction in the adenosine triphosphate (ATP) cost of muscle force production (Bailey et al., 2010), a reduction in the O_2 cost of mitochondrial resynthesis (Larsen et al., 2011) and/or alterations in redox signalling (Whitfield et al., 2016), but further work is required to confirm such mechanisms.

Exercise tolerance and performance

NO_3^- supplementation and the subsequent rise in plasma [NO_2^-] has been correlated with exercise tolerance and/or improvements in performance in healthy (Dreissigacker et al., 2010; Rassaf et al., 2007) and trained (Lansley et al., 2011a) individuals. More specifically, tolerance to severe-intensity [70 % Δ; 70 % of the difference between the power output at the gas exchange threshold (GET) and $\dot{V}O_{2peak}$ (peak O_2 uptake)] CWR cycling improved by 16 % after 6 days of beetroot juice supplementation (5.5 mmol of NO_3^- per day; Bailey et al., 2009). Many authors have since investigated the influence of dietary NO_3^- (5-10 mmol per day for 5-11 days) on exercise tolerance and improvements in time to exhaustion (TTE) achieved during two-legged knee extensor exercise (25 %; Bailey et al., 2010), treadmill running (15 %; Lansley et al., 2011) and severe-intensity cycling (12-21 %; Breese et al., 2013; Kelly et al., 2013; Thompson et al., 2014) have been reported.

Beetroot juice ingestion has been found to improve incremental exercise tolerance during single-legged knee-extensor exercise (Lansley et al., 2011) and cycling (Vanhatalo et al., 2010) in normoxia, hypoxia (+ 5 %; Masschelein et al., 2012) and in those with PAD (+ 17 %; Kenjale et al., 2011). Muggeridge et al. (2014) have also reported that a single dose of BR, ingested 3 h prior to exercise at a simulated altitude of 2500 m, resulted in improvements in 16.1 km cycling time trial performance.

Suggested mechanisms for improvements in exercise performance and tolerance to high-intensity exercise following dietary NO_3^- supplementation may include reductions in muscle metabolic perturbation (Bailey et al., 2010; Vanhatalo et al., 2011) and increases in skeletal muscle Ca^{2+} handling proteins (Hernández et al., 2012) and blood flow particularly in type II fibres (Ferguson et al., 2013; 2015).

A number of studies have, however, reported no improvement in long duration running or cycling performance after acute beetroot juice ingestion, particularly in those who are well trained (Cermak et al., 2012a; Peacock et al., 2012; Wilkerson et al., 2012). This may be due to higher baseline plasma [NO_2^-] due to increased NOS activity (Wilkerson et al., 2012) and an increased capillary density (achieved via training) which is likely to reduce the development of a hypoxic environment in the working skeletal muscle (Jensen, Bangsbo & Hellsten, 2004). There is also evidence to suggest that plasma [NO_2^-] may be reduced during exercise and therefore, the potential for beetroot supplementation to enhance performance reduces as the event continues (Wylie et al., 2013a; Thompson et al., 2015). In addition, NO_3^- supplementation has been found to enhance muscle blood flow, particularly to type II muscle fibres (Ferguson et al., 2013; Hernández et al., 2012) and therefore the effectiveness of NO_3^- ingestion may be attenuated in persons with a lower proportion of these ‘fast-twitch’ fibres, i.e. endurance trained athletes.

A systematic review reported that inorganic dietary NO_3^- ingestion is likely to elicit improvements in exercise tolerance when measured as time to exhaustion achieved during an exercise capacity test, but less likely when measured as performance in a time trial in healthy adults (McMahon et al., 2017). Other reviews have also reported improvements in time to exhaustion following NO_3^- supplementation in older adults (Stanaway et al., 2017) and in athletes (Domínguez et al., 2017).

Differences in supplementation regimes (dose, duration and type of supplement administered), exercise duration and modality and the subject population (recreationally active versus highly trained) may all be factors contributing to intra-study variations and therefore must be considered when investigating the efficacy of dietary NO_3^- ingestion on exercise performance.

The improvements in exercise tolerance following beetroot juice ingestion are more pronounced in hypoxia than normoxia (Kelly et al., 2014). The amelioration of the negative effects of a reduced O_2 availability on O_2 transport and exercise performance after NO_3^- ingestion is important as it may reflect the cardiovascular insufficiency that can be present in conditions, such as PAD, that limit skeletal muscle oxygenation, particularly during exercise (Kenjale et al., 2011). However, the influence of dietary NO_3^- on incremental exercise performance when normal O_2 carrying capacity is reduced, such as in anaemia or after blood donation, has not yet been determined and warrants further investigation.

Factors influencing the effects of dietary NO_3^- supplementation

It is evident that dietary NO_3^- ingestion can elevate markers of NO and elicit favourable effects on cardiovascular health and exercise performance. However, the impact of some daily lifestyle choices and habitual practices can play an important role on the effectiveness of the physiological response to NO_3^- supplementation. Specifically, a paucity of data exist regarding the influence of mouthwash use, blood donation, different NO_3^- food forms and alcohol consumption (particularly red wine) on the ability of dietary NO_3^- to induce cardiovascular benefits.

Mouthwash use

Dietary NO_3^- can elevate precursors of NO, such as NO_2^-, in biological fluids, which is often associated with reductions in systemic BP. It is important to note that circulating NO_2^-, a major storage pool of NO (Cosby et al., 2003), is dependent on the presence and activity of NO_3^- reducing oral bacteria. Therefore, a disturbance in the oral flora may reduce the availability of plasma [NO_2^-] and the possibility of beneficial effects on the vasculature occurring.

Recently, it has been shown that the use of a chlorhexidine-containing mouthwash, which may be considered as part of a daily routine by some persons, can reduce the number of bacteria on the tongue and NO gas produced in the stomach and also attenuate the rise in both salivary and plasma [NO_2^-] after an acute dose (10 $mg{\cdot}kg^{-1}$ in humans; Govoni et al., 2008) or more prolonged ingestion (140 $mg{\cdot}kg^{-1}$ per day, for 5 days, in rats; Petersson et al., 2009) of $NaNO_3$. It is also important to note that the decrease in MAP and DBP recorded after 5 days of NO_3^- ingestion in rats, was abolished after the oral cavity was sprayed twice daily with antibacterial mouthwash (Petersson et al., 2009).

In 2013, Kapil and colleagues showed in healthy volunteers that mouth-rinsing twice daily for 7 days with chlorhexidine-containing mouthwash alongside a low-NO_3^- diet reduced salivary and plasma [NO_2^-] by 90 and 25 %, respectively. The attenuation in these NO metabolites was accompanied by an elevation in seated and ambulatory SBP and DBP. However, the influence of different strength antibacterial mouthwashes on resting and exercise BP, following prolonged natural NO_3^- ingestion has not yet been determined. This information may raise awareness of the influence of commercially available mouthwashes on the ability of NO_3^- supplementation to alter parameters of vascular health, such as BP.

Blood donation

Supplementing the diet with different NO_3^--rich products or increasing the consumption of green leafy vegetables can increase NO bioavailability via the NO_3^--NO_2^--NO pathway. This means of enhancing NO storage pools may be particularly important when NOS activity is compromised (Lundberg et al., 2008) and O_2 availability is reduced (Castello et al., 2006). It is well known that when O_2 transport is limited, by, for example, disease, blood donation or challenging atmospheric conditions, tissue hypoxia may occur (Linarsson et al., 1974) and exercise tolerance is likely to deteriorate (Hogan et al., 1999; Kelly et al., 2014). However, there is evidence to suggest that NO_3^- supplementation can offset performance decrements due to compromised O_2 availability via increases in skeletal muscle blood flow (Casey et al., 2010). In fact, NO_3^--rich beetroot juice has been found to improve muscle oxygenation during incremental exercise (Masschelein et al., 2012) and offset reductions in tolerance to CWR high-intensity knee extensor exercise in hypoxia (Vanhatalo et al., 2011). However, it is unknown whether beetroot juice ingestion can alter the haemodynamic and physiological response to exercise in those persons who choose to donate whole blood and subsequently reduce their O_2 carrying capacity. The results of such research may have important implications for persons with anaemia, those recovering from blood loss and for recreationally active individuals wishing to donate blood.

Food forms

Athletes, coaches and medical professionals are interested in new ways to improve performance and/or cardiovascular health. In addition to seeking out new training and recovery regimens, nutritional interventions are widely utilised among elite and recreationally active individuals. Dietary NO_3^- ingestion, in the form of beetroot juice and NO_3^- salts, has become one of the most popular ergogenic aids in recent times, and there are now a number of different commercially available NO_3^--rich supplements.

However, a number of questions remain unanswered with regard to the NO_3^- food form which may optimise vascular health and performance. Additional research is necessary to allow the development of supplementation guidelines for the use of NO_3^- as a therapeutic and ergogenic aid.

To date, a number of studies have investigated the impact of dietary NO_3^- in various forms, including salts, vegetable juices, gels and bread, on NO bioavailability and BP (Hobbs et al., 2012; Jonvik et al., 2016; Kapil et al., 2010; Muggeridge et al., 2014; Vanhatalo et al., 2010). Whilst NO_3^- supplementation in general may elevate NO bioavailability and often elicit favourable physiological effects, it is evident that the NO_3^- food form (e.g. $NaNO_3$ or beetroot juice) ingested can influence such responses (Flueck et al., 2016; Jonvik et al., 2016; van Velzen et al., 2008). At present, there is no study that has determined the pharmacodynamic pattern of salivary, plasma and urinary $[NO_3^-]$ and $[NO_2^-]$ and BP response to an equimolar dose of NO_3^- administered in different food forms in the same subject cohort over a 24 h period. Further work is necessary to establish the relationship between different NO_3^- vehicles and NO bioavailability in bodily fluids. Establishing the time to peak plasma $[NO_2^-]$ and area under the curve after each NO_3^- source may allow for supplementation regimens to be optimized, particularly when the aim is to sustain a lowered BP over time.

Simultaneous ingestion of salad and alcohol

The beneficial effects on vascular health afforded by the consumption of beetroot juice and/or the adoption of a Mediterranean diet have been chiefly attributed to the high NO_3^- content of the vegetables consumed. It is well established that exogenous NO_3^- is reduced to NO_2^- by bacteria in the oral cavity and once swallowed, this salivary NO_2^- can re-enter the circulation, increasing plasma [NO_2^-]. NO_2^- can also be reduced to NO and other nitrogen oxides, particularly in acidic environments, like the stomach (Benjamin et al., 1994; McKnight et al., 1997), and in the presence of a number of different enzymes and polyphenols (Rocha et al., 2009). NO_3^--rich vegetables and red wine are rich in polyphenols and the acute consumption of both, in isolation (Gago et al., 2007; Webb et al., 2008) and in combination (Rocha et al., 2015), have been reported to promote NO formation. In addition, NO_2^- and alcohol have also been reported to generate ethyl-NO_2^- a potent vasodilator and storage pool of NO. However, the bioavailability of NO in different biological fluids and the BP response to the combination of a vegetable based NO_3^--rich meal and polyphenol-rich red wine and polyphenol-low alcoholic beverage is not known, nor is the magnitude of or time to peak salivary, plasma and urinary [NO_3^-] and [NO_2^-]. This warrants investigation as the consumption of a green leafy salad may serve as a practical and low cost way of acutely improving indices of vascular health. It is also important to determine if there is an additive effect of alcohol, particularly red wine, if consumed in moderation, as part of a typical Mediterranean meal on BP.

Summary

Acute and chronic NO_3^- supplementation has been shown to increase markers of NO bioavailability, lower BP, reduce the O_2 cost of moderate-intensity exercise and improve tolerance to high-intensity exercise. However, it is evident that some factors must be considered carefully when the overall aim is to elicit cardiovascular and exercise-related benefits from NO_3^- consumption. Therefore, elucidating the optimal NO_3^- supplementation regimen for vascular and performance benefits would require determination of the influence of factors, such as: 1) the use of different strength mouthwashes; 2) blood donation; 3) NO_3^- food forms; and 4) polyphenol-rich (red wine) and a low polyphenol alcoholic beverage in combination with a typical Mediterranean salad.

Aims and hypotheses

The aim of this thesis is to provide novel insight into the use of dietary NO_3^- supplementation as a potential therapeutic aid in the face of factors that might influence its effectiveness. The experimental chapters will address the following research questions in young, healthy and normotensive persons:

1) What are the effects of prolonged use of different strength antibacterial mouthwashes, in combination with NO_3^- supplementation, on NO metabolites and resting and exercise BP?
    - *Hypothesis:* NO_3^--rich concentrated beetroot juice supplementation preceded by chlorhexidine-containing mouthwash will significantly attenuate the rise in plasma [NO_2^-] and the expected reductions in BP at rest and during exercise compared with non-chlorhexidine-containing mouthwash and a water control mouthwash. It is also expected that the non-chlorhexidine-containing mouthwash will not significantly alter the variables under investigation when compared with control.

2) What are the effects of short-term NO_3^- ingestion on moderate-intensity and ramp incremental exercise performance following voluntary blood donation?

   - *Hypothesis:* NO_3^--rich concentrated beetroot juice will significantly lower the O_2 cost of moderate-intensity exercise, improve muscle oxygenation status and attenuate the expected reduction in exercise tolerance during ramp incremental exercise following whole blood donation.

3) What are the pharmacokinetic responses of salivary, plasma and urinary [NO_3^-] and [NO_2^-] and BP following consumption of different NO_3^- food forms?

   - *Hypothesis:* Relative to baseline and the control condition, concentrated beetroot juice, non-concentrated beetroot juice, beetroot flapjack and beetroot crystals will all significantly elevate salivary, plasma and urinary [NO_3^-] and [NO_2^-] and reduce BP, but such changes may vary in magnitude and time to peak between the different food forms.

4) What are the pharmacokinetic responses of salivary, plasma and urinary [NO_3^-] and [NO_2^-] and BP following polyphenol-rich and -low alcoholic beverages in combination with a NO_3^--rich meal?

   - *Hypothesis:* Relative to baseline and a low NO_3^- meal with a water control drink, a high NO_3^- meal in combination with red wine, vodka and lemonade or water, will significantly increase salivary, plasma and urinary [NO_3^-] and [NO_2^-] and reduce BP. It was also hypothesised that the NO_3^- and red wine condition will significantly lower BP when compared to the other high NO_3^- conditions due to the increased polyphenol content.

Chapter 3: General Methods

The four experimental chapters (Chapters 4-7) in this thesis required 286 visits to the laboratory by the subjects under investigation, all of which were conducted by the chief researcher (Sinead McDonagh). A further 175 laboratory visits were undertaken by the chief researcher for the determination of salivary, plasma and urinary [NO_3^-] and [NO_2^-]. All of the tests in Chapters 4, 5, 6 and 7 were conducted in an air conditioned physiology laboratory at sea level and at an ambient temperature (between 18-22 °C). Prior to the commencement of data collection, all experimental procedures employed during each experimental chapter were approved by the University of Exeter Ethics Committee.

Health and Safety

The University of Exeter School of Sport and Health Sciences health and safety guidelines were followed during all experimental testing. Specifically, care was taken to ensure that all preparation and data collection areas were clean and safe and provided a suitable environment for the assessment of human subjects. This was achieved by regularly cleaning work surfaces, ergometers, blood pressure cuffs and floors using a dilute Virkon disinfectant. All respiratory apparatus were disinfected after each use in line with the manufacturers' guidelines. During blood sampling and analysis, disposable nitrile gloves were worn by the experimenter and all biohazard material and sharps were disposed into appropriate bins and incinerated at a later date.

Informed Consent

Prior to participation in a study, subjects were given an information sheet to read which included a comprehensive outline of the experimental procedures and the requirements of the tasks to be undertaken. Potential risks and benefits of participation were explained and subjects were informed that whilst their anonymity would be preserved, the results from the study may be published in academic journals and presented at conferences. All participants were free to ask any questions regarding the study procedures and were ensured that they had the right to withdraw from the study at any time, with no disadvantage to themselves. After a minimum of 24 h and when participants were content that they had read and understood the requirements of the study, written informed consent to participate in the study was provided.

Participants

All volunteers for each of the four experiments were recruited from the staff and student population at the University of Exeter. Subjects were healthy, non-smokers, free from disease and did not habitually use antibacterial mouthwash during data collection periods. In Chapter 5, subjects were recreationally active individuals who participated in structured exercise and/or competitive sport regularly. In Chapters 6 and 7, all subjects were instructed to arrive at the laboratory in a fasted and well-hydrated state. Participation in strenuous exercise and alcohol intake were avoided during each experimental period (unless instructed by the researcher) and in the 24 h preceding each visit to the laboratory. Subjects were also asked to refrain from consuming caffeine in the 3 h period prior to each testing session. All tests were conducted at the same time of day (± 2 h) to minimise the effects of diurnal variation on the physiological variables under investigation.

Supplementation

NO_3^- supplementation was administered in many different forms across the Experimental Chapters. The [NO_3^-] and [NO_2^-] of all supplements were determined via chemiluminescence prior to commencement of each study.

Preparation of food sources for determination of NO_3^- and NO_2^- content

Neat 50 µL samples of NO_3^--rich concentrated beetroot juice shots (Chapters 4, 5 and 6), NO_3^--depleted concentrated beetroot juice shots (Chapter 5), non-concentrated beetroot juice, beetroot crystals (Chapter 6), deionised water (Chapters 6 and 7), red wine, lemonade and vodka (Chapter 7 only) were injected into the purge vessel for determination of NO_3^- and NO_2^- content via chemiluminescence (as described in detail later in this chapter). In addition, in Chapters 6 and 7, prior to analysis of the NO_3^- and NO_2^- content of each food form, a 1.5 mL aliquot of each homogenised vegetable (rocket, spinach, green beans and cucumber), fruit (cherry tomatoes) or flapjack was transferred to a heat-resistant microcentrifuge tube. Samples were then heated to 130 °C for 60 min using a heat plate (Grant QBD2, Cambridge, UK) to disintegrate the cell membranes for release of intracellular NO_3^-. Subsequently, samples were centrifuged at 12 000 g and 4 °C for 8 min and the supernatant was removed for analysis via chemiluminescence and diluted with deionised water, where appropriate.

A brief description of each supplementation regimen is provided below.

Supplementation regimens

In Chapters 4 and 5, inorganic NO_3^- was consumed in the form of a concentrated beetroot juice containing ~ 6.2 mmol of NO_3^- (BR; Beet It Sport Stamina Shot, James White Drinks, Ltd., Ipswich, UK). In Chapter 4, subjects were assigned in a double blind, randomised crossover design to mouth-rinse with 3 different strength mouthwashes prior to consuming a 70 mL shot of BR, twice per day, for 6 days. More specifically, subjects were instructed to consume 70 mL of BR in the morning and 70 mL in the evening for 5 days. On day 6, subjects consumed 2 x 70 mL of BR, 2 h before returning to the laboratory for post intervention measures. A minimum of a 10 day washout period separated each of the 3 supplementation periods in this study.

In Chapter 5, after blood donation, subjects were randomly assigned in a double-blind, independent groups, placebo-controlled fashion to consume 7 x 70 mL shots of NO_3^--rich BR or NO_3^--depleted beetroot juice (PL; containing ~0.04 mmol of NO_3^-) over ~ 48 h. During both supplementation periods, subjects were asked to consume 2 x 70 mL of the assigned beverage in the evening 2 days prior to testing, 1 x 70 mL in the morning and again in the evening 1 day prior to testing and a further 2 x 70 mL 2 h prior to testing. On arrival at the laboratory, subjects also consumed a final 70 mL beverage. Each supplementation period was separated by a minimum of 8 days. It is important to note here that the PL drink was similar in appearance, taste and smell and was created by passing the juice, before pasteurisation, through a column containing Purolite A520E ion exchange resin, which selectively removes NO_3^- ions.

Single-blind (personnel performing the physiological measurements were blind), randomised, controlled trials were undertaken in Chapters 6 and 7 with a minimum of 48 h separating each experimental period. In Chapter 6, an acute dose of ~ 5.76 mmol of NO_3^-, in the form of a concentrated beetroot drink (BR; 55 mL of Beet It Sport Stamina

Shot, James White Drinks, Ltd., Ipswich, UK), a non-concentrated beetroot drink (BL; 456 mL of Beet It Organic Beetroot Juice, James White Drinks, Ltd., Ipswich, UK) and a beetroot flapjack (BF; 60g of Beet It Pro Elite Sport Flapjack, James White Drinks, Ltd., Ipswich, UK) were ingested. Subjects also consumed (5g dissolved in 4 oz./114 mL of water; 1.40 mmol of NO_3^-) Concentrated Organic Beetroot Crystals (BC; SuperBeets Canister; Neogenis, now known as HumanN, Texas, US) and a control drink (CON; 70 mL deionised water) which contained negligible NO_3^- and NO_2^- content.

In Chapter 7, subjects were instructed to consume an acute dose of ~ 6.05 mmol of dietary NO_3^- in the form of a green leafy salad (50 g rocket, 88 g spinach and 160 g cucumber) in combination with either a polyphenol-rich red wine [NIT-RW; Montepulciano d'Abruzzo, Tesco Stores Ltd, Welwyn Garden City, U. K; 12.5 % alcohol by volume (ABV)], a low polyphenol alcoholic beverage (NIT-A; 58 mL Red Label Smirnoff Vodka, The Smirnoff Co., London, U.K; 117 mL Tesco Sparkling Lemonade, Tesco Stores Ltd, Welwyn Garden City, U. K; 12.5 % ABV) or a control drink (NIT-CON; 175 mL deionised water). Subjects were also asked to consume a low NO_3^- salad (55 g cucumber, 68 g green beans and 200 g cherry tomatoes; 0.69 mmol NO_3^-) with a control drink (CON; 175 mL deionised water).

In Chapters 4-6, subjects were advised that ingestion of the BR (and BL in Chapter 6) supplement might result in the temporary appearance of red urine (beeturia) and stools. All supplementation regimens used in Chapters 4-7 were well tolerated by all subjects with no adverse effects reported.

Measurement Procedures

Prior to any supplementation or exercise testing, subjects' height, mass and age were recorded.

Blood Pressure

In Experimental Chapters 4, 5, 6 and 7, BP of the brachial artery was measured using an automated sphygmomanometer (Dinamap Pro; GE Medical Systems, Tampa, FL) after a period of rest. Specifically, subjects were seated in a quiet room, and after 10 minutes of solitary rest, five BP measurements were recorded and the mean of the final four measurements were used for data analysis. In Chapter 4, BP was also measured after 10 minutes of supine rest using the automated device, and a manual sphygmomanometer (Accoson, Blood Pressure Cuff, Essex, England) and stethoscope (Spirit Dual Head Stethoscope CK-605T, Spirit Medical Co. Ltd., New Taipei City, Taiwan) were used to determine BP during treadmill walking (during 4-6 and 8-10 minutes of exercise).

The reliability of measuring SBP was determined by repeating BP assessments of the brachial artery, in 10 subjects, on five separate days. Subjects arrived at the laboratory on each day in a fasted state and were asked to rest, in a seated position, in a quiet room for 10 minutes. BP was measured five times and the mean of the final four SBP measurements was calculated and recorded. The coefficient of variation for the intra-test (using BP measurements taken on the same day) was 1.4 %. The inter-test (using BP measurements taken on different days) coefficient of variation was 1.1 %.

Heart rate

In each experimental chapter, after 10 minutes of rest in a seated (or supine; Chapter 4 only) position, heart rate (HR) was recorded using an automated device. In addition, in Chapter 5, HR was recorded every 5 s during moderate-intensity and incremental exercise using short-range telemetry (Polar RS400, Polar Electro Oy, Kempele, Finland).

Blood sampling

In Chapters 4, 5 and 6 (at 24 h post ingestion of each supplement), blood samples were obtained from the antecubital fossa via venepuncture only. In Chapters 6 and 7, a cannula (Insyte-WTM, Becton-Dickinson, Madrid, Spain) was inserted into the antecubital vein of each subject to allow regular blood sampling during each experimental period. The cannula was infused with 0.9 % saline at 10 mL/h to ensure patency. All samples were drawn into 7.5 mL lithium-heparin tubes (Vacutainer, Becton-Dickinson, NJ, USA) and centrifuged for 10 minutes at 3000 *g* and 4 ºC within 2 minutes of collection. The plasma was then extracted, aliquoted into 3 Eppendorf tubes and stored at -80 ºC for later determination of [NO_3^-] and [NO_2^-].

Saliva and urine sampling

In Chapters 4, 6 and 7, saliva samples were collected by expectoration, without stimulation, over a period of 5 minutes, prior to and after NO_3^- supplementation. The saliva samples were subsequently aliquoted into 1.5 mL Eppendorf tubes. In Chapters 6 and 7, midstream urine samples were also collected at baseline and at a number of time points after NO_3^- ingestion. Each sample was aliquoted into 3 x 1.5 mL Eppendorf tubes.

Both the saliva and urine samples were frozen immediately at -80 ºC for later determination of [NO_3^-] and [NO_2^-] via chemiluminescence.

NO_3^- and NO_2^- concentration

Plasma (Chapters 4-7), saliva (Chapters 4, 6 and 7) and urine (Chapters 6 and 7) samples were analysed for [NO_3^-] and [NO_2^-] using gas-phase chemiluminescence. This process is dependent on the reduction of NO_3^- and NO_2^- to NO gas. Prior to and during analyses, all glassware, utensils and surfaces were regularly rinsed with deionized water to remove residual NO_2^-.

Before the determination of plasma [NO_3^-], the neat plasma was first deproteinized. In Chapters 4-6, 400 µL of zinc sulphate ($ZnSO_4$; 10 % w/v) and 400 µL of sodium hydroxide (NaOH; 0.5 M) were added to 200 µL of plasma and vortexed for 30 s and then left to stand at room temperature for 15 minutes. Subsequently, the plasma samples were centrifuged for 5 minutes at 4000 rpm and the supernatant was removed for analysis of NO_3^-. In Chapter 7, plasma samples were deproteinized for both [NO_3^-] and [NO_2^-] (to prevent protein deposit build up and damage to the NO analyser) using a cold ethanol precipitation technique. Specifically, 1000 µL of ethanol (at 0 ºC) were added to 500 µL of plasma and vortexed for 30 s before being left to stand on ice for 30 minutes. Subsequently, samples were centrifuged at 18 600 *g* for 5 minutes. The supernatant was removed and used for analysis. For [NO_3^-], the supernatant was injected into a gas sealed purge vessel containing vanadium trichloride (VCl_3; 0.8 % w/v) in hydrochloric acid (HCl; 1M) at 95 ºC. An additional gas bubbler containing NaOH (1 M) was installed to prevent damage to the nitric oxide analyser from acid vapour.

For NO_2^- reduction, untreated (Chapters 4-6) and deproteinized (Chapter 7) plasma was injected into the glass purge vessel, which contained 5 mL glacial acetic acid and 1 mL sodium iodide (NaI; 4 % w/v) at 35 ºC.

In Chapters 4, 6 and 7, saliva and urine samples were centrifuged for 10 minutes at 18 600 *g* prior to analysis. The supernatants from all saliva samples were diluted with deionised water by a factor of 100 prior to determination of [NO_3^-] and [NO_2^-]. Urine samples were also diluted using deionised water, by a factor of 100 (Chapter 6: [NO_3^-] only; Chapter 7: [NO_2^-] only) and 1000 (Chapter 7: [NO_3^-] only). The same analysis technique as stated for plasma (above) was employed for saliva and urine samples.

The NO produced from the reduction of NO_3^- and NO_2^- was measured using a NO analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK). The reaction of NO with ozone in the chemiluminescent chamber yielded nitrogen dioxide, which on production, emits a light at the infra-red region on the electromagnetic spectrum. This light was detected by a red-sensitive photomultiplier tube, housed in the NO analyser, and was amplified to produce an analogue mV output signal. [NO_3^-] and [NO_2^-] were determined by plotting the signal area against a calibration plot of 25 nM to 1 μM sodium NO_2^- ($NaNO_2$) and 100 nM to 10 uM $NaNO_3$, respectively. The coefficients of variation for duplicate samples for [NO_3^-] and [NO_2^-] after a NO_3^- dose and using the above techniques were 0.49 and 3.87 % for plasma, 0.33 and 0.66 % for saliva and 0.00 and 0.23 % for urine, respectively.

Exercise testing procedures

Chapter 5 includes a detailed description of the exercise tests undertaken in this thesis. In addition, the reliability of pulmonary gas exchange measurements was established using bouts of moderate-intensity exercise performed on two separate days in 11 participants.

On each visit, participants completed a constant work step test, consisting of a 3 minute baseline cycling period at 20 W, followed by a sudden transition to a work rate eliciting 80 % of the work-rate associated with the GET for 5 minutes, with each bout separated by 10 minutes of passive rest. Participants were asked to cycle at the same preferred cadence (~ 80 rpm) on each visit. The coefficient of variation for steady state $\dot{V}O_2$ was 1.3 % at an absolute power output of 90 W.

Statistical analyses

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS). More specific details of the statistical procedures performed in each experiment are outlined in the 'Statistical Analyses' section of Chapters 4-7. Data are presented as mean ± SD unless otherwise stated. Statistical significance was accepted at $P < 0.05$.

The effects of chronic nitrate supplementation and the use of strong and weak antibacterial agents on plasma nitrite concentration and exercise blood pressure

Original Article

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Chapter 4: The effects of chronic nitrate supplementation and the use of strong and weak antibacterial agents on plasma nitrite concentration and exercise blood pressure

Abstract

Chlorhexidine containing mouthwash (STRONG), which disturbs oral microflora, has been shown to diminish the rise in plasma nitrite concentration ($[NO_2^-]$) and attenuate the reduction in resting blood pressure (BP) typically seen after acute nitrate (NO_3^-) ingestion. We aimed to determine whether STRONG and weaker antiseptic agents attenuate the physiological effects of chronic NO_3^- supplementation using beetroot juice (BR). Twelve healthy volunteers mouth rinsed with STRONG, non-chlorhexidine mouthwash (WEAK) and deionised water (CON) three times a day, and ingested 70 mL BR (6.2 mmol NO_3^-), twice a day, for 6 days. BP (at rest and during 10 min of treadmill walking) and plasma and salivary $[NO_3^-]$ and $[NO_2^-]$ were measured prior to and on day 6 of supplementation. The change in salivary $[NO_3^-]$ 4 h post final ingestion was higher ($P < 0.05$) in STRONG (8.7 ± 3.0 mM) compared to CON (6.3 ± 0.9 mM) and WEAK (6.0 ± 3.0 mM). In addition, the rise in plasma $[NO_2^-]$ at 2 h was lower in STRONG compared with WEAK (by 89 ± 112 nM) and CON (by 200 ± 174 nM) and in WEAK compared with CON (all $P < 0.05$). Changes in resting BP were not different between conditions ($P > 0.05$). However, during treadmill walking, the increase in systolic and mean arterial BP was higher 4 h after the final NO_3^- bolus in STRONG compared with CON ($P < 0.05$) but not WEAK. The results indicate that both strong and weak antibacterial agents suppress the rise in plasma $[NO_2^-]$ observed following the consumption of a high NO_3^- diet and the former can influence the BP response during low-intensity exercise.

Key words: beetroot juice; nitrate; antibacterial mouthwash; blood pressure; nitric oxide; exercise

Chapter 4: The effects of chronic nitrate supplementation and the use of strong and weak antibacterial agents on plasma nitrite concentration and exercise blood pressure

Introduction

There is now an abundance of evidence that a diet rich in vegetables, particularly those with a high concentration of inorganic nitrate (NO_3^-), is favourable to cardiovascular health and is associated with longevity [12, 19, 44]. NO_3^-, a relatively inert molecule, can be reduced *in vivo* to nitrite (NO_2^-) and the potent vasodilator and blood flow regulator, nitric oxide (NO) [20, 21, 39]. Dietary supplementation with NO_3^--rich beetroot juice (BR) can significantly increase plasma [45, 47] and salivary NO_2^- concentrations ($[NO_2^-]$) [17] and lead to reductions in systolic (SBP) and diastolic (DBP) blood pressure (BP) [30, 40, 45].

Ingested dietary NO_3^- is rapidly absorbed in the upper gastrointestinal tract with approximately 25% of this circulating NO_3^- being taken up and concentrated by the salivary glands [39]. Mammalian cells lack NO_3^- reductase activity, and the fundamental step in reducing NO_3^- to NO_2^- is facilitated within the saliva by facultative anaerobic bacteria residing on the dorsal surface of the tongue (14, 17]. Once swallowed, some NO_2^- is further reduced to NO in the acidic environment of the stomach [5, 34] but the remainder enters the systemic circulation, increasing plasma $[NO_2^-]$ [33, 45]. The reduction of NO_2^- to NO is promoted by a number of different enzymes and proteins including deoxyhaemoglobin [10] and nitric oxide synthase (NOS) [42]. This NO_3^--NO_2^--NO pathway is believed to be an important alternative to the classic oxygen (O_2)-dependent NOS-linked production of NO, particularly in acidic and hypoxic environments when NO production via the NOS pathway may be compromised [35].

If the oral microflora is disturbed by the use of antibacterial mouthwash, the expected increase in plasma $[NO_2^-]$ and decrease in resting BP following the consumption of

NO_3^- may not manifest [17, 24, 38]. An attenuation in NO_2^- formation was observed in rats that were treated twice daily with a chlorhexidine containing mouthwash, despite consuming NO_3^- supplemented drinking water for one week [38]. Consequently, the lowering of BP expected with NO_3^- ingestion was absent [38]. In healthy humans, the expected rise in salivary [NO_2^-] after the consumption of an acute bolus of dietary NO_3^- was absent and the elevation in plasma [NO_2^-] was reduced by ~ 75 %, following mouthwash administration when compared with the control condition [17]. Also in healthy individuals, mouth rinsing twice daily for 7 days with a commercially available chlorhexidine containing mouthwash resulted in a reduction in salivary and plasma [NO_2^-] of ~ 90 % and ~ 25 %, respectively [24]. This was accompanied by an increase in seated and ambulatory SBP and DBP after only one day’s use of the chlorhexidine containing mouthwash, compared with baseline values [24]. More recently, Bondonno and colleagues [9] reported an increase in salivary [NO_3^-], a reduction in salivary [NO_2^-] and an elevation in SBP in treated hypertensive persons after 3 days of rinsing with an antibacterial wash. Surprisingly, plasma [NO_2^-] was unaffected by the chronic use of an antimicrobial rinse in this population.

Individuals often consume a diet rich in fruit and vegetables, particularly those foodstuffs high in NO_3^-, to protect against cardiovascular [22, 23] and gastrointestinal [15] complications. In addition, NO_3^- is often ingested prior to exercise as it has been reported to improve muscle efficiency [3, 30] and continuous [3, 27] and intermittent [48] exercise performance. However, the effect of prolonged chlorhexidine containing mouthwash alongside ‘natural’ NO_3^- ingestion (i.e., via BR), on BP during low-intensity exercise in a healthy cohort has not been evaluated.

It is important to establish whether regular use of chlorhexidine or weaker antiseptic agents (such as those mouth rinses that do not contain chlorhexidine as the principal antibacterial agent) might attenuate the beneficial effects of NO_3^- achieved via the consumption of BR or other naturally occurring foodstuffs on BP during rest and exercise when NO_3^- supplementation is continued over several days. Therefore, the purpose of this study was to determine the plasma and salivary $[NO_3^-]$ and $[NO_2^-]$ and the haemodynamic response to different types of mouth rinses prior to BR ingestion over 6 days in a healthy population. Specifically, the effects of mouthwashes containing strong (chlorhexidine; STRONG), minimal (no chlorhexidine; WEAK) and no (water; CON) antibacterial properties on indirect measures of oral NO_3^- reductase effectiveness and parameters of vascular health were investigated. It was hypothesised that relative to CON and WEAK, BR supplementation preceded by STRONG mouthwash, would attenuate the rise in plasma $[NO_2^-]$ and the associated reductions in BP at rest and during exercise. It was also hypothesised that, relative to CON, WEAK would not alter the aforementioned variables.

Methods

Subjects

Twelve healthy, recreationally active participants (males, *n* = 6; females, *n* = 6), volunteered to participate in this study (mean ± SD; females: age 22 ± 2 years, body mass 69 ± 8 kg, height 1.74 ± 0.55 m; males: age 24 ± 2 years, body mass 80 ± 7 kg, height 1.82 ± 0.89 m), none of whom habitually smoked tobacco, consumed dietary supplements or used mouthwash. The study was approved by the Institutional Research Ethics Committee.

Prior to testing, and after the requirements of the study, associated risks and potential benefits of participation were explained, written informed consent was obtained. In addition, this study was performed in accordance with the International Journal of Sports Medicine’s ethical standards [18].

Subjects were instructed to arrive at the laboratory in a fully rested and hydrated state, at least 3 h postprandial, and to avoid strenuous exercise in the 24 h preceding each testing session. In addition, subjects were asked to avoid alcohol consumption and chewing gum throughout each supplementation period and to refrain from caffeine intake in the 3 h preceding each laboratory visit. All subjects were also asked not to use antibacterial mouthwash, unless instructed by the researcher, for the duration of the study. Each subject recorded habitual diet and exercise undertaken during the first supplementation period and were asked to replicate these during the second and third supplementation periods. All subjects were fully familiar with laboratory testing procedures having participated previously in similar studies within our laboratory. Exclusion criteria were the presence of known cardiovascular disease and hypertension and the use of antihypertensive medication and antibiotics.

Experimental Overview

Subjects were asked to report to the laboratory on six occasions, over an eight week period. The first visit included baseline measurements of BP (during seated and supine rest and during treadmill walking), heart rate (HR), arterial stiffness, and plasma and salivary [NO_3^-] and [NO_2^-]. Each subject was then randomly assigned, in a double-blind, crossover fashion to mouth rinse three times daily with either a strong, chlorhexidine containing mouthwash (STRONG; Corsodyl®, GlaxoSmithKline, Brentford, England), a weak, non-chlorhexidine containing antibacterial mouthwash (WEAK; Vademecum med®, Schwarzkopf & Henkel, Düsseldorf, Germany), or deionised water (CON). Specifically, subjects were asked to mouth rinse 15 min prior to BR ingestion (as previously described by Govoni et al. [17]), twice a day, and before consuming a lunch time meal, each day, for six days. Subsequent measurements were performed prior to each supplementation period and on day six of each supplementation period. All tests were conducted at the same time of day (± 2 h) to minimise the effects of diurnal variation on the physiological variables under investigation.

Experimental Protocol

During each visit to the laboratory, following 10 min of solitary seated rest in a quiet room, BP of the brachial artery was measured using an automated sphygmomanometer (Dinamap Pro; GE Medical Systems, Tampa, FL, USA). Five measurements were recorded and the mean of the final four measurements was used for data analysis. Five further BP measurements were recorded after 10 min of supine rest. Arterial stiffness was measured three times, using a non-invasive automated pulse-wave velocity (PWV) device (Complior SP; Alam Medical, Vincennes, Paris, France) and the mean of the three values was reported at each time point. Electrodes were placed on the carotid, femoral, and radial arteries, and the pulse transit time was calculated and recorded. The position of each electrode was measured in relation to the nearest bony landmark to allow for precise reproduction of the position of the electrodes in subsequent tests. A resting venous blood sample (~ 4 mL) was then drawn from an antecubital vein into lithium-heparin tubes (Vacutainer, Becton-Dickinson, NJ, USA). The samples were centrifuged for 10 min at 3000 *g* and 4 ˚C, within 2 min of collection, and the plasma was then extracted. A saliva sample (~ 1 mL) was collected by expectoration, without stimulation, over a period of 5 min. Plasma and saliva samples were frozen at -80 ˚C for later determination of [NO_3^-] and [NO_2^-] using a modified chemiluminescence technique as previously described [47]. Immediately prior to analysis, saliva samples were centrifuged for 10 min at 18 600 *g*. The supernatants were then removed and diluted by a factor of 100 with deionised water.

A BP and walking protocol was completed 2 h and 4 h post ingestion of the final bolus of dietary NO_3^- which was preceded by 2 min of mouth rinsing with either STRONG, WEAK or CON. Subjects completed 10 min of treadmill walking at 4 $km \cdot h^{-1}$ (Woodway PPS 55 Sport slat-belt treadmill, Woodway GmbH, Weil am Rhein, Germany). BP of the brachial artery was measured during treadmill walking (during 4-6 and 8-10 min) using a manual sphygmomanometer (Accoson, Blood Pressure Cuff, Essex, England) and stethoscope (Spirit Dual Head Stethoscope CK-605T, Spirit Medical Co. Ltd., New Taipei City, Taiwan). HR was measured using short-range radiotelemetry (Polar S610, Polar Electro Oy, Kempele, Finland).

The duration of each phase of the protocol, during each visit to the laboratory, was ~1 h, with each measurement occurring approximately every 10 min. Therefore, on day 6 of each supplementation period, seated and supine BP and arterial stiffness were measured 2 h 15 min, 2 h 30 min and 2 h 35 min after the final nitrate bolus, respectively. Blood and saliva samples were collected at 2 h 40 min and 2 h 45 min respectively, and exercising BP was measured, at two time points, from 2 h 50 to 3 h post final nitrate ingestion. The same data collection timing protocol was repeated from 4 h post supplementation of nitrate and during each baseline visit to the laboratory.

Supplementation

All 12 subjects completed STRONG, WEAK and CON conditions, with each 6 day supplementation period separated by a minimum of 10 days wash-out. After the initial visit, all subjects were assigned in a double-blind, randomised, crossover design to mouth rinse with a strong antibacterial mouthwash (STRONG; Corsodyl®, containing 0.2 % chlorhexidine digluconate), a weak antibacterial mouthwash [WEAK; Vademecum med®, containing ~ 2.5 mL of Vademecum med® concentrate in 500 mL deionised water (which includes peppermint oil, clove oil, sodium benzoate, chamomile oil, sage, menthol and alcohol)], and a mouthwash containing no antibacterial agents (CON; deionised water), prior to consuming a 70 mL shot of BR (BR; beetroot juice containing ~ 6.2 mmol of NO_3^-, “Beet It Sport Stamina Shot”; James White Drinks Ltd., Ipswich, UK), twice a day, for six days. Subjects were also asked to mouth rinse 15 min prior to consuming a lunch time meal. On the day following pre-supplementation measures (visits 1, 3 and 5), subjects were instructed to consume 70 mL of BR in the morning (~ 10 a.m) and 70 mL in the evening (~ 7 p.m) each day, for five days. On day six (visits 2, 4 and 6), subjects consumed 2 x 70 mL of BR, 2 h prior to returning to the laboratory. Two 10 mL volumes of STRONG, WEAK and CON were each gargled for 1 min (2 min of mouth rinsing overall), 15 min prior to each BR ingestion and the lunch time meal throughout the supplementation period.

Statistical Analyses

Differences in BP, HR, pulse-wave velocity, and plasma and salivary [NO_3^-] and [NO_2^-], were assessed using a two-way (condition x time) repeated-measures ANOVA. Significant main and interaction effects were further explored using simple contrasts via

Fisher's LSD. Statistical analyses were performed using SPSS version 19.0 (Chicago, IL, USA). Data are presented as mean ± SD, unless otherwise stated. Statistical significance was accepted at $P < 0.05$. A statistical trend was defined as $P < 0.10$.

Results

Subjects' self-reported adherence to the BR supplementation protocol, mouth rinsing regimen and avoidance of potential confounding factors (such as the consumption of alcohol) was 100 % for each of three supplementation periods. All subjects reported that their physical activity and dietary patterns were similar throughout each supplementation period. The ingestion of BR was well tolerated with no negative side effects. Subjects did, however, report beeturia (red urine) and red stools.

Plasma [NO_3^-] and [NO_2^-]

The effects of BR supplementation and different mouth rinses on plasma [NO_3^-] and [NO_2^-] are presented (as the change relative to baseline) in Fig. 1. There was a significant main effect by time on plasma [NO_3^-] (Fig. 1A; $P < 0.01$), and a significant main effect by condition and time and an interaction effect on plasma [NO_2^-] (Fig. 1C; all $P < 0.01$).

At baseline, before any mouth rinsing or ingestion of BR, plasma [NO_3^-] was not different between conditions (CON: 17 ± 5; WEAK: 18 ± 10; STRONG: 18 ± 7 µM; $P > 0.05$). Following BR supplementation, plasma [NO_3^-] was elevated above baseline at 2 and 4 h in CON (2 h: 301 ± 56 and 4 h: 250 ± 53 µM), WEAK (2 h: 298 ± 69 and 4 h: 246 ± 50 µM) and STRONG (2 h: 301 ± 102 and 4 h: 243 ± 79 µM) (Fig. 1A; all $P <$

0.05). The increase in plasma [NO_3^-] above baseline was not significantly different between CON, WEAK and STRONG at 2 or 4 h post final BR ingestion (Fig. 1A; all $P > 0.05$).

At baseline, before any mouth rinsing or ingestion of BR, plasma [NO_2^-] was not significantly different between conditions (CON: 70 ± 24; WEAK: 76 ± 31; STRONG: 80 ± 37 nM; $P > 0.05$). Following BR supplementation, plasma [NO_2^-] was significantly increased from baseline at 2 and 4 h in CON (2 h: 380 ± 219 and 4 h: 323 ± 205 nM), WEAK (2 h: 269 ± 186 and 4 h: 278 ± 250 nM) and STRONG (2 h: 180 ± 141 and 4 h: 114 ± 112 nM) (all $P < 0.05$). Follow up tests revealed that at 2 h, the elevation in plasma [NO_2^-] was lower in STRONG compared to CON (by 200 ± 174 nM) and WEAK (by 89 ± 112 nM) (Fig. 1C; both $P < 0.05$). Similarly, the change in plasma [NO_2^-] was lower (by 110 ± 157 nM) in WEAK compared to CON at 2 h (Fig. 1C; $P < 0.05$). At 4 h, plasma [NO_2^-] was higher in CON and WEAK, compared to STRONG (Fig. 1C; $P < 0.01$).

Salivary [NO_3^-] and [NO_2^-]

The effects of BR supplementation and different mouth rinses on salivary [NO_3^-] and [NO_2^-] are presented (as the change relative to baseline) in Fig. 1. There were significant main effects by condition and time for both salivary [NO_3^-] (Fig. 1B; all $P < 0.05$) and [NO_2^-] (Fig. 1D; all $P < 0.05$) and an interaction effect on salivary [NO_2^-]. At resting baseline, before any mouth rinsing or supplementation of BR, salivary [NO_3^-] was not significantly different between conditions (CON: 0.3 ± 0.2; WEAK: 0.2 ± 0.3; STRONG: 0.3 ± 0.3 mM; all $P > 0.05$). Following BR supplementation, salivary [NO_3^-] was significantly elevated above baseline at 2 and 4 h in CON (2 h: 7.8 ± 1.9 mM and 4 h: 6.3 ± 0.9 mM; both $P < 0.05$), WEAK (2 h; 7.4 ± 4.0 and 4 h: 6.0 ± 3.0 mM; both $P < 0.05$) and STRONG (2 h: 10.6 ± 3.6 and 4 h: 8.7 ± 3.0 mM; both $P < 0.05$). At 2 h, the increase in salivary [NO_3^-] relative to baseline, was greater in STRONG compared to CON ($P < 0.05$) but not WEAK ($P > 0.05$). Similarly, at 4 h, the increase in salivary [NO_3^-] relative to baseline, was significantly larger in STRONG compared to CON and WEAK (Fig. 1B; both $P < 0.05$).

At baseline, before any mouth rinsing or supplementation, salivary [NO_2^-] was not significantly different between conditions (CON: 0.2 ± 0.1; WEAK: 0.1 ± 0.1; STRONG: 0.1 ± 0.1 mM; all $P > 0.05$). Following BR supplementation, salivary [NO_2^-] was significantly elevated (Fig. 1D; all $P < 0.05$) above baseline at 2 and 4 h in CON (2 h: 1.4 ± 1.1 and 4 h: 1.3 ± 0.9 mM), WEAK (2 h: 1.2 ± 0.8 and 4 h: 0.9 ± 0.6 mM) and STRONG (2 h: 0.2 ± 0.6 and 4 h: 0.4 ± 1.0 mM). The increase in salivary [NO_2^-] compared to baseline was significantly lower at 2 h in STRONG compared to CON ($P < 0.05$). At 4 h, salivary [NO_2^-] was lower after STRONG compared to CON and WEAK (Fig. 1D; all $P < 0.05$).

Pulse Wave Velocity and Heart Rate

There were no significant main effects by condition or time, and no interaction effect for carotid-femoral and carotid-radial PWV (Table 1; $P > 0.05$). The mean change in HR relative to presupplementation baseline during rest and low-intensity exercise following chronic nitrate supplementation and prior mouth rinsing with STRONG, WEAK and CON are reported in Table 1. There was a main effect by time on HR during 10 min of treadmill walking ($P < 0.05$). Further analyses showed that HR increased significantly from baseline to 4 h in STRONG (*P* < 0.05), but was not altered in WEAK or CON (both *P* < 0.05).

Blood Pressure

Supine BP. The effects of BR supplementation and different mouth rinses on supine SBP, DBP and MAP are presented in Fig. 2. At baseline, the mean supine SBP, DBP and MAP across all conditions was 111 ± 8, 59 ± 5 and 76 ± 6 mmHg, respectively. There was a significant main effect by time (*P* < 0.05) on SBP (Fig. 2A) and MAP (Fig. 2C), but no main effect by condition or interaction effect (*P* > 0.05). Supine SBP tended to increase (by 4 ± 7 mmHg) from 2 h to 4 h in STRONG (Fig. 2A; *P* = 0.07). Supine MAP increased significantly from 2 to 4 h in CON (by 2 ± 3 mmHg) and in STRONG (by 2 ± 4 mmHg), (*P* < 0.05). No significant main effects by condition or time, or an interaction effect were present for supine DBP (all *P* > 0.05).

Seated BP. The effect of BR supplementation and different mouth rinses on seated SBP, DBP and MAP are presented in Fig. 2. At baseline, the mean seated SBP, DBP and MAP across all conditions was 113 ± 8, 62 ± 5 and 79 ± 6 mmHg, respectively. There were no significant main effects by condition or time, and no interaction effect for SBP (all *P* > 0.05). There was, however, a significant main effect by time on seated DBP (Fig. 2E) and MAP (Fig 2F; *P* < 0.05). In the CON condition seated DBP was higher, relative to baseline, at 4 h (by 3 ± 4 mmHg) compared to 2 h (Fig. 2E; *P* < 0.05). Seated DBP in WEAK was significantly reduced (by 2 ± 3 mmHg) at 2 h compared to baseline (Fig. 2E; *P* < 0.05). In addition, the reduction in seated DBP in WEAK was significantly different from a small rise (by 1 ± 5 mmHg) in seated DBP in the STRONG condition (Fig. 2E; *P* < 0.05). Follow-up analyses also showed that the slight reduction in MAP at 2 h was different from the rise in MAP at 4 h in CON (Fig. 2F; $P < 0.05$).

Exercising BP. The effect of BR supplementation and different mouth rinses on exercising SBP, DBP and MAP recorded at 4-6 min and 8-10 min during walking are presented in Fig 3. At baseline, the mean exercising SBP, DBP and MAP across all conditions was 121 ± 10, 66 ± 6 and 84 ± 6 mmHg and 121 ± 10, 64 ± 5 and 84 ± 6 mmHg, at 4-6 and 8-10 min of treadmill walking, respectively. There was a significant main effect by condition on SBP recorded at 4-6 min and 8-10 min during treadmill walking (Fig 3; all $P < 0.05$). Post hoc analyses revealed that exercising SBP at 4-6 min was significantly elevated (by 6 ± 10 mmHg) compared to baseline in STRONG at 4 h ($P < 0.05$). This increase in exercising SBP in STRONG was significantly different from the change in exercising SBP between baseline and 4 h in CON (-1 ± 8 mmHg; $P < 0.05$). Follow up tests also showed a trend for exercising SBP at 8-10 min to increase (by 6 ± 10 mmHg; $P = 0.08$) at 4 h (from baseline) in STRONG. This increase in exercising SBP was, however, significantly different from the change in exercising SBP between baseline and 4 h in CON (Fig. 3D; $P < 0.05$). At 4 h in the STRONG condition there was a trend for exercising MAP to be elevated at 4-6 min (3 ± 6 mmHg; $P = 0.10$) and there was a significant increase in MAP at 8-10 min (3 ± 6 mmHg; $P < 0.05$). At 4 h, the increase in exercising MAP at 8-10 min in STRONG was significantly different from the exercising MAP in CON at 8-10 min (Fig. 3F; $P < 0.05$).

Discussion

The principal finding of this study, consistent with our hypothesis, was that supplementation with BR alongside regular rinsing with STRONG over a 6 day period significantly attenuated the rise in plasma and salivary [NO_2^-] when compared with WEAK and CON. Contrary to our hypothesis, however, the increase in plasma [NO_2^-] was also significantly blunted in the WEAK condition compared with CON. Nitrate supplementation was largely ineffective in reducing BP in the current study; however, during treadmill walking, BP tended to be higher after rinsing with STRONG mouthwash. Specifically, an original finding of this study was that SBP and MAP were significantly increased during walking at 4 h after the final BR ingestion on the sixth day of supplementation in STRONG compared with CON. We note that the design of our study does not permit us to distinguish between the effects of mouthwashes on acute compared to more chronic BR supplementation, *per se*; rather, our study investigated acute supplementation on the final day of a 6 day supplementation period.

Plasma and Salivary [NO_3^-] and [NO_2^-]

BR supplementation increased plasma [NO_3^-] to a similar extent in the CON, WEAK and STRONG conditions. Similar [NO_3^-] kinetics were reported by Govoni and colleagues [17] when comparing chlorhexidine containing mouthwash with the control condition after acute supplementation with sodium NO_3^-. In the current study, plasma [NO_3^-] rose by ~ 1900 % relative to baseline at 2 h and remained elevated by ~ 1500 % at 4 h post final BR ingestion. The slight reduction in plasma [NO_3^-] between 2 and 4 h follows a pattern similar to that reported by Wylie et al. [47] after acute ingestion of a similar dose of NO_3^--rich BR. Previous studies using oral supplementation with NO_3^- salts have also reported increases in plasma [NO_3^-], but of a lesser magnitude (~ 400-600 %) [7, 30].

In the present study, BR supplementation resulted in a significant increase in plasma [NO_2^-] at all time points, regardless of condition. The absence of antibacterial properties of the mouthwash in the CON condition resulted in an elevated plasma [NO_2^-] by ~ 500 % at 2 h and 4 h. Numerous other reports have also shown increases in plasma [NO_2^-] after NO_3^- supplementation; however, the size of these increases have typically been smaller (50-150 %) [2, 3, 7, 28, 30, 31, 41]. The larger elevations noted in the present study are likely due to the higher NO_3^- dose (~12.4 $mmol \cdot day^{-1}$) compared to those reported in previous BR supplementation studies (5-6 $mmol \cdot day^{-1}$) [2, 3, 27, 41]. Wylie et al. [48] reported similar elevations in plasma [NO_2^-] of ~ 400 % after ingestion of ~29 mmol of NO_3^- over a 36 h period.

Consistent with our hypothesis, the increase in plasma [NO_2^-] in STRONG was significantly lower than that observed in the WEAK and CON conditions. These results agree with those of others who noted an attenuation in the rise of plasma [NO_2^-] after rinsing with chlorhexidine prior to acute NO_3^- ingestion, in both humans [17] and rats [38]. The findings in the current study may be explained by the significantly higher salivary [NO_3^-] and significantly lower salivary [NO_2^-] in the STRONG condition compared with CON. This implies that mouth rinsing with chlorhexidine prior to a BR load attenuated the conversion of NO_3^- to NO_2^- by commensal anaerobes in the oral cavity [17]. A recent study reported that when subjects used a chlorhexidine containing mouthwash twice daily, for 7 days, in conjunction with a low NO_3^- diet, salivary and plasma [NO_3^-] were significantly elevated and salivary and plasma [NO_2^-] were reduced by 90 and 25 %, respectively [24]. The results from the present study, and those of others, highlight the importance of the oral microflora in determining plasma [NO_2^-] [24, 45].

It is noteworthy that a blunting of the rise of plasma [NO_2^-] (by ~ 29 %, when compared with CON) was also present at 2 h when a weak, non-chlorhexidine containing mouthwash (WEAK) was administered prior to BR ingestion. The change in salivary [NO_3^-] from baseline in WEAK was similar to that in CON at both 2 h and 4 h. Salivary [NO_2^-] decreased (~ 20 % from baseline) in WEAK, but this reduction was not significantly different when compared with CON. These findings follow a similar trend to the results of Govoni et al. [17]. The results for WEAK suggest that there may be another ingredient in this mouthwash that actively disrupts the conversion of salivary NO_3^- to NO_2^-. Previous research has suggested that rinsing with 10 % ethanol alone can influence the bacterial composition of the oral microflora [37]. It may be speculated therefore that the other active ingredient in the WEAK mouthwash was alcohol although we cannot rule out possible influences of other ingredients such as peppermint, clove or chamomile oils.

Supine and Seated Blood Pressure

Several studies have reported a significant reduction in both SBP and DBP after acute [25, 31, 40, 41, 45] and chronic [2, 30, 41] NO_3^- supplementation. However, others have reported reductions in DBP [29] or SBP [28] only. The present study showed no significant reduction in resting supine BP across all conditions. These results are consistent with those of Larsen et al. [31] who also reported no significant change in SBP and DBP after 30 min of supine rest following NO_3^- supplementation. The absence of a reduction in supine BP in the current study may be explained, at least in part, by the relatively low baseline BP (SBP: 111, DBP: 59, MAP: 76 mmHg) values in our subjects. Kapil et al. [25] have previously reported a negative correlation between the baseline BP and the reduction in BP in response to NO_3^- supplementation.

BR supplementation in conjunction with WEAK resulted in a significant decrease in seated DBP, but not SBP or MAP at 2 h, when compared to baseline. In addition, the decrease in DBP with WEAK was significantly different from the small rise in DBP noted with STRONG. This may be explained by the difference in plasma $[NO_2^-]$ between the two conditions, with STRONG being significantly lower than WEAK. STRONG did not alter seated SBP, DBP or MAP. Petersson et al. [38] have reported significant reductions in DBP and MAP in rats after consuming NO_3^- supplemented drinking water. However, such reductions were no longer present when rats were treated with chlorhexidine prior to NO_3^- consumption. The results of the present study suggest that disrupting the oral commensal bacteria in the mouth, by regular use of STRONG mouthwash, attenuates the conversion of NO_3^- to NO_2^- and the potential for BR to lower BP [29, 45].

Exercising Blood Pressure and Heart Rate

This is the first study to assess the effects of BR supplementation on BP during low-intensity exercise, either with or without the use of antibacterial mouthwash. Ambulatory BP has been recognised as an important predictor of adverse cardiovascular events [43]. Monitoring BP over a 24 h period allows physicians and physiologists to determine BP during normal daily activities, such as walking and sleeping. However, with this approach, the activity taking place at the time at which BP measurements are recorded is dependent on participant recall. It has recently been reported that chlorhexidine use in the absence of NO_3^- supplementation resulted in a small but significant increase in ambulatory SBP (2-3.5 mmHg) [24]. In the present study, the CON condition did not significantly alter BP during low-intensity exercise relative to baseline. However, rinsing with STRONG prior to BR consumption resulted in significantly elevated SBP at 4 h during treadmill walking, compared to baseline. Additionally, SBP and MAP were higher in STRONG at 2 h and 4 h post final ingestion when compared with WEAK and CON. Typically, physical exertion tends to increase SBP due to the increase in cardiac output. The higher BP in STRONG, although relatively small, may be meaningful as it has been suggested that exaggerated increases in SBP and DBP during exercise are associated with a higher risk of developing hypertension, independent of other risk factors such as body mass index, fasting blood glucose and parental history of hypertension [13, 36].

There was a significant increase in HR during treadmill walking from pre to 4 h post BR ingestion when preceded by STRONG. Previous studies have reported no differences in HR following NO_3^- compared to placebo ingestion during either rest or exercise [2, 3, 16, 30]. The explanation for these findings is not clear. However, it appears that STRONG increases the cardiovascular demand of low-intensity aerobic exercise by increasing both HR and MAP.

Arterial Stiffness

NO is known to contribute to the regulation of arterial elasticity in humans [26]. Arterial stiffness may be measured via PWV and carotid-femoral PWV is generally accepted as an appropriate, non-invasive and reproducible method of determining arterial stiffness [32]. Bahra and colleagues [1] found that acute inorganic NO_3^- supplementation resulted in an improvement in arterial compliance (measured using an aortic PWV device). In contrast, the results of the current study suggest that chronic supplementation with BR, a natural source of NO_3^-, does not affect arterial stiffness. These equivocal findings may be due to the differences in techniques employed to determine arterial compliance. Previous research has suggested that inhibiting endogenous NO production with L-NMMA results in a significant increase in PWV [26] which is associated with an increase in cardiovascular morbidity and mortality [4, 8, 46]. The results of the present study suggest that BR supplementation, preceded by different strength antibacterial mouthwashes, does not affect arterial compliance. Although plasma [NO_2^-] was blunted in WEAK and STRONG conditions, indicating a potential reduction in the bioavailability of NO, it appears that the availability of NO was not altered sufficiently to significantly affect vascular tone.

Implications

The widespread use of antibacterial mouthwash in the prevention and treatment of plaque, gum disease [11] and malodor [6] may have a detrimental impact upon vascular health. The results from the present study suggest that chronic use of strong (chlorhexidine) and weak (no chlorhexidine) antibacterial mouth rinse prior to ingestion of NO_3^--rich foodstuffs (BR) can disturb the oral microflora and attenuate the expected rise in plasma [NO_2^-]. In the present study, chlorhexidine containing mouthwash resulted in a greater rise in BP during low-intensity exercise. An elevated BP during exercise is a significant risk factor for future hypertension and increases the likelihood of an adverse cardiovascular event [36]. An important novel observation in the current study is that alcohol (present in both rinses), or some other component of mouthwash, may potentially impact the physiological response to BR. Therefore, the use of mouthwashes should be considered carefully when the goal is to derive putative cardiovascular or exercise-related benefits from the consumption of high NO_3^- foodstuffs.

Conclusion

In summary, this study has shown that regular mouth rinsing with a chlorhexidine containing mouthwash and also a non-chlorhexidine containing mouthwash, attenuated the rise in plasma [NO_2^-] following chronic supplementation with NO_3^--rich beetroot juice. BR did not significantly reduce BP during seated and supine rest or during treadmill walking. However, prior rinsing with a chlorhexidine containing mouthwash led to a greater increase in BP during low-intensity exercise compared to the control condition. Our study adds to the growing body of literature [17, 24, 38] indicating that antibacterial mouthwashes have the potential to counteract the beneficial effects on cardiovascular health afforded by the consumption of NO_3^- in the diet.

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Figure Legends

Figure 1: Change (Δ) relative to presupplementation baseline in plasma [NO_3^-] (A) and [NO_2^-] (C) and salivary [NO_3^-] (B) and [NO_2^-] (D) following oral rinsing with deionised water (CON; black filled bars), non-chlorhexidine containing mouthwash (WEAK; light grey filled bars) and chlorhexidine containing mouthwash (STRONG; dark grey filled bars) at 2 and 4 h post ingestion of the final BR dose. Data are presented as group mean ± SE. Significant main effect by time shown as * for 2-way ANOVA ($P < 0.05$). Significant main effect by condition shown as $^{¥}$ for 2-way ANOVA ($P < 0.05$). Significant interaction effect (condition x time) shown as § for 2-way ANOVA ($P < 0.05$). $^{\$}$ Significantly different from baseline; ^{a} significant difference from 2 h WEAK; ^{b} significant difference from 2 h STRONG; ^{c} significant difference from 4 h CON; ^{d} significant difference from 4 h WEAK; ^{e} significant difference from 4 h STRONG ($P < 0.05$).

Figure 2: Change (Δ) relative to presupplementation baseline during supine rest for systolic blood pressure (SBP; A), diastolic blood pressure (DBP; B) and mean arterial pressure (MAP; C) and seated rest, SBP (D), DBP (E) and MAP (F) following rinsing with deionised water (CON; black filled bars), non-chlorhexidine containing mouthwash (WEAK; light grey filled bars) and chlorhexidine containing mouthwash (STRONG; dark grey filled bars) at 2 and 4 h post ingestion of the final BR dose. Data are presented as group mean ± SE. Significant main effect by time shown as * for 2-way ANOVA ($P < 0.05$). $^{\$}$ Significantly different from baseline; ^{b} significant difference from 2 h STRONG; ^{c} significant difference from 4 h CON; ^{d} significant difference from 4 h WEAK; ^{e} significant difference from 4 h STRONG ($P < 0.05$).

Figure 3: Change (Δ) relative to presupplementation baseline in exercising systolic blood pressure (SBP; A, D), diastolic blood pressure (DBP; B, E) and mean arterial pressure (MAP; C, F) measured during 4-6 min and 8-10 min of treadmill walking following rinsing with deionised water (CON; black filled bars), non-chlorhexidine containing mouthwash (WEAK; light grey filled bars) and chlorhexidine containing mouthwash (STRONG; dark grey filled bars) at 2 and 4 h post ingestion of the final BR dose. Data are presented as group mean ± SE. Significant main effect by time shown as * for 2-way ANOVA ($P < 0.05$). Significant main effect by condition shown as $^{¥}$ for 2-way ANOVA ($P < 0.05$). $^{\$}$ Significantly different from baseline; ^{a} significantly different from 2 h WEAK; ^{d} significantly different from 4 h WEAK; ^{e} significantly different from 4 h STRONG ($P < 0.05$).

Table 1. Change (Δ) relative to presupplementation baseline in heart rate during rest and low-intensity exercise and absolute pulse wave velocity responses during supine rest following nitrate supplementation in combination with pre-rinsing with water (control), a chlorhexidine containing mouthwash, or a non-chlorhexidine containing mouthwash

	CON	WEAK	STRONG
△Heart Rate ($b{\cdot}min^{-1}$)			
Seated			
2 h	-1 ± 15	$6 \pm 8^{*}$	1± 8
4 h	-3 ± 15	0 ± 5	-3 ± 7
Supine			
2 h	1 ± 11	$4 \pm 4^{*}$	-1 ± 7
4 h	-3 ± 13	$0 \pm 4^{\$}$	-4 ± 6
Low-intensity Exercise			
2 h	0 ± 5	0 ± 5	4 ± 9
4 h	4 ± 10	2 ± 5	$6 \pm 7^{*}$
PWV ($m{\cdot}s^{-1}$)			
Carotid: Radial			
Baseline	8.2 ± 1.6	8.1 ± 1.8	8.1 ± 1.6
2 h	8.1 ± 1.1	8.1 ± 1.5	7.7 ± 1.1
4 h	8.1 ± 1.1	8.4 ± 1.6	8.1 ± 1.2
Carotid: Femoral			
Baseline	6.7 ± 1.3	6.6 ± 0.7	6.5 ± 1.1
2 h	6.3 ± 0.8	6.5 ± 1.0	6.3 ± 1.1
4 h	6.5 ± 1.0	6.7 ± 0.8	6.7 ± 1.4

Values are means ± SD. PWV, pulse wave velocity; CON, deionised water; WEAK, non-chlorhexidine containing mouthwash; STRONG, chlorhexidine containing mouthwash. Significantly different from baseline, $P < 0.05$. $^{\$}$ Significantly different from STRONG, $P < 0.05$.

Figure 1.

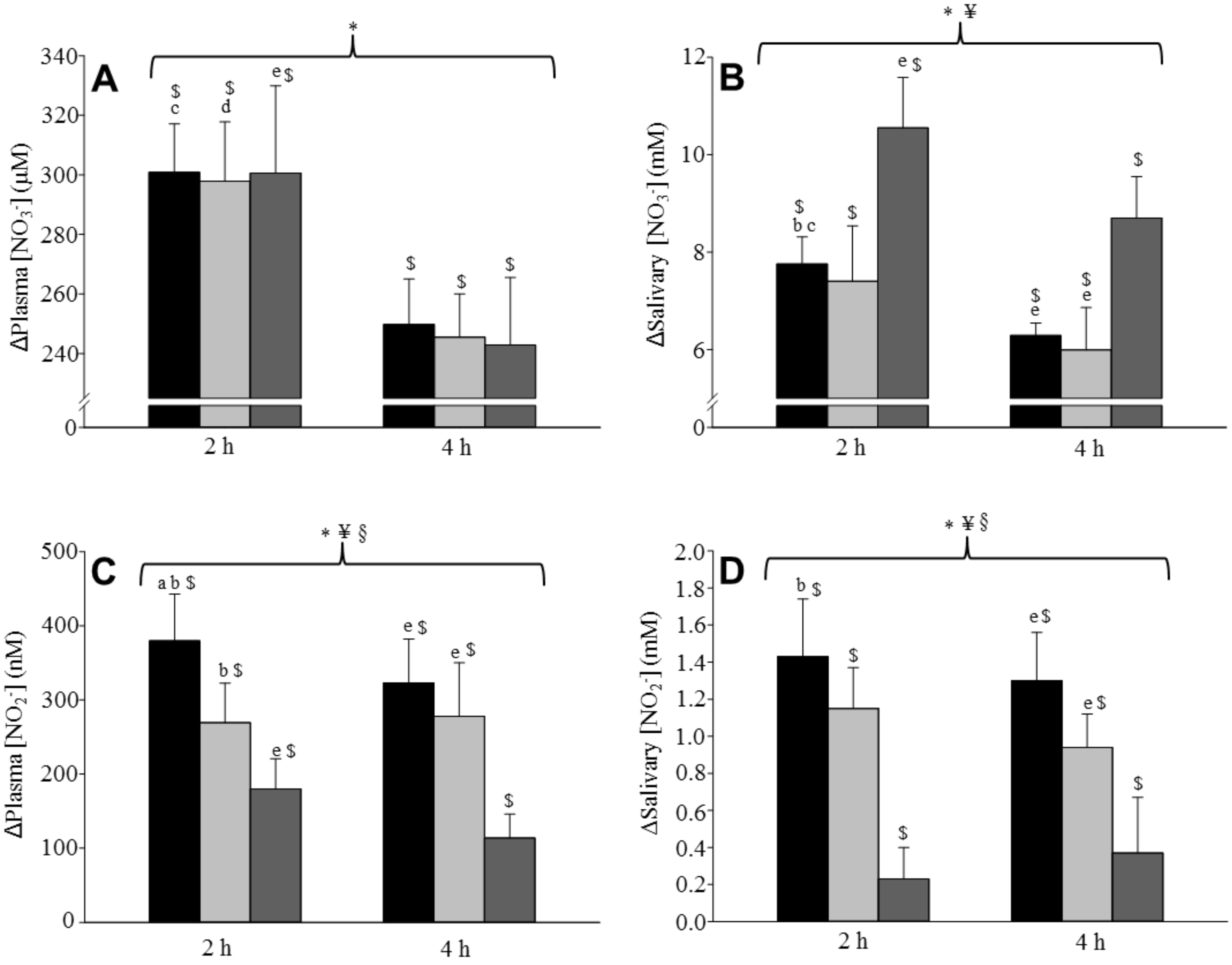
A
ΔPlasma [NO$_3^-$] (μM)
0
240
260
280
300
320
340
*
$ c
$ d
e $
$
$
$
2 h
4 h
B
ΔSalivary [NO$_3^-$] (mM)
0
6
8
10
12
* ¥
$ b c
$
e $
$ e
$ e
$
2 h
4 h
C
ΔPlasma [NO$_2^-$] (nM)
0
100
200
300
400
500
* ¥ §
a b $
b $
e $
e $
e $
$
2 h
4 h
D
ΔSalivary [NO$_2^-$] (mM)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
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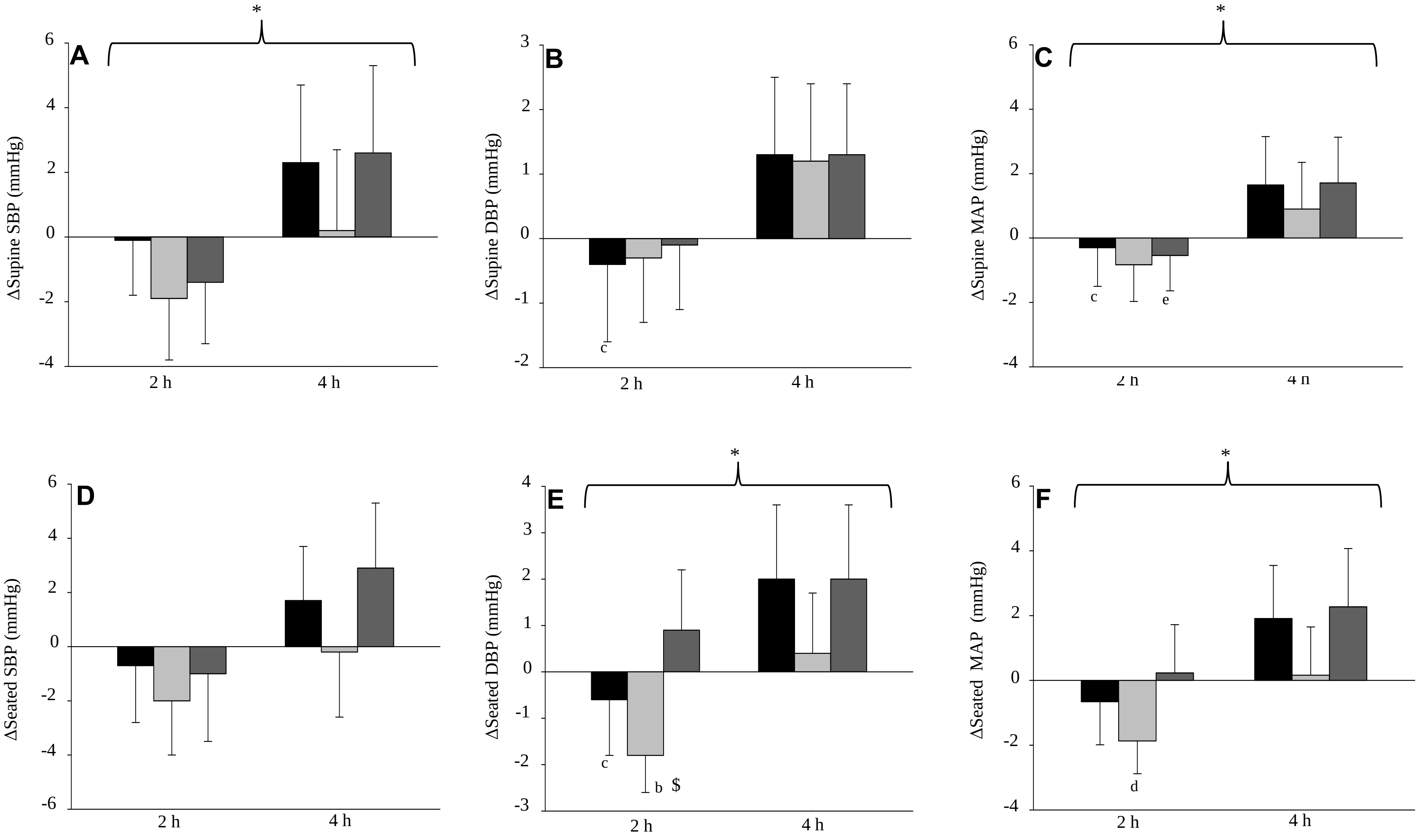
Figure 2.


A
ΔSupine SBP (mmHg)
6
4
2
0
-2
-4
*
2 h
4 h
B
ΔSupine DBP (mmHg)
3
2
1
0
-1
-2
c
2 h
4 h
C
ΔSupine MAP (mmHg)
6
4
2
0
-2
-4
*
c
e
2 h
4 h
D
ΔSeated SBP (mmHg)
6
4
2
0
-2
-4
-6
2 h
4 h
E
ΔSeated DBP (mmHg)
4
3
2
1
0
-1
-2
-3
*
c
b
$
2 h
4 h
F
ΔSeated MAP (mmHg)
6
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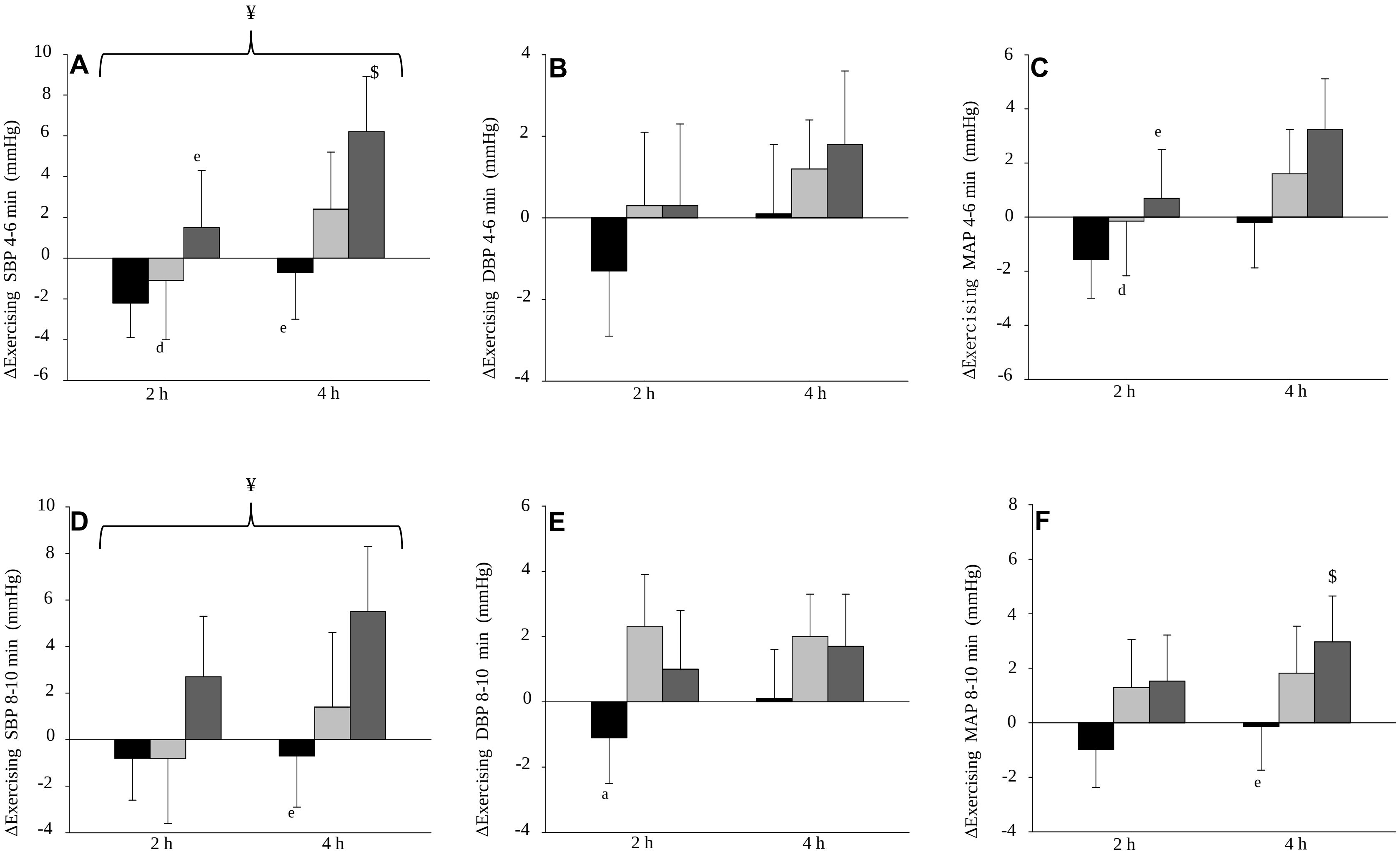
2
0
-2
-4
*
d
2 h
4 h

Figure 3.


A
¥
$
e
d
e
ΔExercising SBP 4-6 min (mmHg)
10
8
6
4
2
0
-2
-4
-6
2 h
4 h
B
ΔExercising DBP 4-6 min (mmHg)
4
2
0
-2
-4
2 h
4 h
C
e
d
ΔExercising MAP 4-6 min (mmHg)
6
4
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-6
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¥
e
ΔExercising SBP 8-10 min (mmHg)
10
8
6
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-4
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ΔExercising DBP 8-10 min (mmHg)
6
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2
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-2
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ΔExercising MAP 8-10 min (mmHg)
8
6
4
2
0
-2
-4
2 h
4 h

Dietary nitrate supplementation attenuates the reduction in exercise tolerance following blood donation

Original Article

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Running head: Nitrate, blood donation and exercise performance

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Chapter 5: Dietary nitrate supplementation attenuates the reduction in exercise tolerance following blood donation

Abstract

We tested the hypothesis that dietary nitrate-rich beetroot juice (BR) supplementation could partially offset deteriorations in O_2 transport and utilization, and exercise tolerance, after blood donation. Twenty-two healthy volunteers performed moderate-intensity and ramp incremental cycle exercise tests prior to and following the withdrawal of ~450 mL of whole blood. Before donation, all subjects consumed 7 x 70 mL of nitrate-depleted beetroot juice shots (PL) in the 48 h preceding the exercise tests. During the 48 h after blood donation, subjects consumed 7 shots of either BR (each containing 6.2 mmol nitrate; $n = 11$) or PL ($n = 11$) before repeating the exercise tests. [Hemoglobin] and hematocrit were reduced by ~ 8-9 % following blood donation ($P < 0.05$), with no difference between the BR and PL groups. When compared with pre-donation, steady-state $\dot{V}O_2$ during moderate-intensity exercise was ~ 4 % lower post-donation in BR ($P < 0.05$) but was unchanged in PL. The ramp test peak power decreased from pre-donation (PL: 341 ± 70 *vs.* BR: 331 ± 68 W) to post-donation (PL: 324 ± 69 *vs.* BR: 322 ± 66 W) in both groups ($P < 0.05$). However, the decrement in performance was significantly less in BR (2.7 %) compared with PL (5.0 %; $P < 0.05$). Nitrate supplementation reduced the O_2 cost of moderate-intensity exercise and attenuated the decline in ramp incremental exercise performance following blood donation. These results have implications for improving functional capacity following blood loss.

New and Noteworthy: Dietary nitrate supplementation with beetroot juice lowered the O_2 cost of moderate-intensity exercise, better preserved muscle oxygenation and attenuated the decline in incremental exercise test performance following donation of

450 mL whole blood. These results have implications for improving functional capacity following blood loss.

Key words: blood withdrawal; beetroot juice; O_2 transport; O_2 uptake; exercise performance; nitric oxide

Chapter 5: Dietary nitrate supplementation attenuates the reduction in exercise tolerance following blood donation

Introduction

The peak rate of pulmonary oxygen uptake ($\dot{V}O_{2peak}$) is an important determinant of exercise capacity and is influenced by the interaction of several central and peripheral factors (6, 53, 64). $\dot{V}O_{2peak}$ and exercise performance can be altered by manipulating the capability of the cardiovascular system to transport O_2 to contracting skeletal muscles during exercise (5, 11, 18, 51, 57, 67). For example, interventions involving the infusion of erythrocytes (18, 19) or the stimulation of erythropoiesis (57, 67) to enhance hemoglobin concentration ([Hb]), increase $\dot{V}O_{2peak}$ during maximal exercise. Conversely, limiting O_2 transport to working muscle by reducing [Hb] via whole blood withdrawal consistently results in a lowered $\dot{V}O_{2peak}$ (11, 18, 47, 54). During sub-maximal exercise, however, Panebianco et al. (47) reported no change in $\dot{V}O_2$ at two and seven days post 450 mL blood donation, despite significant reductions in [Hb]. Compensatory adjustments in cardiovascular control, such as increases in heart rate (HR) and cardiac output ($\dot{Q}$), offset the lower [Hb] and enable muscle O_2 delivery to be maintained during low-intensity exercise after blood donation (19, 27, 51).

The gaseous physiological signaling molecule, nitric oxide (NO), plays a key role in the regulation of vascular tone. NO can be synthesised via the oxidation of L-arginine in a reaction catalysed by the NO synthases (NOS; 32) or it can be produced via the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) and subsequently NO (8). Recently, dietary NO_3^- supplementation has been employed to augment plasma [NO_2^-] and the potential for O_2-independent NO synthesis (4, 38, 65). This NO_3^--NO_2^--NO pathway may be particularly important when NOS activity is compromised (20, 42), O_2 availability is limited (14, 25, 34, 35) and pH is low (44). Limitations in systemic O_2 transport can result in tissue hypoxia and greater metabolic perturbation (41, 60), which can contribute to reduced exercise tolerance (1), as is commonly observed at altitude (2) and in a number of disease states (35, 68). There is evidence to suggest that NO and NO_2^- can combat an insufficient muscle O_2 supply by increasing muscle blood flow via hypoxia-induced vasodilatation (13, 61). Therefore, it is possible that dietary NO_3^- supplementation could ameliorate deteriorations in exercise performance when ‘normal’ O_2 availability is reduced, during for example, high-intensity exercise, in hypobaric hypoxia or after blood donation.

We and others have reported that, in healthy subjects, dietary NO_3^- supplementation can significantly impact the physiological responses to exercise (4, 15, 38, 59). Specifically, a reduction in the O_2 cost of moderate-intensity exercise has been reported after supplementation with both sodium NO_3^- (38, 39, 40) and NO_3^--rich beetroot juice (BR; 3, 4, 15, 59, 69). In addition, a significantly increased time to task failure (TTF), indicating improved exercise tolerance, has been reported following BR ingestion when recreationally-active, but not highly trained, subjects completed severe-intensity (3, 4, 37) and ramp incremental exercise (59). These alterations may be due to a NO_2^- or NO-related reduction in the ATP cost of muscle contraction (3), greater mitochondrial efficiency (40), changes in muscle redox status (66), and/or enhanced muscle blood flow, particularly to type II fibres (21, 22). Such changes could be particularly advantageous after whole blood withdrawal when [Hb] is reduced and O_2 transport is challenged (11, 18, 54). Indeed, BR supplementation has been shown to reduce muscle metabolic perturbation during exercise in normobaric hypoxia and to restore exercise tolerance and oxidative function to the values observed in normoxia (60, 61). In addition, it has been reported that, when the fraction of inspired O_2 is lowered to 11-13%, BR supplementation can improve muscle oxygenation status (43), reduce $\dot{V}O_2$

during sub-maximal exercise (34, 46), and enhance TTF during incremental exercise (43). BR supplementation has also been reported to increase arterial O_2 saturation following dynamic apnea (i.e., breath-hold diving), which supports an O_2 sparing effect of NO_3^- ingestion (48). Collectively, these studies suggest that NO_3^- ingestion may enhance the physiological response to exercise when O_2 availability is limited, by sparing muscle O_2 demand and/or better preserving muscle O_2 supply. However, it is not known whether the reductions in O_2 carrying capacity and exercise performance subsequent to the withdrawal of whole blood can be offset by BR supplementation. If so, this may have important implications for clinical conditions in which [Hb] is lowered, for example in anemia, following surgery or involuntary blood loss, or in athletes wishing to donate blood without compromising training.

The purpose of the present study was to determine whether 48 h of BR supplementation following 450 mL of whole blood withdrawal alters the physiological responses to sub-maximal and maximal intensity cycle exercise. It was hypothesized that BR supplementation would lower the O_2 cost of moderate-intensity exercise, improve muscle oxygenation status, and attenuate the expected reduction in TTF during ramp incremental exercise following blood donation.

Methods

Subjects

Twenty-two recreationally active and pre-registered National Health Service (NHS) blood donors (males, $n = 14$; females, $n = 8$) volunteered to participate in this study, which was approved by the Institutional Research Ethics Committee and conformed to the ethical principles of the Declaration of Helsinki. None of the subjects were tobacco smokers or habitual users of dietary supplements. All subjects provided written informed consent prior to the commencement of the study, after the experimental procedures, associated risks and potential benefits of participation had been explained.

Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, at least 3 h postprandial, and to avoid strenuous exercise in the 24 h preceding each visit. In addition, subjects were asked to avoid alcohol consumption, chewing gum and antibacterial mouthwash throughout each supplementation period and to avoid caffeine intake in the 3 h preceding each laboratory visit. Each subject recorded habitual diet and exercise undertaken during the first supplementation period and were asked to replicate these habits during the second supplementation period. Prior to data collection, subjects were fully familiarized with the exercise testing procedures. This minimized any possible learning effects during the study. Exclusion criteria were the presence of known cardiovascular disease, hypertension and anemia, the use of antihypertensive medication and antibiotics, and having major surgery or giving blood within 6 months of the study commencing.

Experimental Overview

Subjects were asked to report to the laboratory on three separate occasions over a ten day period. The first visit included a 5 min bout of moderate-intensity cycle exercise at 80 W, followed by a ramp incremental test to task failure with no dietary supplementation. This served as the pre-intervention familiarization test. Hematocrit

(Hct), [Hb], plasma [NO_3^-] and [NO_2^-], pulmonary $\dot{V}O_2$ dynamics, muscle oxygenation status, HR, blood lactate concentration ([lactate]), blood glucose concentration ([glucose]) and TTF during ramp incremental exercise were measured during the first visit and repeated during each visit to the laboratory. Prior to visit 2, subjects consumed 7 shots of NO_3^--depleted beetroot juice (PL) over ~ 48 h. On the final day of supplementation, subjects completed the same moderate-intensity exercise bout and ramp incremental test on a cycle ergometer as was performed at pre-intervention. Two days before the final visit to the lab, subjects attended a National Health Service (NHS) blood donation clinic. Each subject lay supine on a bed before ~ 450 mL of whole blood was drawn from an antecubital vein over a 15 min period. The blood withdrawal was performed by the NHS as part of the national blood donation service. Following blood donation, each subject was randomly assigned, in a double-blind, placebo controlled fashion to consume 7 shots of either NO_3^--rich beetroot juice (BR; n = 11; mean ± SD; females, n = 4: age 23 ± 3 years, body mass 67 ± 4 kg, height 1.76 ± 0.05 m; males, n = 7: age 26 ± 5 years, body mass 81 ± 12 kg, height 1.80 ± 0.10 m) or NO_3^--depleted beetroot juice as a placebo (PL; n = 11; mean ± SD; females, n = 4: age 22 ± 3 years, body mass 77 ± 11 kg, height 1.75 ± 0.10 m; males, n = 7: age 28 ± 7 years, body mass 77 ± 8 kg, height 1.79 ± 0.10 m) over the next ~ 48 h. Visit 3 occurred on the final day of supplementation with the exercise tests conducted 2 h following final supplement ingestion. All tests were performed at the same time of day (± 2 h) to minimise diurnal variation on the physiological variables under investigation.

Exercise tests

During the first visit to the laboratory subjects performed a short bout of low-intensity exercise at 80 W, followed by a ramp incremental exercise test to task failure on an electrically-braked cycle ergometer (Lode Excalibur Sport, Gronigen, The Netherlands) for determination of $\dot{V}O_{2peak}$ and gas exchange threshold (GET). The protocol began with 3 min of 'unloaded' baseline cycling at 20 W, followed by 5 min at 80 W and 10 min of passive rest. Subsequently, 3 min of baseline cycling at 20 W was performed and then the power output was increased linearly by 30 $W{\cdot}min^{-1}$ until the subject was unable to continue. The subjects cycled at a self selected cadence (~80 rpm), and this cadence, along with saddle and handle bar configuration, was recorded and replicated for subsequent tests. Pulmonary gas exchange was measured breath-by-breath and averaged into 10-s bins. $\dot{V}O_{2peak}$ was taken as the highest 30-s mean value attained during the test. The GET was determined as described previously (59). The work rate that would require 80% of the GET (moderate-intensity exercise) was calculated, taking into account the mean response time for $\dot{V}O_2$ during ramp exercise (59).

Subjects returned to the laboratory on two further occasions. The second visit was preceded by PL supplementation (n = 22) and the third visit, ~ 48 h post blood donation, was preceded by 2 days of either BR (n = 11) or PL (n = 11) supplementation. The final visit was conducted 48 h post donation to allow restoration of total blood volume (23) and to minimize the risk of a syncopal episode occurring during maximal exercise. On each of these two laboratory visits, subjects completed a single 5-min bout of moderate-intensity exercise (at 80 % of the GET) and a ramp incremental test to task failure, separated by 10 min of passive rest. The incremental test was terminated when cadence fell more than 10 rpm below the chosen cadence, despite strong verbal encouragement.

TTF was recorded to the nearest second and the power output achieved at the point of test termination was recorded as the peak power output (PPO). Feedback on performance was only provided once all experimentation for the entire study had been completed.

Measurements

During each visit to the laboratory, a venous blood sample (~ 4 mL) was drawn from an antecubital vein into lithium-heparin tubes (Vacutainer, Becton-Dickinson, NJ, USA) and centrifuged for 10 min at 3000 *g* and 4 ˚C, within 2 min of collection. Subsequently, the plasma was extracted and frozen at -80 ˚C for later determination of [NO_3^-] and [NO_2^-] using a modified chemiluminescence technique (7) as previously described (69). Blood samples from a pre-warmed fingertip were collected into four 30 μL heparinized microhematocrit tubes (Hawksley and Sons Ltd, Lancing, Sussex, England) which underwent microcentrifugation for 1 min for the determination of Hct (1560 Micro-haematocrit reader, Hawksley and Sons Ltd, Lancing, Sussex, England). In addition, blood from the same fingertip was collected into four microcuvettes for determination of [Hb] (HemoCue AB, Ängelholm, Sweden).

Pulmonary gas exchange and ventilation were measured breath-by-breath throughout all exercise tests. Subjects wore a nose clip and breathed through a mouthpiece and impeller turbine assembly (Jaeger Triple V). The inspired and expired gas volume and gas concentration signals were sampled continuously at 100 Hz, with the latter using paramagnetic (O_2) and infrared (carbon dioxide; CO_2) analyzers (Oxycon Pro, Jaeger, Hoechberg, Germany) via a capillary line connected to the mouthpiece. These analyzers were calibrated before each test with gases of known concentration, and the turbine volume transducer was calibrated using a 3-litre syringe (Hans Rudolph, Kansas City, MO, USA). The volume and concentration signals were time-aligned by accounting for the delay in capillary gas transit and analyzer rise time relative to the volume signal. Pulmonary O_2 uptake ($\dot{V}O_2$), CO_2 output ($\dot{V}CO_2$), minute ventilation ($\dot{V}E$) and respiratory exchange ratio (RER) were calculated and displayed breath-by-breath. HR was measured at rest and during all cycle tests using short-range radiotelemetry (Polar S610, Polar Electro Oy, Kempele, Finland). A fingertip blood sample was collected into a capillary tube over the 20 s preceding the step transition in work rate to moderate-intensity exercise and the incremental test. Capillary samples were also collected during the final 20 s of the moderate-intensity exercise bout and following exhaustion in the ramp test. These samples were analyzed within 60 s of collection to determine blood [lactate] (YSI *2300*, Yellow Springs Instruments, Yellow Springs, OH, USA).

The oxygenation status of the *m. vastus lateralis* of the right leg was monitored using near-infrared spectroscopy (NIRS; model NIRO 300, Hamamatsu Photonics KK, Hiugashi-ku, Japan). Four different wavelength laser diodes provided the light source (776, 826, 845 and 905 nm) and a photomultiplier tube in the spectrometer was used to detect the light returning from the tissue. The intensity of incident and transmitted light was recorded continuously throughout exercise at 2 Hz and used to estimate the change in concentration from baseline for oxygenated, deoxygenated, and total tissue Hb and myoglobin. The NIRS data therefore represent a relative change based on the optical density measured in the first data point collected. The deoxyhemoglobin concentration ([HHb]) was assumed to represent the balance between local O_2 supply and utilization and therefore to provide an estimate of changes in O_2 extraction within the field of interrogation (28, 36). Prior to the cycling exercise, the right leg was cleaned and shaved around the belly of the muscle, the probes were placed in the holder and attached to the skin with an adhesive 20 cm above the fibular head. An elastic bandage was wrapped around the subject’s leg to secure the holder and wires in place and to minimize the possibility of extraneous light influencing the signal. Pen marks were made around the probe holder to allow for precise reproduction of the position of the probe in subsequent tests. The probe gain was set at rest with the subject in a seated position and the leg extended at down stroke on the cycle ergometer. NIRS data were collected continuously throughout the moderate-intensity and incremental exercise tests.

Supplementation

After completion of the familiarization test, subjects consumed 7 shots of NO_3^--depleted beetroot juice (PL; beetroot juice containing ~ 0.04 mmol NO_3^- per 70 mL; Beet It Sport Stamina Shot, James White Drinks, Ltd., Ipswich, UK) over ~ 48 h before completing the pre-donation control trial (PL-Pre and BR-Pre for the PL and BR groups, respectively). This was done in order to control for the antioxidants and polyphenols that exist in both the NO_3^--rich and NO_3^--depleted beverages. The PL was created by passing NO_3^--rich BR through a Purolite A520E ion-exchange resin which selectively removes NO_3^- (37). After blood donation, subjects were randomly assigned, in a double-blind, placebo-controlled fashion, to consume 7 shots of either NO_3^--rich (BR; beetroot juice containing ~ 6.2 mmol NO_3^- per 70 mL; Beet It Sport Stamina Shot, James White Drinks, Ltd., Ipswich, UK; *n* = 11) or NO_3^--depleted beetroot juice (PL; beetroot juice containing ~ 0.04 mmol NO_3^- per 70 mL; Beet It, James White Drinks, Ltd., Ipswich, UK; *n* = 11) over ~ 48 h (PL-Post and BR-Post for the PL and BR groups, respectively).

During both supplementation periods subjects were instructed to consume 2 x 70 mL of the beverage in the evening (~7 p.m.) two days prior to testing, and 1 x 70 mL in the morning (~10 a.m.) and 1 x 70 mL in the evening (~7 p.m.) one day prior to testing. On each experimental day, subjects consumed a further 2 x 70 mL, 2 h prior to testing and 1 x 70 mL on arrival at the laboratory. The supplementation periods were separated by a mean of 8 days (BR: 7 ± 5 days, PL: 9 ± 5 days).

Data Analyses

The breath-by-breath $\dot{V}O_2$ data collected during the exercise tests were initially examined to exclude errant breaths caused by, for example, coughing, swallowing and sighing, and those values lying more than four standard deviations (SDs) from the local mean were removed. $\dot{V}O_{2baseline}$ was defined as the mean $\dot{V}O_2$ measured over the last 60 s of baseline cycling and end-exercise $\dot{V}O_2$ was defined as the mean $\dot{V}O_2$ measured over the last 30 s of exercise. The baseline and end-exercise $\dot{V}CO_2$, RER, $\dot{V}E$ and HR values were calculated in the same manner.

To provide information on muscle oxygenation, the changes in [HHb] and the tissue oxygenation index (TOI; calculated as the fraction of oxygenated [Hb] compared to total [Hb]) during moderate-intensity exercise were assessed at baseline (60 s preceding the transition to moderate-intensity exercise), in 10 s time bins surrounding 60 s, 120 s, 240 s, and at end-exercise (mean response over the final 30 s of exercise). During ramp incremental exercise, the changes in [HHb] and TOI were assessed at baseline, in 10 s time bins surrounding 120 s, 240 s, 360 s and at task failure.

Blood lactate accumulation (Δ blood [lactate]) was calculated as the difference between blood [lactate] at end-exercise and blood [lactate] at baseline. Similarly, the change in blood glucose concentration (Δ blood [glucose]) was calculated as the difference between blood [glucose] at end-exercise and blood [glucose] at baseline.

Statistical Analyses

Differences in Hct, [Hb], plasma [NO_3^-] and [NO_2^-], pulmonary $\dot{V}O_2$ dynamics, HR, blood [lactate], NIRS-derived variables and TTF were assessed using a mixed model ANOVA. Significant main and interaction effects were further explored using Fisher's LSD. Independent t-tests were used to assess the relative change between the BR and PL treatment groups. Pearson's product moment correlation coefficient was used to explore relationships between changes in [Hb] and Hct and changes in TTF. Statistical analyses were performed using SPSS version 19.0 (Chicago, IL, USA). Data are presented as mean ± SD, unless otherwise stated. Statistical significance was accepted at $P < 0.05$.

Results

Subjects' self-reported adherence to the supplementation regimen prior to and post blood donation was 100 %. All subjects reported that their physical activity and dietary patterns were similar throughout each of the supplementation periods. The ingestion of BR and PL supplements were well tolerated and no negative side effects were reported. Subjects did, however, report beeturia (red-stained urine).

[Hb] and Hct

The group mean [Hb] and Hct data prior to and following blood donation and BR or PL ingestion are displayed in Table 1. There was a significant main effect by time for both [Hb] and Hct ($P < 0.01$) but no main effect by group and no interaction effect ($P > 0.05$). Prior to donation, [Hb] and Hct were not different between the BR and PL treatment groups. [Hb] and Hct were both significantly reduced from pre to post donation ($P < 0.05$), with no differences between PL and BR groups ($P > 0.05$).

Plasma [NO_3^-] and [NO_2^-]

The group mean plasma [NO_3^-] and [NO_2^-] pre and post blood donation in the BR and PL groups are shown in Table 1. There was a significant main effect by time and group and an interaction effect on plasma [NO_3^-] and [NO_2^-] ($P < 0.01$). Prior to blood donation, neither plasma [NO_3^-] nor [NO_2^-] were different between groups ($P > 0.05$). Following blood donation, there was a substantial increase in plasma [NO_3^-] and [NO_2^-] in the BR group ($P < 0.05$). A small (~ 11 %) rise in plasma [NO_3^-] ($P < 0.05$) was also observed in the PL group but there was no change in plasma [NO_2^-] ($P > 0.05$).

$\dot{V}O_2$ response to moderate-intensity and incremental exercise

Moderate-intensity exercise

The pulmonary gas exchange and ventilatory responses to moderate-intensity exercise pre and post blood donation in PL and BR groups are reported in Table 2 and the group mean $\dot{V}O_2$ response profiles in BR and PL groups pre and post blood donation are shown in Figure 1. There was a significant main effect by time ($P < 0.01$) but no main effect by condition and no interaction effect ($P > 0.05$) for the $\dot{V}O_2$ measured during the baseline cycling period and at end-exercise. Prior to donation, there were no differences in baseline or end-exercise $\dot{V}O_2$ between BR and PL groups ($P > 0.05$). Follow-up tests revealed that both baseline $\dot{V}O_2$ ($P < 0.01$) and end-exercise $\dot{V}O_2$ ($P < 0.05$) were reduced in the BR group post-donation compared with pre-donation.

The $\dot{V}CO_2$, $\dot{V}E$, RER, blood [lactate] and blood [glucose] data during moderate-intensity exercise are reported in Table 2. Prior to donation, there were no differences in these variables at baseline or at end-exercise between the BR and PL groups ($P > 0.05$) and there were no significant main effects by condition or time and no interaction effects ($P > 0.05$).

Ramp incremental exercise

The effects of blood donation and BR and PL supplementation on the ramp incremental test parameters are reported in Table 3 and illustrated in Figures 2 and 3.

There was a significant main effect by time on $\dot{V}O_{2peak}$ ($P < 0.05$), but no main effect by condition or an interaction effect ($P > 0.05$). There were no differences between the groups at baseline ($P > 0.05$). Follow-up tests indicated that, from pre to post donation, there was a significant reduction (0.19 $L \cdot min^{-1}$; ~ 5 %) in $\dot{V}O_{2peak}$ in the PL group ($P < 0.05$) but not in the BR group (0.12 $L \cdot min^{-1}$; ~ 3 %; $P > 0.05$). There was a significant main effect by time and an interaction effect ($P < 0.05$) but no main effect by condition ($P > 0.05$) for PPO and TTF. Post hoc tests revealed a significant reduction in PPO and

TTF in both PL and BR groups from pre to post donation (*P* < 0.01). There were no differences in PPO or TTF between the groups prior to blood donation (*P* > 0.05). However, the reduction in PPO and TTF following blood donation was more pronounced in PL compared with BR (5 % vs. 3 %; *P* < 0.05). The change in [Hb] and Hct from pre to post donation was correlated with the change in TTF during ramp incremental exercise in PL (r = 0.58; *P* = 0.06, and r = 0.70; *P* < 0.05, respectively) but not BR (r = -0.10; *P* > 0.05 and r = -0.41; *P* > 0.05, respectively).

There was a significant interaction effect, but no main effects by time or group, for peak $\dot{V}CO_2$. Specifically, peak $\dot{V}CO_2$ was reduced in the PL group (*P* < 0.05), but was unaffected in the BR group (*P* > 0.05). There was no main effect by time or condition nor an interaction effect for peak $\dot{V}E$ (*P* > 0.05). There was a significant main effect by time and an interaction effect for peak RER (*P* < 0.05). Despite no difference at baseline, post hoc tests revealed an increase in peak RER in the BR group from pre to post donation (*P* < 0.01).

NIRS measurements

Moderate-intensity exercise

There were no differences for total Hb (THb) between or within conditions during the moderate-intensity exercise bout. The [HHb] and TOI values measured during moderate-intensity exercise are reported in Table 4. There were no main effects by condition or time and no interaction effect for baseline [HHb] (*P* > 0.05). There was a significant main effect by time for [HHb] from pre to post donation at 60 s, 120 s, 240 s and end-exercise (*P* < 0.05), but no main effect by condition or an interaction effect at any time point ($P > 0.05$). Post hoc tests revealed a trend toward an increase in [HHb] in the PL group, but not the BR group, from pre to post donation at 120 s and 240 s of moderate exercise ($P < 0.10$). There were no main effects by time or interaction effects for TOI at 60 s, 120 s, 240 s and end-exercise ($P > 0.05$). However, there was a trend toward a main effect by condition for all time points ($P < 0.10$). Follow-up tests revealed that blood donation resulted in reductions in TOI in the PL group at 60 s, 120 s and 240 s during moderate exercise, respectively ($P < 0.05$; Table 4).

Ramp incremental exercise

There were no differences for THb between or within conditions during ramp incremental exercise. The [HHb] and TOI values measured during ramp incremental exercise are reported in Table 4 and the [HHb] profile is shown in Figure 4. There was a significant main effect by time ($P < 0.05$) but no main effect by condition or an interaction effect ($P > 0.05$) for [HHb] at 120 s and 240 s during ramp incremental exercise. Post hoc tests showed that [HHb] increased from pre to post donation at 240 s in PL ($P < 0.05$) but not BR ($P > 0.05$; Table 4). There was a significant main effect by time ($P < 0.05$) and a trend for an interaction effect for [HHb] at 360 s ($P < 0.10$) and at end-exercise ($P < 0.05$) during the incremental exercise test. Post hoc tests revealed that [HHb] increased significantly from pre to post donation in the PL group at both 360 s and end-exercise ($P < 0.05$; Table 4). The change in [HHb] from pre to post donation was higher in PL versus BR at end-exercise ($P < 0.05$) and tended to be higher at 360 s ($P<0.10$).

Discussion

The principal original findings in this study, consistent with our hypotheses, were that NO_3^--rich beetroot juice ingestion lowered the O_2 cost of moderate-intensity exercise, better preserved muscle oxygenation during moderate and ramp incremental exercise and attenuated the reduction in ramp incremental exercise test performance and $\dot{V}O_{2peak}$ following blood donation. These results indicate that dietary NO_3^- supplementation can ameliorate decrements in exercise performance in a situation (i.e. reduction in blood O_2-carrying capacity) which would be expected to compromise physiological function during exercise.

Effects of blood donation on [Hb] and Hct

The standard NHS blood bank donation (~ 450 mL) reduced [Hb] and Hct by a similar magnitude in the PL and BR groups. These results concur with previous studies that have investigated the influence of whole blood withdrawal on [Hb]. For example, Gordon et al. (27) and Mora-Rodriguez et al. (45) reported ~ 8 % and ~ 7 % reductions in [Hb], 24 and 48 h post blood donation, respectively. The ~ 8 % reduction in Hct in the present study is also similar to the values reported by Burnley et al. (11) and Gordon et al. (27) who reported a ~ 7-8 % decrease in Hct one day after 450 mL blood donation. The reduction in blood O_2 carrying capacity, secondary to the lower [Hb] and Hct, can result in a reduction in muscle O_2 delivery and muscle O_2 diffusing capacity during maximal exercise, with significant implications for exercise performance (5, 11, 18, 47, 54).

Effects of nitrate supplementation on plasma [NO_3^-] and [NO_2^-]

The ingestion of NO_3^--rich BR significantly elevated plasma [NO_3^-] and [NO_2^-] when compared with baseline values. These findings are in agreement with earlier studies which also examined the influence of BR supplementation in young, healthy subjects (4, 34, 69). A small but significant rise in plasma [NO_3^-] was also noted in the PL group post donation. This may be explained by a slight hemoconcentration or an upregulation in NOS activity consequent to the reduction in whole body iron concentration after donating blood (62). Plasma [NO_2^-] rose by ~ 800 % in the BR group from pre to post donation, suggesting appreciably enhanced NO bioavailability. Numerous other studies have also reported increases in plasma [NO_2^-] after BR supplementation, but the percentage increases attained were approximately half of those reported in this study (56, 69). This finding is likely a result of the higher dose of NO_3^- ingested (~ 43 mmol over 48 h) when compared with previous short-term BR supplementation studies. Interestingly, unlike in some earlier studies (4, 38, 59, 69), BR supplementation did not reduce resting blood pressure (BP) despite the elevated plasma [NO_2^-] (mean arterial pressure, pre- vs. post-donation: 81 ± 7 vs. 80 ± 7 mmHg). Similar BP values pre- vs. post-donation in the PL group indicates that total blood volume was restored 48 h following blood donation. The lack of effect of BR on BP in the present study may be related to the relatively low baseline BP values of the study participants (115/64 mmHg) and the relatively large number of female participants. It has been reported that females are less sensitive than males to the influence of NO_3^- supplementation on BP and that the extent of BP reduction with NO_3^- supplementation is correlated with the baseline BP (33).

Effects of blood donation and nitrate supplementation on the physiological responses to moderate-intensity exercise

The $\dot{V}O_2$ during both the unloaded baseline period and in the steady state of moderate-intensity exercise was significantly reduced (by ~ 4 %) in the BR group, but not the PL group, after blood donation. A similar reduction in the O_2 cost of moderate-intensity exercise has been reported by Bailey et al. (4) after six days of non-concentrated NO_3^--rich BR ingestion and by Larsen et al. (38) after three days of $NaNO_3$ supplementation. The present findings are consistent with those of Kelly et al. (34) who observed that, in hypoxia, BR supplementation resulted in a decrease in both baseline and steady-state $\dot{V}O_2$ when compared with placebo. It has also been reported that acute (46) and 6 days (43) BR ingestion resulted in significant reductions in $\dot{V}O_2$ during submaximal cycling exercise in hypoxia (15 % and 11 % O_2, respectively). Acute BR supplementation has also been reported to better preserve arterial O_2 saturation following dynamic apnea (48).

The lowering of the O_2 cost of submaximal exercise after NO_3^- supplementation may be due to a number of mechanisms, including a reduction in the ATP cost of muscle force production (4) and/or an improvement in mitochondrial efficiency (40) and/or changes in redox signalling (66). In addition to changes in muscle contractile or metabolic efficiency, muscle O_2 delivery or its intramuscular distribution may be altered following NO_3^- supplementation (21, 22). Exercise, particularly in hypoxia or under conditions that may limit O_2 carrying capacity, such as blood donation, acts as a potent stimulus for vasodilatation and delivery of O_2 to working muscle (12, 13). Both NO and O_2 compete for the binding site at cytochrome-*c* oxidase (COX) in the mitochondrial electron transport chain (9). An elevation in NO availability via NO_3^- supplementation, perhaps especially in conditions limiting O_2 delivery, increases the likelihood of NO binding to COX and therefore inhibiting O_2 consumption at the mitochondrion (10). As a result, NO may modify the intramuscular distribution of O_2 and improve the oxygenation status of muscle fibres that are situated further away from the capillaries (29, 55, 63). Compared to placebo, BR supplementation has been reported to enable a greater maximal rate of mitochondrial ATP resynthesis (Q_{max}) and result in faster muscle phosphocreatine recovery kinetics following exercise in hypoxia (60, 61), indicating improved muscle O_2 availability at least in the immediate post-exercise period (61).

In the present study, TOI was significantly reduced and [HHb] tended to be higher during moderate-intensity exercise post- compared to pre-donation in the PL group, suggesting that muscle O_2 availability was lower and a greater muscle fractional O_2 extraction was necessary to achieve the required $\dot{V}O_2$ (24, 36). These changes were attenuated in the BR group, consistent with our hypothesis that BR supplementation would better preserve muscle oxygenation during moderate-intensity exercise when compared with PL. These results are consistent with Masschelein et al. (43) who reported that BR resulted in a greater muscle TOI and lower [HHb] during submaximal exercise in normobaric hypoxia. Collectively, these studies indicate that under conditions which may impair blood O_2 carrying capacity, such as following blood donation (present study) or in normobaric hypoxia (43), BR ingestion promotes a better matching between muscle O_2 delivery and O_2 demand, i.e. less O_2 extraction is required for the same moderate-intensity work rate, perhaps due to the lower exercise $\dot{V}O_2$ (34) or to preferential alterations in muscle perfusion (21, 22, 61). An increased ratio of O_2 delivery to O_2 consumption at a given work rate would be expected to retard the rate of fatigue development and to improve exercise performance.

Effects of blood donation and nitrate supplementation on the physiological responses to incremental exercise

As expected, blood donation and the associated reduction in O_2 carrying capacity resulted in a significant reduction in PPO and TTF during ramp incremental exercise. Panebianco et al. (47) also reported a significant reduction in PPO during incremental exercise, 2 days post blood donation. An important original finding in the present study was that ingestion of BR in the 48 hours post blood donation partly negated the decrement in performance when compared with PL. Specifically, the reduction in PPO and TTF following blood donation was significantly more pronounced in the PL group compared with BR. Interestingly, the reduction in TTF in the PL group was quite well correlated with the reduction in [Hb] (r = 0.58, $P = 0.06$) and Hct (r = 0.70, $P < 0.05$) following blood donation, whereas in the BR group, the correlations were weaker and non-significant ([Hb]: r = -0.10; Hct: r = -0.41; both $P > 0.05$), implying that BR supplementation compensated for the lower [Hb] and Hct. These findings are consistent with those of Masschelein et al. (43) who reported that, compared to PL, BR ingestion significantly attenuated the reduction in TTF when incremental exercise was performed in hypoxia.

$\dot{V}O_{2peak}$ was reduced by 5 % from pre to 48 h post donation in the PL group. Similarly, Burnley et al. (11) reported a 4 % decrease in $\dot{V}O_{2peak}$ during severe-intensity exercise 24 h following blood donation. This reduction was proportional to the reduced [Hb] and thus the ability to deliver O_2 to the working skeletal muscle during maximal exercise. In the present study, the reduced $\dot{V}O_{2peak}$ in the PL group following blood donation occurred in conjunction with an increased muscle [HHb], which may be interpreted as an increase in muscle fractional O_2 extraction in an (ultimately unsuccessful) attempt to offset the effects of a reduced [Hb] and lower muscle O_2 delivery (51, 54). In contrast, $\dot{V}O_{2peak}$ and [HHb] during the incremental test were not significantly altered by blood donation in the BR group. These results may indicate that the O_2 sparing effect of BR ingestion (Figure 2B), coupled perhaps with altered perfusion distribution (21, 22, 61), enabled muscle oxygenation to be better preserved during incremental exercise, such that an increased muscle fractional O_2 extraction was not mandated to achieve a given $\dot{V}O_{2peak}$. Ferguson et al. (21, 22) have reported that, in rats, BR supplementation can enhance vascular conductance and blood flow to working muscle and elevate the microvascular partial pressure of O_2 (PO_{2mv}), particularly in type II fibres. If similar effects occur in humans, this may enhance the blood-myocyte O_2 exchange gradient during higher intensity exercise, better preserving muscle oxygenation status, homeostasis and performance. It is also possible that a portion of the preserved ramp incremental test performance following blood donation with BR compared to PL may be attributable to effects of NO_3^- on muscle contractile function (50), perhaps particularly in type II fibers (31).

The mechanistic bases for the positive effects of BR ingestion on vascular and metabolic function in this and other situations warrants further investigation. In particular, while it is widely believed that the effects may be attributed to greater NO bioavailability or bioactivity, it is presently unclear precisely how this NO pool is stored and transported. NO is a highly reactive molecule with a short-half life *in vivo* and its rapid reaction with, for example, O_2 or heme proteins (30) suggests that the free transport of NO may be limited in plasma and within cells. It has been proposed that NO_2^- itself represents a principal means of 'NO' storage and transport, with the one electron reduction of NO_2^- to NO in blood and other tissues being facilitated, amongst many other factors including xanthine oxidoreductase, by deoxyhemoglobin and deoxymyoglobin, which will naturally be present in greater abundance in contracting skeletal muscle (16, 42). However, BR ingestion likely also increases the production and storage of other reactive nitrogen species. In particular, low molecular weight thiol groups may react with nitrogen oxides to yield s-nitrosothiol species (SNOs) which can be transported in the blood as s-nitrosohemoglobin (HbSNO) (17). It has recently been reported that the reduction in blood pressure following NO_3^- or NO_2^- ingestion in a rat model of hypertension was more closely related to plasma [s-nitrosothiol] than to plasma [NO_2^-] (49) and that s-nitrosothiol bioactivity derived through βCys93 may be essential for hypoxic vasodilation by erythrocytes (70). In contrast, in humans, Gladwin et al. (26) reported a significant arterial-venous NO_2^- gradient during forearm exercise and concluded that SNOs and HbSNO do not play a significant role in the regulation of vascular tone. The role of SNOs and HbSNO in the physiological effects of nitrate ingestion in humans remains to be clarified. Equally, the precise mechanisms by which an elevation of tissue [NO_2^-] following NO_3^- ingestion influences metabolic and vascular control at rest and during exercise remains unclear. While it is possible that NO_2^- itself is bioactive (58), unresolved questions include the triggers and time course for the possible reduction of NO_2^- to NO, and the nature of both NO transport to, and storage within, biological targets. Resolution of these issues will likely require synthesis of experimental data deriving from ‘competing’ hypotheses.

Perspectives

This study has shown for the first time that despite a significant reduction in [Hb] post blood withdrawal, BR supplementation lowered the O_2 cost of moderate-intensity exercise, better preserved muscle oxygenation during moderate-intensity and ramp incremental exercise, and attenuated the reduction in $\dot{V}O_{2peak}$ and incremental exercise test performance. These results may have significant implications for athletes who wish to give blood without significant detriment to training, individuals with clinical conditions which reduce blood O_2 carrying capacity, such as anemia, and in conditions resulting in acute blood loss such as surgery or military combat. In this context, it is of interest that transfusion of stored blood may impair vasodilatory capacity, an effect that might be linked to the loss of NO bioavailability that occurs during blood storage (17, 52). Treating banked blood to better maintain NO stores might lead to improved functional outcomes following transfusion. In conclusion, BR supplementation attenuates the decline in functional capacity arising from blood donation.

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Figure Legends

Figure 1: Pulmonary oxygen uptake ($\dot{V}O_2$) response following BR and PL supplementation prior to and following blood donation during a step increment to a moderate-intensity work rate. Responses prior to blood donation are shown as solid, filled circles, while responses post blood donation are shown as open, unfilled circles. The dotted vertical line represents the abrupt imposition of the moderate work rate from a baseline of ‘unloaded’ cycling. *A:* Group mean $\dot{V}O_2$ response to moderate-intensity exercise following PL ingestion. *B:* Group mean $\dot{V}O_2$ response to moderate-intensity exercise following BR ingestion. *C:* Steady state $\dot{V}O_2$ following PL and BR supplementation relative to pre blood donation baseline. The O_2 cost of moderate-intensity exercise was reduced following BR supplementation and blood donation compared with pre donation values, $^*P < 0.05$.

Figure 2: Group mean pulmonary $\dot{V}O_2$ response to incremental exercise prior to blood donation and following BR and PL supplementation after blood donation. Responses prior to blood donation are shown as solid, filled circles, while responses post blood donation are shown as open, unfilled circles. The dotted vertical line represents the onset of the ramp incremental test from a baseline of ‘unloaded’ cycling. The $\dot{V}O_{2\text{peak}}$ was reduced in the PL group ($*= P < 0.05$), but not the BR group, after blood donation. TTF was reduced in both groups post donation ($^{\#} = P < 0.05$), however, the reduction in TTF was greater in the PL group when compared with the BR group ($^{\$} = P < 0.05$).

Figure 3. Group mean time to task failure (TTF) in the ramp incremental test prior to and post blood donation, following BR and PL supplementation. Responses prior to blood donation are shown as solid, filled bars, while responses post donation are shown as open, unfilled bars. The TTF was reduced in both groups post donation (*= $P < 0.05$); however, the reduction in TTF was greater in the PL group when compared with the BR group ($^{\#}$=$P < 0.05$).

Figure 4. Group mean changes in deoxyhaemoglobin ([HHb]) prior to and post blood donation, following BR and PL ingestion. Responses prior to blood donation are shown as solid, filled circles, while responses post blood donation are shown as open, unfilled circles. The dotted vertical line represents the onset of the ramp incremental test from a baseline of ‘unloaded’ cycling. [HHb] increased significantly from pre to post donation in the PL group at 360 s and end-exercise (*=$P < 0.05$). [HHb] was not altered from pre to post donation in the BR group. TTF was reduced in both groups post donation ($^{\#} = P < 0.05$), however, the reduction in TTF was greater in the PL group when compared with the BR group ($^{\$} = P < 0.05$).

Table 1: Blood pressure, resting heart rate, plasma nitrate and nitrite concentrations, hemoglobin concentration and hematocrit prior to and following blood donation in the PL and BR groups.

	PL		**BR**	
	Pre	**Post**	**Pre**	**Post**
Blood pressure (mmHg)				
Systolic	119 ± 7	118 ± 9	115 ± 11	$113 \pm 11^{*}$
Diastolic	69 ± 7	67 ± 7	64 ± 7	63 ± 7
Mean Arterial	86 ± 6	84 ± 8	81 ± 7	80 ± 7
Resting HR (b·min^{-1})	62 ± 9	66 ± 9	66 ± 11	$71 \pm 10^{*}$
Plasma $[NO_3^-]$ (µM)	45 ± 11	$50 \pm 14^{*}$	47 ± 17	$845 \pm 350^{*\$}$
Plasma $[NO_2^-]$ (nM)	73 ± 18	72 ± 21	81 ± 29	$619 \pm 363^{*\$}$
[Hb] (g·L^{-1})	149 ± 12	$132 \pm 18^{*}$	148 ± 15	$137 \pm 19^{*}$
Hct (%)	45 ± 2	$41 \pm 4^{*}$	45 ± 3	$42 \pm 5^{*}$

Values are mean ± SD. PL, Placebo group; BR, Nitrate group; Pre, pre-donation; Post, post-donation ; HR, heart rate; $[NO_2^-]$, nitrite concentration; $[NO_3^-]$, nitrate concentration; [Hb], hemoglobin concentration; Hct, hematocrit. *Significantly different from pre in the same condition ($P < 0.05$). $^{\$}$Significantly different from post supplementation value in the PL group ($P < 0.05$).

Table 2: Ventilatory and gas exchange dynamics, and blood lactate and glucose concentrations during moderate-intensity exercise prior to and following blood donation in the PL and BR groups

	PL		**BR**	
	Pre	**Post**	**Pre**	**Post**
$\dot{V}O_2$ (L·min^{-1})				
Baseline	1.01 ± 0.17	0.97 ± 0.20	0.96 ± 0.20	0.87 ± 0.21$^{\#}$
End exercise	1.72 ± 0.50	1.69 ± 0.53	1.65 ± 0.32	1.59 ± 0.34$^{\#}$
$\dot{V}CO_2$ (L·min^{-1})				
Baseline	0.88 ± 0.19	0.86 ± 0.19	0.89 ± 0.19	0.81 ± 0.19$^{\#}$
End exercise	1.60 ± 0.52	1.56 ± 0.50	1.53 ± 0.29	1.54 ± 0.29
RER				
Baseline	0.88 ± 0.08	0.90 ± 0.06	0.89 ± 0.05	0.92 ± 0.09
End exercise	0.94 ± 0.06	0.93 ± 0.06	0.93 ± 0.04	0.96 ± 0.06$^{\#}$
$\dot{V}E$ (L·min^{-1})				
Baseline	25 ± 5	24 ± 5	24 ± 5	22 ± 5$^{\#}$
End exercise	42 ± 11	40 ± 11	38 ± 6	38 ± 6
Δ **Blood [lactate]** (mM)	0.0 ± 0.3	0.1 ± 0.4	0.1 ± 0.3	0.1 ± 0.4
Δ**Blood [glucose]** (mM)	0.1 ± 0.7	- 0.2 ± 0.7	0.00 ± 0.3	0.1 ± 0.5

Values are mean ± SD. PL, Placebo group; BR, Nitrate group; Pre, pre-donation; Post, post-donation; [Bla], blood lactate concentration; [glu], blood glucose concentration; HR, heart rate. $^{\#}$Significantly different from pre in the same condition ($P < 0.05$).

Table 3: Physiological responses to ramp incremental exercise prior to and following blood donation in the PL and BR groups.

	PL		**BR**	
	Pre	**Post**	**Pre**	**Post**
$\dot{V}O_2$peak (L·min^{-1})	3.84 ± 0.91	3.65 ± 0.85*	3.52 ± 0.65	3.40 ± 0.73
$\dot{V}O_2$peak (mL·kg^{-1}·min^{-1})	49.9 ± 11.0	47.4 ± 10.0*	46.6 ± 6.0	44.9 ± 6.0
Peak power (W)	341 ± 70	324 ± 69*	331 ± 68	322 ± 66*
GET (L·min^{-1})	1.76 ± 0.40	1.68 ± 0.43	1.64 ± 0.44	1.63 ± 0.44
GET (W)	117 ± 29	109 ± 27	116 ± 35	112 ± 24
$\dot{V}CO_2$peak (L·min^{-1})	4.69 ± 1.12	4.44 ± 0.97*	4.26 ± 0.68	4.36 ± 0.77
RER peak	1.22 ± 0.06	1.22 ± 0.05	1.22 ± 0.06	1.29 ± 0.06*
$\dot{V}E$peak (L·min^{-1})	156 ± 44	150 ± 43*	134 ± 28	137 ± 32
HRpeak (b·min^{-1})	177 ± 16	181 ± 9	178 ± 12	179 ± 10
Δ Blood [lactate] (mM)	6.1 ± 1.4	5.5 ± 1.2	6.1 ± 1.9	6.8 ± 2.5
Δ Blood [glucose] (mM)	-0.2 ± 0.7	0.0 ± 1.1	-0.2 ± 0.4	0.0 ± 1.1

Values are mean ± SD. PL, Placebo group; BR, Nitrate group; Pre, pre-donation; Post, post-donation; GET, Gas exchange threshold; [Bla], blood lactate concentration; [glu], blood glucose concentration; HR, heart rate. *Significantly different from pre in the same condition ($P < 0.05$).

Table 4: Near-infrared spectroscopy-derived [HHb] and TOI dynamics during moderate-intensity and ramp incremental exercise prior to and following blood donation in the PL and BR groups.

	PL		**BR**	
	Pre	**Post**	**Pre**	**Post**
	Moderate-intensity exercise			
[HHb]				
Baseline (AU)	-4.4 ± 3.0	-2.3 ± 3.1	-3.1 ± 3.7	-1.9 ± 2.5
60 s (AU)	-1.2 ± 2.3	2.3 ± 5.0	-0.1 ± 5.0	0.6 ± 3.9
120 s (AU)	-0.9 ± 3.0	3.5 ± 6.2	-0.1 ± 4.9	1.0 ± 3.7
240 s (AU)	-0.7 ± 3.9	2.3 ± 5.2	0.1 ± 4.9	1.1 ± 3.6
End (AU)	0.0 ± 4.4	2.5 ± 4.9	0.0 ± 4.9	1.0 ± 3.4
TOI				
Baseline (%)	65.3 ± 3.4	63.4 ± 3.3*	68.2 ± 4.3	70.1 ± 5.8
60 s (%)	61.9 ± 4.9	57.7 ± 5.0*	64.6 ± 6.5	65.6 ± 8.5
120 s (%)	61.9 ± 4.8	57.1 ± 5.7*	64.8 ± 6.1	65.6 ± 8.8
240 s (%)	60.7 ± 6.6	58.1 ± 4.8*	64.8 ± 6.5	65.8 ± 8.9
End (%)	61.4 ± 6.4	57.8 ± 5.0	65.3 ± 6.3	65.8 ± 8.9
	Ramp incremental exercise			
[HHb]				
Baseline (AU)	-6.2 ± 4.1	-3.4 ± 3.6	-5.1 ± 4.1	-2.6 ± 2.5
120 s (AU)	-3.3 ± 5.4	-0.1 ± 5.0	-2.7 ± 5.0	-0.7 ± 3.3
240 s (AU)	-0.8 ± 6.2	3.3 ± 5.8*	-0.6 ± 5.8	1.4 ± 4.4
360 s (AU)	2.0 ± 9.4	7.3 ± 9.1*	1.5 ± 6.6	3.4 ± 5.8
End (AU)	6.2 ± 11.3	12.8 ± 10.1*	3.8 ± 7.6	5.3 ± 7.2
TOI				
Baseline (%)	66.5 ± 3.9	67.3 ± 7.1	71.5 ± 3.9	72.5 ± 4.7
120 s (%)	63.3 ± 5.1	64.6 ± 8.6	68.6 ± 5.5	69.5 ± 6.9
240 s (%)	60.8 ± 6.5	60.7 ± 9.2	65.8 ± 7.5	65.9 ± 9.7
360 s (%)	57.3 ± 11.5	55.4 ± 12.3	61.9 ± 8.6	61.7 ± 11.4
End (%)	49.5 ± 12.6	47.6 ± 14.9	57.1 ± 7.0	57.2 ± 10.9

Values are mean ± SD. PL, Placebo group; BR, Nitrate group; Pre, pre-donation; Post, post-donation; [HHb], deoxygenated haemoglobin concentration; TOI, tissue oxygenation index; AU, arbitrary units. *Significantly different from pre in the same condition ($P < 0.05$).

Figure 1.

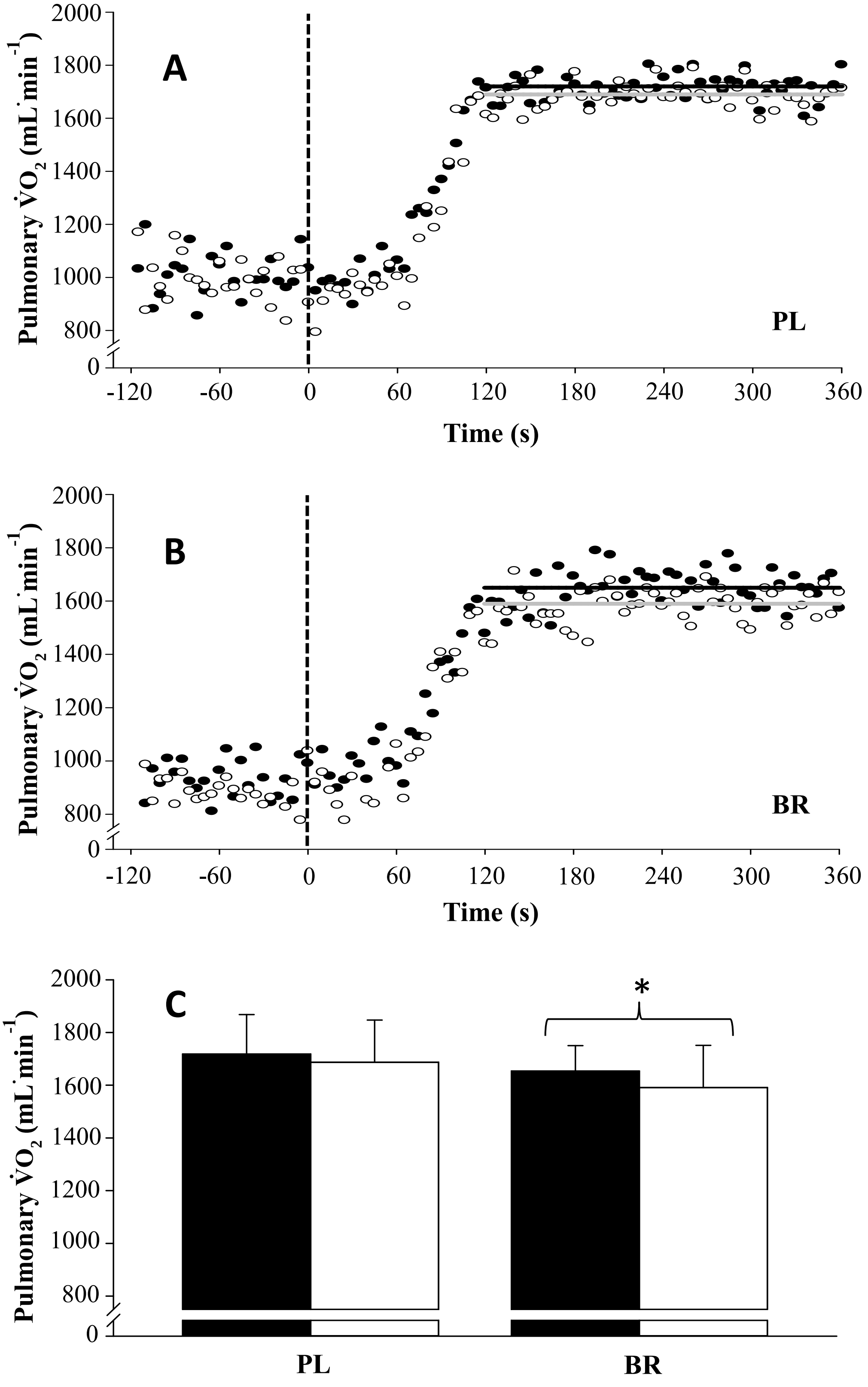
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Figure 2.

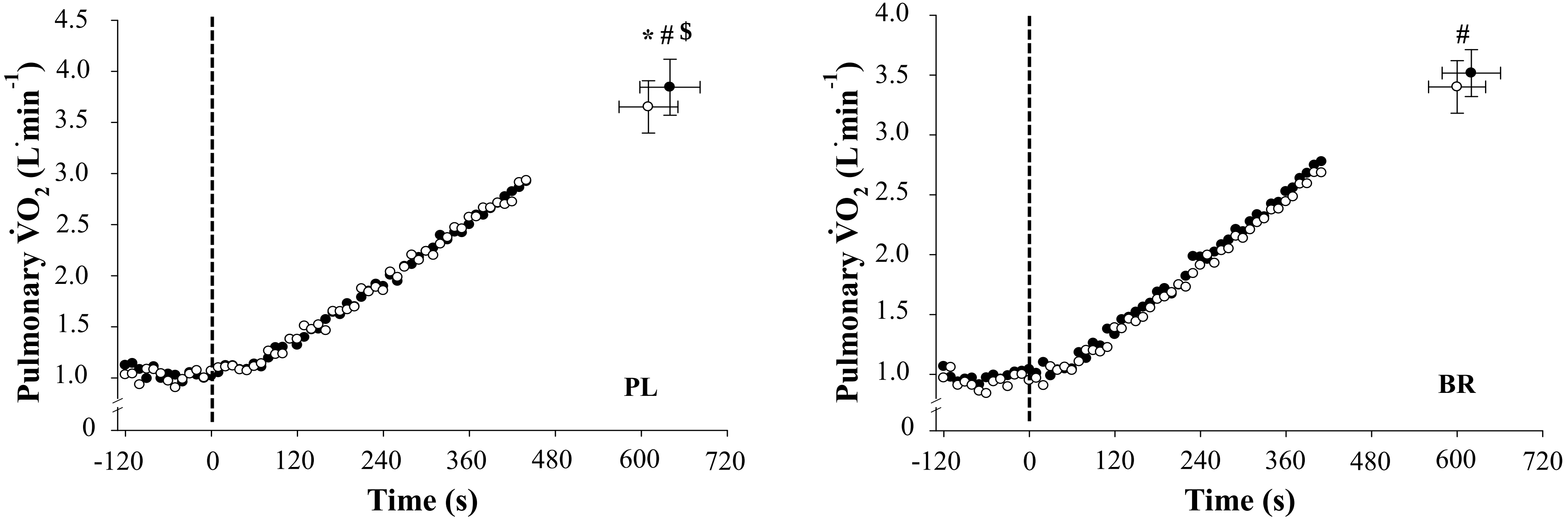
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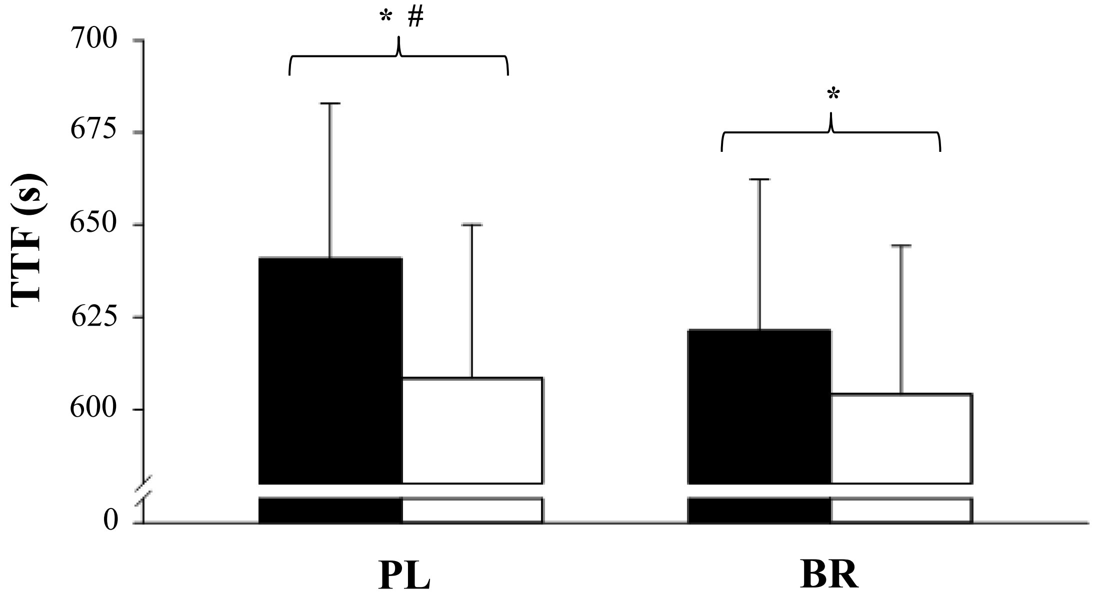
Figure 3.



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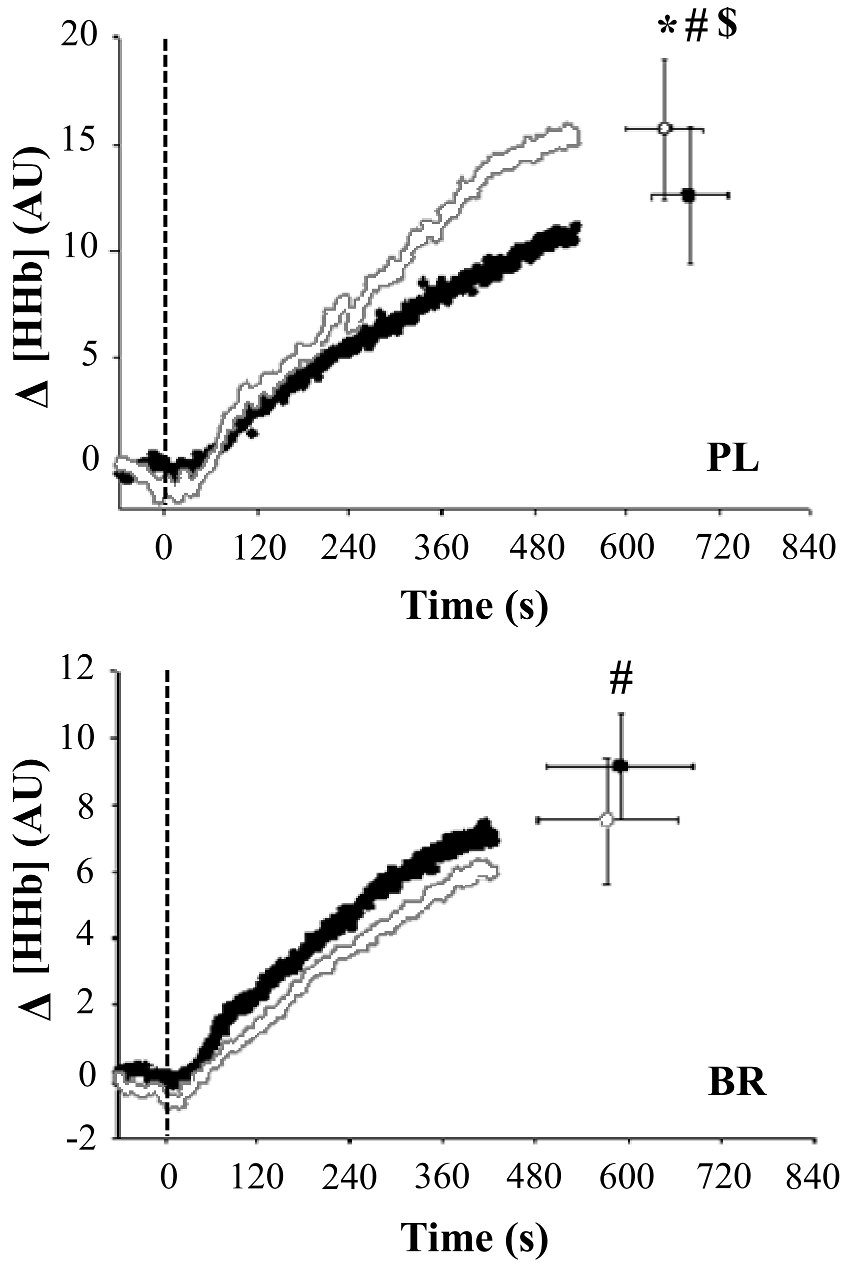
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Δ [HHb] (AU)
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Time (s)
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BR
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120
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Time (s)

Influence of dietary nitrate food forms on nitrate metabolism and blood pressure in healthy normotensive adults

Original Article

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Chapter 6: Influence of dietary nitrate food forms on nitrate metabolism and blood pressure in healthy normotensive adults

ABSTRACT

Inorganic nitrate (NO_3^-) supplementation has been shown to improve cardiovascular health indices in healthy adults. The purpose of this study was to investigate how the vehicle of NO_3^- administration can influence NO_3^- metabolism and the subsequent blood pressure response. Ten healthy males consumed an acute equimolar dose of NO_3^- (~ 5.76 mmol) in the form of a concentrated beetroot juice drink (BR; 55 mL), a non-concentrated beetroot juice drink (BL; 456 mL) and a solid beetroot flapjack (BF; 60 g). A drink containing soluble beetroot crystals (BC; ~ 1.40 mmol NO_3^-) and a control drink (CON; 70 mL deionised water) were also ingested. BP and salivary, plasma and urinary [NO_3^-] and [NO_2^-] were determined before and up to 24 h after ingestion. All NO_3^--rich vehicles elevated salivary, plasma and urinary nitric oxide metabolites compared with baseline and CON ($P < 0.05$). The peak increases in plasma [NO_2^-] were greater in BF (371 ± 136 nM) and BR (369 ± 167 nM) compared to BL (283 ± 93 nM; all $P < 0.05$) and BC (232 ± 51 nM). BR, but not BF, BL and BC, reduced systolic (~ 5 mmHg) and mean arterial pressure (~ 3-4 mmHg; $P < 0.05$), whereas BF reduced diastolic BP (~ 4 mmHg; $P < 0.05$). Although plasma [NO_2^-] was elevated in all conditions, the consumption of a small, concentrated NO_3^--rich fluid (BR) was the most effective means of reducing BP. These findings have implications for the use of dietary NO_3^- supplements when the main objective is to maintain or improve parameters of cardiovascular health.

Word count: 247

Key words: dietary nitrate, nitrite, blood pressure, pharmacokinetics, cardiovascular health

1.1 INTRODUCTION

Nitric oxide (NO), an important signalling molecule, plays an essential role in the regulation of physiological processes, such as blood flow distribution (Shen et al., 1994), blood pressure (BP) control (Webb et al., 2008), muscle contractility, mitochondrial respiration, and glucose and calcium homeostasis (Stamler & Meissner, 2001). NO was previously thought to be generated exclusively via oxidation of the amino acid, L-arginine, in a reaction catalysed by a family of NO synthase (NOS) enzymes (Stuehr et al., 1991). However, it was later found that nitrate (NO_3^-) and nitrite (NO_2^-), originally known as inert end products of endogenous NO production (Moncada & Higgs, 1993), can be recycled *in vivo* to form NO, particularly in hypoxic and acidic environments (van Faassen et al., 2009; Modin et al., 2001).

NO_3^- can also be obtained through the diet, in the form of green leafy vegetables, beetroot juice and salts (Bryan & Hord, 2010) and can be serially reduced to form NO_2^- and NO in a manner that does not depend on NOS activity (Benjamin et al., 1994). Once ingested, NO_3^- is rapidly absorbed in the upper gastrointestinal tract (van Velzen et al., 2008) and approximately one quarter passes into the entero-salivary circulation and is concentrated in the saliva (Lundberg & Govoni, 2004; Spiegelhalder et al., 1976). Facultative anaerobes in the oral cavity reduce NO_3^- to NO_2^- (Duncan et al., 1995) and when swallowed, this NO_2^- can be further reduced to NO and other reactive nitrogen intermediates in the acidic environment of the stomach (Benjamin et al., 1994). It is also clear that a small portion of the NO_2^- can be absorbed into the systemic circulation where it can either directly (Dejam et al., 2004) or indirectly, via its reduction to NO (van Velzen et al., 2008), mediate physiological effects, such as the lowering of BP (e.g.

Ashworth et al., 2015; Bailey et al., 2009; Larsen et al., 2006; Vanhatalo et al., 2010; Webb et al., 2008). With hypertension [systolic blood pressure (SBP) / diastolic blood pressure (DBP); > 140/90 mmHg] being the leading cause of cardiovascular morbidity and mortality and affecting over one billion people worldwide (Chobanian et al., 2003; Wang et al., 2006), dietary NO_3^- consumption has emerged as a potential prophylactic means for reducing the risk of high BP, stroke and coronary heart disease (Joshipura et al., 2001). Studies have reported that both acute and chronic ingestion of NO_3^- can reduce BP (Ashworth et al., 2015; Bailey et al., 2009; Larsen et al., 2006; Vanhatalo et al., 2010; Webb et al., 2008), an effect that is closely related to the rise in plasma NO_2^- concentration ($[NO_2^-]$). While it has been reported that the increase in plasma $[NO_2^-]$ and reductions in BP after NO_3^- ingestion follow a dose-response relationship (Kapil et al., 2010; Wylie et al., 2013), little is known about other factors that may also influence the increase in NO bioavailability and cardiovascular health benefits of NO_3^- consumption.

NO_3^- can be administered in many different forms. Studies have previously reported an increase in NO metabolites and a reduction in BP after the consumption of NO_3^- via non-concentrated beetroot juice (250-500 mL; e.g. Webb et al., 2008; Vanhatalo et al., 2010), concentrated beetroot juice (70-140 mL; e.g. Wylie et al., 2013), beetroot bread (Hobbs et al., 2012), NO_3^--rich whole green vegetables (Ashworth et al., 2015) and their juices (Jonvik et al., 2016), Swiss chard and rhubarb extract gels (Muggeridge et al., 2014) and capsulated NO_3^- salts (Kapil et al., 2010). The dietary NO_3^- vehicle may potentially influence NO_3^- metabolism and the subsequent lowering of BP, by, for example, altering the uptake of NO_3^- and/or NO_2^- into the systemic circulation. However, while the aforementioned studies demonstrate that different dietary NO_3^-

vehicles are effective at increasing NO bioavailability and lowering BP, inter-study differences in the baseline BP of the participants and the dose of NO_3^- administered does not allow for the influence of the NO_3^- vehicle, *per se*, to be elucidated. Recently, McIlvenna et al. (2017) reported that the plasma pharmacokinetic profile for plasma [NO_3^-] and [NO_2^-] and changes in BP were similar over a 6 h period following the ingestion of equimolar doses of a NO_3^--rich chard gel and a concentrated beetroot juice. In addition, Flueck et al. (2016) reported that reductions in resting BP and O_2 consumption during moderate-intensity exercise were somewhat greater when an equimolar dose of NO_3^- was administered as concentrated beetroot juice compared to $NaNO_3$. Similarly, Jonvik et al. (2016) found that beverages made from beetroot, spinach and rocket raised plasma [NO_3^-] and [NO_2^-] to a similar extent but reduced SBP to a greater extent than a beverage containing $NaNO_3$. However, no study to date assessed the plasma [NO_3^-] and [NO_2^-] and BP responses to the consumption of a range of different, commercially-available NO_3^--rich products, including those in solid and liquid forms over a 24 h period. Establishing if there are differences in the physiological response to the various available dietary NO_3^- food forms is important for informing the optimal supplementation strategy for beneficial effects.

Therefore, the purpose of this study was to determine the pharmacodynamic and pharmacokinetic response to an equimolar dose of NO_3^- administered in three different NO_3^- vehicles using the same subject population. Specifically, we examined plasma, salivary and urinary [NO_3^-] and [NO_2^-] and the BP response to the acute consumption of an equimolar dose of NO_3^- (~ 5.76 mmol) administered in the form of a low-volume NO_3^--rich beetroot juice concentrate (BR; 55 mL), a high volume non-concentrated beetroot juice drink (BL; 456 mL), and a solid beetroot flapjack (BF; 60 g; all Beet It, James White Drinks Ltd, Ipswich, UK). We also examined the same variables after the consumption of diluted beetroot crystals (BC; 114 mL) SuperBeets, Neogenis, now known as HumanN, Texas, US) which, at the dose recommended by the manufacturer, contains a lower NO_3^- (1.40 mmol) content than the other products but also a small amount of NO_2^- (~ 0.07 mmol). We also took the opportunity to assess the validity of a commonly used non-invasive test for estimating NO availability (NO Test Strips, Berkeley Test®, CA, USA), by comparing its results with determinations of salivary and plasma $[NO_3^-]$ and $[NO_2^-]$ derived via the gold standard chemiluminescence technique. It was hypothesised that relative to pre-supplementation baseline and a water control (CON), BR, BL, BF and BC would result in significant elevations in plasma, salivary and urinary $[NO_3^-]$ and $[NO_2^-]$ and reductions in systemic BP, but that such changes may vary between the different vehicles.

1.2 METHODS

1.2.1 Subjects

Ten healthy, normotensive (mean ± SD; resting systolic BP (SBP) 112 ± 9 mmHg, diastolic BP (DBP) 66 ± 6 mmHg, mean arterial pressure (MAP) 81 ± 6 mmHg) males (age 24 ± 5 years, body mass 74 ± 8 kg, height 1.77 ± 0.10 m) volunteered to participate in this study. None of the subjects habitually smoked tobacco, consumed dietary supplements or used antibacterial mouthwash. The study was approved by the University of Exeter Research Ethics Committee. Prior to testing and after the requirements of the study and potential risks and benefits of participation were explained, written informed consent was obtained.

Subjects were instructed to arrive at the laboratory in a fully rested and hydrated state, at least 8 h post prandial. Subjects were asked to avoid caffeine consumption and participation in strenuous exercise in the 24 h period prior to each laboratory visit. All subjects were also asked to refrain from alcohol consumption and the use of antibacterial mouthwash and chewing gum for the duration of the study. Each subject recorded diet and exercise undertaken during the 24 h period post ingestion of the first supplement and were asked to replicate these during the remaining four supplementation periods. Subjects were asked to abstain from high NO_3^- foods throughout each 24 h supplementation period. Exclusion criteria were the presence of known cardiovascular disease and hypertension and the use of antihypertensive medication and antibiotics.

1.2.2 Experimental Overview

Subjects were asked to attend the laboratory on ten separate occasions over a three week period. Prior to the first visit to the laboratory, each subject was randomly assigned, in a single-blind, crossover fashion to consume an acute dose of ~ 5.76 mmol dietary NO_3^- in the form of a concentrated beetroot drink (55 mL of Beet It Sport Stamina Shot, James White Drinks, Ltd., Ipswich, UK), a non-concentrated beetroot drink (456 mL of Beet It Organic Beetroot Juice, James White Drinks, Ltd., Ipswich, UK) and a beetroot flapjack (60 g of Beet It Pro Elite Sport Flapjack, James White Drinks, Ltd., Ipswich, UK). In addition, subjects consumed the recommended dose (5 g dissolved in 114 mL of water; 1.40 mmol NO_3^- and ~ 0.07 mmol NO_2^-) of Concentrated Organic Beetroot Crystals (SuperBeets Canister; Neogenis, now known as HumanN, Texas, US), and a control drink (70 mL deionised water) which contained negligible NO_3^- and NO_2^- content.

Owing to the NO_2^- content of the SuperBeets product, we did not attempt to match the NO_3^- content of this product with the other products tested in this study but rather provided the supplement as per the manufacturer's instructions. All 10 subjects completed BR, BL, BF, BC and CON conditions, with each acute supplementation being separated by a minimum of a 48 h wash-out period. All supplements were presented to the subjects at room temperature. On the day of supplementation BR, BC and CON were consumed immediately after instruction, whereas BL and BF were ingested at regular intervals over a 5 and 10 min period, respectively. During each visit, saliva, blood and urine samples were collected and BP and indirect measures of NO availability (Berkeley Test®, CA, USA) were recorded prior to and over the 24 h period post NO_3^- ingestion. All tests began at the same time of day, typically at 9 am (± 1 h), to minimise diurnal variation on the physiological variables under investigation. The personnel performing the physiological measurements were not aware of the type of supplement being consumed by the subjects.

1.2.3 Experimental Protocol

Throughout each 24 h experimental period, a low NO_3^- diet was provided, water consumption was standardised and subjects were asked to remain seated in the laboratory during the first 6 h to avoid influencing the physiological variables under investigation. During each visit to the laboratory, BP of the brachial artery and heart rate (HR) were measured using an automated sphygmomanometer (Dinamap Pro; GE medical Systems, Tampa, FL, USA) prior to NO_3^- supplementation and at 1, 2, 3, 4, 5, 6 and 24 h post NO_3^- supplementation. Five BP and HR measurements were recorded following each 10 min period of seated rest in a quiet room and the mean of the final four measurements were used for data analysis.

Blood samples were obtained prior to ingestion of NO_3^- and at 15 min, 30 min, 1, 2, 3, 4, 5 and 6 h post ingestion. All samples (~ 6 mL) were drawn from a cannula (Insyte-WTM, Becton-Dickinson, Madrid, Spain) inserted into the subject’s antecubital vein. At 24 h, a single, resting venous blood sample (~ 6 mL) was drawn from an antecubital vein. All samples were drawn into lithium-heparin tubes (Vacutainer, Becton-Dickinson, NJ, USA) and centrifuged for 10 min at 3000 *g* and 4 ºC within 2 min of collection and the plasma was then extracted. Saliva samples (~ 1 mL) were collected by expectoration, without stimulation, over a period of 5 min before NO_3^- consumption and at 1, 2, 3, 4, 5, 6 and 24 h post consumption. Indirect measures of NO bioavailability were measured at the same time points using NO Test Strips (Berkeley Test®, CA, USA), as per the manufacturer’s guidelines. Specifically, the saliva collection pad on the NO Test Strip was used to swab the tongue and oral cavity over a 5 s period. The two ends of the strip were then folded in half and the saliva collection pad was pressed firmly against the NO test pad (on the opposite end of the strip) for 10 s. The NO test pad was then monitored for changes in colour and after 45 s, the intensity of the colour displayed on the NO test pad was compared with the associated colour chart on the packaging (Berkeley Test®, CA, USA) and recorded. The NO Test Strips are based on a modified Griess reagent reaction, which identifies the presence of NO_2^- in the saliva. The changes in colour are, in theory, directly proportional to increases in salivary NO_2^- (i.e. the darker the pink colour displayed on the Test Strip, the more salivary NO_2^- present) and categorised as depleted, low, threshold, target and high levels of NO bioavailability. In addition, midstream urine samples were collected at baseline and at 3, 6 and 24 h post NO_3^- ingestion.

Plasma, saliva and urine samples were frozen at -80 ºC for later determination of [NO_3^-] and [NO_2^-] using a modified chemiluminescence technique, as previously described (Wylie et al., 2013a). Briefly, [NO_2^-] was determined by its reduction to NO in the presence of acetic acid and sodium iodide. [NO_3^-] was determined by the reduction of NO metabolites ([NO_x] = [NO_3^-] + [NO_2^-]) to NO in the presence of vanadium (III) chloride and hydrochloric acid and the subsequent subtraction of [NO_2^-]. Immediately prior to analysis of saliva (for [NO_3^-] and [NO_2^-]) and urine (for [NO_3^-] only) samples were centrifuged for 10 min at 18,600 *g*. The supernatants were then removed and diluted by a factor of 100 with NO_3^- and NO_2^- free deionised water. The same data collection protocol was repeated during each visit to the laboratory.

1.2.4 Statistical Analyses

Differences in BP, HR, NO indicator strip results and plasma, salivary and urinary [NO_3^-] and [NO_2^-] were assessed using a 2-way (condition x time) repeated-measures ANOVA. Significant main and interaction effects were further explored using Fisher's LSD. Relationships between variables were assessed via Pearson's product-moment correlation coefficient. Statistical analyses were performed using SPSS version 19.0 (Chicago, IL, USA). Plasma [NO_3^-] and [NO_2^-] incremental area under the curve (iAUC) from baseline until 6 h (0 – 6 h) was calculated for BR, BL, BF and BC using the trapezium model (GraphPad Prism, GraphPad, San Diego, CA). Differences in iAUC between conditions were assessed using a 1-way ANOVA with significant main effects further explored using Fisher's LSD. Data are presented as mean ± SD, unless otherwise stated. Statistical significance was accepted at $P<0.05$.

1.3 RESULTS

All subjects reported that they adhered to the prescribed dietary regime and abstained from physical activity during each of the 24 h experimental periods. The ingestion of the four NO_3^- vehicles were well tolerated with no negative side effects. Subjects did, however, report beeturia (red urine) after BL consumption only.

The plasma, salivary and urinary $[NO_3^-]$ and $[NO_2^-]$ prior to and post ingestion of all supplements is presented in Fig. 1.

1.3.1 Salivary $[NO_3^-]$ and $[NO_2^-]$

There were significant main effects for time and condition and interaction effects for salivary $[NO_3^-]$ and $[NO_2^-]$ ($P < 0.05$). At baseline, there were no differences in salivary $[NO_3^-]$ and $[NO_2^-]$ between the conditions ($P > 0.05$). The elevations in salivary $[NO_3^-]$ in BR, BL, BF and BC were significantly higher than CON 1 h post ingestion and remained elevated above CON until 6 h post ingestion ($P < 0.05$). Salivary $[NO_3^-]$ was also higher in BR and BL 24 h after consumption when compared with CON ($P < 0.05$). The peak elevation above baseline in salivary $[NO_3^-]$ occurred at 1 h (5.52 ± 1.23 mM) post administration of BR and this rise in salivary $[NO_3^-]$ was significantly higher than BF (3.61 ± 1.30 mM) and BC (1.21 ± 0.36 mM) at the same time point ($P < 0.05$). Salivary $[NO_3^-]$ remained higher in BR compared to BF and BC at 2, 3, 4 and 6 h post ingestion ($P < 0.05$) Salivary $[NO_3^-]$ was also lower in BC compared with all other

NO_3^--rich conditions at 1, 2, 3, 4, and 5 h post consumption and at 6 h when compared with BL ($P < 0.05$). The peak elevation above baseline in salivary [NO_3^-] occurred at 2 h in BL (4.53 ± 1.59 mM), BF (3.73 ± 1.49 mM) and BC (1.65 ± 0.99 mM). At 4 h, the rise in salivary [NO_3^-] was significantly higher in BR (4.19 ± 1.92 mM) when compared with BL (3.36 ± 1.52 mM; $P < 0.05$).

The increases in salivary [NO_2^-] in BR, BL, BF and BC were significantly higher than CON at 2 h and remained elevated above CON until 6 h post ingestion ($P < 0.05$). The peak elevation above baseline in salivary [NO_2^-] occurred at 2 h in BR (0.63 ± 0.53 mM), BL (0.49 ± 0.38 mM), BF (0.34 ± 0.22 mM) and BC (0.14 ± 0.08 mM). The rise in salivary [NO_2^-] was significantly higher in BR versus BF at 1, 3, 4 and 6 h post ingestion ($P < 0.05$). In addition, the rise in salivary [NO_2^-] was higher in BL (0.40 ± 0.26 mM) versus BF (0.29 ± 0.20 mM) at 1 h, and BR was higher than BL at 5 h (0.47 ± 0.36 vs. 0.25 ± 0.18 mM) and 6 h (0.49 ± 0.27 vs. 0.27 ± 0.23 mM) post NO_3^- consumption ($P < 0.05$). Salivary [NO_2^-] was lower in BC when compared with all other NO_3^--rich conditions from 1-6 h post ingestion ($P < 0.05$).

1.3.2 Plasma [NO_3^-] and [NO_2^-]

There were significant main effects for time and condition and interaction effects for plasma [NO_3^-] and [NO_2^-] ($P < 0.05$). At baseline, there were no differences in plasma [NO_3^-] and [NO_2^-] between the conditions ($P > 0.05$). Plasma [NO_3^-] was significantly elevated in BR, BL and BF when compared with CON from 15 min to 24 h post NO_3^- ingestion ($P < 0.05$). Plasma [NO_3^-] was also significantly higher in BC when compared with CON from 30 min – 6 h post ingestion ($P < 0.05$). The peak elevation above baseline in plasma [NO_3^-] occurred at 2 h in BR (270 ± 40 µM), BL (228 ± 32 µM), BF (211 ± 28 µM) and BC (99 ± 20 µM). Plasma [NO_3^-] in BR was higher than in BL (15 min – 5 h; $P < 0.05$), BF (15 min – 6 h; $P < 0.05$) and BC (15 min - 24 h; $P < 0.05$). In addition, plasma [NO_3^-] was significantly higher in BL compared to BF at 15 min (98 ± 30 µM vs. 57 ± 22 µM) and 1 h (200 ± 44 µM vs. 167 ± 30 µM) post NO_3^- consumption ($P < 0.05$). Plasma [NO_3^-] was lower in BC when compared with all other NO_3^--rich conditions from 15 min-24 h post ingestion ($P < 0.05$). Plasma [NO_3^-] iAUC 0-6 h was significantly greater in BR (67.6 ± 10.3 µM • min), BL (52.3 ± 7.5 µM • min) and BF (48.9 ± 7.2 µM • min) compared to BC (11.7 ± 3.9 µM • min; all $P < 0.05$). Furthermore, plasma [NO_3^-] iAUC 0-6 h was greater in BR compared to BF and BL (both $P < 0.05$), and tended to be greater in BL compared to BF ($P = 0.053$).

Plasma [NO_2^-] was significantly elevated in BL (125 ± 44 nM) compared with CON (76 ± 16 nM) and BR (98 ± 43 nM) at 15 min post ingestion ($P < 0.05$). In addition, plasma [NO_2^-] was higher in BC versus all conditions at 15 min and versus BL and BR at 30 min post ingestion ($P < 0.05$). Plasma [NO_2^-] was also higher in BR, BL and BF when compared with CON from 30 min until 6 h post supplement ingestion ($P < 0.05$). The peak elevation above baseline in plasma [NO_2^-] occurred at 15 min in BC (232 ± 51 nM), at 2 h in BF (371 ± 136 nM) and at 3 h in BR (369 ± 167 nM) and BL (283 ± 93 nM). The rise in plasma [NO_2^-] was significantly lower in BL when compared with BF at 1 and 2 h post ingestion and when compared with BR at 4 h post ingestion ($P < 0.05$). In addition, plasma [NO_2^-] was lower in BC when compared with all other NO_3^--rich conditions from 2 - 5 h post ingestion and lower at 1 and 6 h compared with BR and BF and BR and BL, respectively ($P < 0.05$). Plasma [NO_2^-] iAUC 0-6 h was significantly greater in BR (72.2 ± 49.7 µM • min), BL (54.4 ± 28.9 µM • min) and BF (70.9 ± 46.3

µM • min) compared to BC (28.4 ± 17.8 µM • min; all $P < 0.05$). There was no difference in plasma [NO_2^-] iAUC 0-6 h between BR and BF, but plasma [NO_2^-] iAUC tended to be greater in BR and BF compared to BL (both $P = 0.08$).

1.3.3 Urinary [NO_3^-] and [NO_2^-]

There were significant main effects for time and condition and interaction effects for urinary [NO_3^-] and [NO_2^-] ($P < 0.05$). Urinary [NO_3^-] was significantly elevated in BR, BL, BF and BC compared with CON at 3 and 6 h post ingestion ($P < 0.05$). Peak increases above baseline in urinary [NO_3^-] occurred at 6 h in BR (5.63 ± 2.18 mM), BL (4.43 ± 1.81 mM), BF (4.23 ± 1.66 mM) and BC (1.63 ± 0.37 mM). Urinary [NO_3^-] was lower in BC when compared with all other NO_3^--rich conditions at 3 and 6 h post ingestion and at 24 h when compared with BR ($P < 0.05$).

Urinary [NO_2^-] was significantly elevated in BR, BL, BF and BC when compared with CON at 3 and 6 h post NO_3^- ingestion ($P < 0.05$). Urinary [NO_2^-] was also elevated in BR at 24 h post ingestion when compared with CON ($P < 0.05$). Peak elevations above baseline in urinary [NO_2^-] occurred at 3 h in BR (7.54 ± 4.76 µM), BL (10.1 ± 6.15 µM), BF (2.98 ± 1.26 µM) and BC (3.68 ± 10.5 µM). However, the peak rise in urinary [NO_2^-] was significantly lower in BF when compared with BR and BL ($P < 0.05$) and BC was lower than BR and BL ($P < 0.05$). In addition, at 6 h post ingestion the rise in urinary [NO_2^-] in BL (6.14 ± 2.65 µM) was significantly higher than BR (2.72 ± 1.00 µM), BF (2.91 ± 2.31 µM) and BC (1.38 ± 0.48 µM), ($P < 0.05$). BR consumption also resulted in a significant elevation in urinary [NO_3^-] and [NO_2^-] at 24 h when compared with CON and BL ($P < 0.05$).

1.3.4 Nitric Oxide Indicator Strips

The NO indicator strip results in response to acute ingestion of BR, BL, BF and BC are presented in Fig. 2. There were significant main effects for time and condition and an interaction effect for the NO indicator strips ($P < 0.05$). At baseline, there were no differences in NO indicator strip results between the conditions ($P > 0.05$). Consumption of BR, BL, BF and BC resulted in a significant elevation in NO indicator strip results from 1 – 6 h post ingestion when compared with baseline ($P < 0.05$). Peak increases above pre-supplementation baseline in the NO indicator strips occurred at 1 h in BR (Target) and at 2 h in BL (Threshold), BF (Threshold) and BC (Low). The NO strip results were higher at 1, 2, 3, 4, 5 and 6 h post BR, BL, BF and BC when compared with CON. At 24 h, NO indicator results were also higher in BL than CON ($P < 0.05$). In addition, at 1 h, the rise in NO indicator strip result was higher in BR (Target) when compared with BL (Threshold). NO strip results were lower in BC when compared with all other NO_3^--rich conditions at 2 and 4 h post ingestion and when compared with BR and BL at 1, 3 and 5 h post ingestion ($P < 0.05$). There was a significant correlation between the change in plasma [NO_2^-] and the change in NO indicator strip result across all conditions (r = 0.48, $P < 0.05$). There was also a significant correlation between salivary [NO_2^-] and the NO indicator strip result across all conditions (r = 0.57, $P < 0.05$).

1.3.5 Blood Pressure and Heart Rate

The change in SBP and MAP following the acute ingestion of the four NO_3^- vehicles are displayed in Fig. 3. There was a significant main effect for time and condition and an interaction effect for SBP ($P < 0.05$). In the BR condition, SBP was significantly reduced from baseline (115 ± 10 mmHg) to 3 h post ingestion (110 ± 9 mmHg; $P < 0.01$). The change in SBP in the BR condition was significantly greater when compared with CON from 1 h until 5 h post NO_3^- ingestion ($P < 0.05$). In addition, the reduction in SBP in the BR condition was significantly greater when compared with BF, BL and BC at 3 h post ingestion ($P < 0.05$). BR also resulted in a greater reduction in SBP when compared with BL and BC at 1 and 2 h post ingestion, respectively ($P < 0.05$). There was a significant correlation between the change in SBP and the change in plasma [NO_2^-] in BR (r = -0.29, $P < 0.05$). There was a significant main effect for time ($P < 0.05$), but no main effect for condition ($P > 0.05$) or an interaction effect for DBP ($P > 0.05$). Follow up analyses revealed that at 1 h, DBP in BR (63 ± 5 mmHg) and BF (62 ± 6 mmHg) was significantly lower than pre-supplementation baseline (BR: 66 ± 5 mmHg; BF 66 ± 7 mmHg) and CON (65 ± 8 mmHg; all $P < 0.05$). An interaction effect was noted for MAP ($P<0.05$). Follow up analyses revealed a significant reduction in MAP in the BR condition at 1 h (80 ± 6 mmHg) and 3 h (79 ± 6 mmHg) when compared with baseline (83 ± 6 mmHg; $P < 0.05$) and CON (1 h; 81 ± 8 mmHg, 3 h; 82 ± 9 mmHg; $P < 0.05$). In addition, MAP was lower 1 h after BF ingestion and 4 h after BR ingestion when compared with CON ($P < 0.05$).

There was a significant main effect for time ($P < 0.05$) and an interaction effect for HR ($P < 0.05$). There were no differences in HR between conditions at baseline ($P > 0.05$). However, HR was elevated in CON (65 ± 8 $b{\cdot}min^{-1}$), BR (68 ± 9 $b{\cdot}min^{-1}$) and BF (65 ± 5 $b{\cdot}min^{-1}$) at 1 h post ingestion when compared with baseline (CON: 61 ± 7; BR: 62 ± 8; BF: 62 ± 7 $b{\cdot}min^{-1}$; all $P < 0.05$). BR and BF ingestion also resulted in an elevated HR at 5 h (67 ± 8 $b{\cdot}min^{-1}$; $P < 0.05$) and 6 h (65 ± 7 $b{\cdot}min^{-1}$; $P < 0.05$) post consumption, respectively. In contrast, HR was reduced at a number of time points across the 24 h period in the CON, BL and BC conditions (*P < 0.05*).

1.4 DISCUSSION

This study is the first to compare the pharmacodynamic and pharmacokinetic response to an equimolar dose of NO_3^- administered in several different forms over 24 h as well as the ingestion of a small dose of NO_2^--containing crystals in another commercially-available supplement. We investigated the influence of an acute dose of dietary NO_3^- (~ 5.76 mmol NO_3^-) in the form of a small concentrated volume of fluid (BR), a large non-concentrated volume of fluid (BL), a solid (BF; flapjack) and dissolvable crystals (BC; 1.40 mmol NO_3^-) on plasma, salivary and urinary [NO_3^-] and [NO_2^-] and on resting BP over a 24 h period. The principal finding in this study was that the ingestion of concentrated beetroot juice was the most effective means of increasing markers of NO bioavailability and reducing systemic BP when compared with non-concentrated beetroot juice, beetroot flapjack and beetroot crystals.

1.4.1 Salivary [NO_3^-] and [NO_2^-]

The NO_3^- supplements were successful in elevating salivary [NO_3^-] and [NO_2^-] over a 24 h period. The largest elevation in salivary [NO_3^-] occurred 1 h after concentrated beetroot juice-ingestion, with there being a ~ 700 % increase above baseline. The peak elevation in non-concentrated beetroot juice, beetroot flapjack and beetroot crystals occurred at 2 h post ingestion, increasing by ~ 670, ~ 270 and ~ 130 % above baseline values, respectively. The kinetic profiles for salivary [NO_3^-] were similar to those for plasma [NO_3^-], with concentrated beetroot juice providing the greatest increase in plasma [NO_3^-], followed by non-concentrated beetroot juice, beetroot flapjack and then beetroot crystals. This similarity suggests that the differences in salivary [NO_3^-] may have been mediated by between-vehicle differences in absorption of NO_3^- into the systemic circulation. Specifically, these data imply that the absorption of NO_3^- into the systemic circulation was greater when NO_3^- was ingested in a fluid, compared to a solid, food form and that this difference subsequently altered the concentration of NO_3^- in saliva. It is possible that the differences in NO_3^- absorption may be explained, in part, by the different rates of gastric emptying between liquids and solids (Jian et al., 1979). However, if gastric emptying were to play an important role in salivary NO bioavailability, it may have been expected that the larger, non-concentrated volume of fluid would have increased the rate of gastric emptying and resulted in a faster time to peak salivary [NO_3^-] compared to that of the concentrated fluid (Noakes et al., 1991), which was not the case in the present study. It is important to note here that the smaller salivary [NO_3^-] elevation in the beetroot crystal condition was likely due to the smaller dose of NO_3^- administered when compared with the other NO_3^--rich vehicles (1.40 vs. 5.76 mmol NO_3^-).

Consistent with earlier reports that there is a direct relationship between dietary NO_3^- consumption and salivary [NO_2^-] (Spiegelhalder et al., 1976), concentrated beetroot juice, non-concentrated beetroot juice and beetroot flapjack increased salivary [NO_2^-] to a similar extent in the present study, peaking at 2 h post consumption and following a similar kinetic pattern to salivary [NO_3^-] over the time course of the experiment. In contrast, the smaller dose of NO_3^-administered in the beetroot crystal condition resulted in a smaller rise in salivary [NO_2^-] when compared with the other vehicles. McDonagh et al. (2015) have also shown increases in salivary [NO_3^-] and [NO_2^-] after chronic ingestion of the same concentrated beetroot juice shot but the magnitude of increase was much greater than in the present study, which may be linked to the longer supplementation period.

The peak rise in salivary [NO_2^-] was lower with the beetroot flapjack compared to concentrated beetroot juice and non-concentrated beetroot juice, but no difference in salivary [NO_2^-] was found between non-concentrated beetroot juice and concentrated beetroot juice. These salivary [NO_2^-] responses mirror those of salivary [NO_3^-], suggesting that the increase in salivary [NO_2^-] following the consumption of each supplement is proportional to the amount of NO_3^- available for bacterial reduction to NO_2^- in the oral cavity. However, it is important that other factors that may contribute to the differences in salivary [NO_2^-] response between conditions are not disregarded. Indeed, salivary flow-rate, oral pH and temperature and the activity of bacterial NO_3^- reductases (Djekoun-Bensoltane et al., 2007) have the potential to alter the salivary [NO_2^-] response following NO_3^- ingestion, and it is possible that some of these may have been altered by the ingestion of the different NO_3^- vehicles.

1.4.2 *Plasma [NO_3^-] and [NO_2^-]*

The acute consumption of concentrated beetroot juice, non-concentrated beetroot juice, beetroot flapjack and beetroot crystals elevated plasma [NO_3^-] and [NO_2^-] above baseline values across the 24 h period. Peak plasma [NO_3^-] occurred at 2 h post ingestion, rising by ~ 680, 530, 500 and 150 % in concentrated beetroot juice, beetroot flapjack, non-concentrated beetroot juice and beetroot crystals, respectively. Previous studies have reported similar increases (~ 400 – 600 %) in plasma [NO_3^-] after supplementation with NO_3^- salts (Bescós et al., 2012; Larsen et al., 2007). In fact, the overall pharmacokinetic profile of plasma [NO_3^-] after ingestion of the three James White beetroot supplements (~ 5.76 mmol NO_3^-) in this study is similar to previous work by Webb et al. (2008) and Wylie et al. (2013). Specifically, there was a rapid increase in plasma [NO_3^-] within 30 min, attainment of peak values at 2 h (Larsen et al., 2010), and then a steady decline beyond 4 h post NO_3^- consumption.

Peak plasma [NO_2^-] was ~ 490, 520, 240 and 210 % above baseline in the concentrated beetroot juice, beetroot flapjack, non-concentrated beetroot juice and beetroot crystal conditions, respectively. These values are higher than those typically reported (~ 50 – 140 %) after beetroot juice supplementation, despite a similar dose (5 - 6 mmol) of NO_3^- being administered (Lansley et al., 2011; Vanhatalo et al., 2010). These differences may be explained by variations in the presence and/or activity of NO_3^- reductases between the subject populations (Govoni et al. 2008; Webb et al. 2008) and/or differences in dietary control between studies (Vanhatalo et al., 2010). It is important to note, however, that the rise in plasma [NO_2^-] in the non-concentrated beetroot juice condition was similar to those values previously reported after ingestion of 0.5 L of the same non-concentrated NO_3^--rich beetroot juice drink (Lansley et al., 2011; Vanhatalo et al., 2010). Interestingly, the iAUC and the peak increase in plasma [NO_2^-] were similar in the concentrated beetroot juice and beetroot flapjack conditions, despite the rise in salivary [NO_2^-] being lower with the beetroot flapjack. This may be explained by a greater exposure time of the more solid supplement to the NO_3^- reducing bacteria found in the oral cavity (via swallowing and chewing), particularly during the initial stages of digestion, although we cannot rule out possible effects in the stomach and upper gastrointestinal tract.

The peak rise in plasma [NO_2^-] has been consistently reported to occur between 2 and 3 h following dietary NO_3^- intake (Webb et al., 2008; Wylie et al., 2013). In the current study, peak plasma [NO_2^-] occurred at 2 h in beetroot flapjack and at 3 h in concentrated beetroot juice and non-concentrated beetroot juice. In contrast, peak plasma [NO_2^-] occurred 15 min post ingestion of beetroot crystals owing to the small dose of NO_2^- that was present in this supplement. Plasma [NO_2^-] has been reported previously to peak between 15 and 30 minutes post consumption of an acute NO_2^- bolus (Kevil et al., 2011).

The time lag between the appearance of peak [NO_3^-] and [NO_2^-] in plasma emphasises the importance of the entero-salivary circulation and the role of the oral bacteria in reducing NO_3^- to NO_2^- (Kapil et al., 2012; Webb et al., 2008). It is important to note here that although plasma [NO_2^-] peaked within the typical time frame in the beetroot flapjack condition, there was a rapid elevation from 30 min – 1 h, suggesting that the kinetic response to the solid supplement was somewhat faster than either of the liquid supplements containing the same NO_3^- and NO_2^- content (concentrated beetroot juice and non-concentrated beetroot juice) used in this study. Muggeridge and colleagues (2014) also found that using a more solid NO_3^- vehicle than a typical vegetable juice, such as a Swiss chard and rhubarb extract gel (Science in Sport GO + Nitrates, Lancashire, U.K.) resulted in a faster pharmacokinetic response for plasma [NO_2^-]. In contrast, McIlvenna et al. (2017) reported similar plasma [NO_3^-] and [NO_2^-] after ingestion of concentrated beetroot juice and the chard gel. Interestingly, these authors reported that the concentrated beetroot juice elevated total plasma nitroso species ([RXNO]) to a greater extent than the chard gel (McIlvenna et al., 2017).

Investigating the influence of a more diverse range of NO_3^- vehicles on plasma [RXNO] in addition to [NO_3^-] and [NO_2^-] may be useful in future work. Consistent with the proposed explanation for the peak plasma [NO_2^-] response (see above), this relatively faster time-to-peak in the beetroot flapjack condition, may also be explained by the solid food form spending more time in the oral cavity, or effects in the stomach and upper gastrointestinal tract.

Overall, differences in dietary administration may play a key role in the pharmacokinetic response to NO_3^- supplementation. Specifically, the different NO_3^- food stuffs, particularly the more compact or dense products, may modify exposure time to oral bacteria, alter the uptake of NO_3^- into the systemic circulation, change salivary flow rate and/or possibly alter the oral and gastric pH, resulting in different pharmacokinetic responses when compared to those values typically reported after consumption of NO_3^--rich beetroot juice and salts (Kapil et al., 2010; Webb et al., 2008; Wylie et al., 2013).

1.4.3 NO Indicator Strips

This is the first study to validate the Berkeley Test® nitric oxide indicator strips as a means for estimating changes in NO bioavailability, via salivary [NO_2^-], following the consumption of different NO_3^- supplements. Modi et al. (2016) have previously reported that the test strips were significantly correlated with salivary [NO_2^-] in non-supplemented subjects. Overall, the strips identified elevations and subsequent falls in

NO biomarkers over the 24 h experimental period after NO_3^- ingestion. The strips were able to identify the dose-response differences between the higher NO_3^- products (5.76 mmol NO_3^-) and the beetroot crystal product (1.40 mmol NO_3^-), but not the more subtle changes in NO bioavailability after the equimolar doses of NO_3^-. The NO strip test results were positively correlated with an increase in both salivary and plasma [NO_2^-]. Therefore, Berkeley Test® nitric oxide indicator strips may provide a practical way of estimating salivary [NO_2^-] following NO_3^- ingestion, for example, in field studies or when more direct approaches, such as chemiluminescence, are not available.

1.4.4 Urinary [NO_3^-] and [NO_2^-]

In this study, the four NO_3^- vehicles resulted in an increase in urinary [NO_3^-] and [NO_2^-] when compared with pre-supplementation baseline and CON. The pharmacokinetic profile revealed that the peak urinary [NO_3^-] occurred at 6 h in all NO_3^- loaded conditions, with concentrated beetroot juice resulting in the highest [NO_3^-] when compared with non-concentrated beetroot juice, beetroot flapjack and beetroot crystals, increasing by 6-fold, versus 3-, 3.5- and 2- fold, respectively. Several studies have previously shown increases in urinary NO_3^- excretion after ingestion of pharmaceutically (Bartholomew & Hill, 1984; Bescós et al., 2012) and naturally (Hobbs et al., 2012; Oldreive et al., 2001) derived NO_3^-. Pannala et al. (2003) reported similar urinary NO_3^- kinetics to this study after ingestion of a high-nitrate meal (containing ~ 3.9 mmol NO_3^-). These authors reported that urinary NO_3^- increased by 4-fold and this peak excretion occurred between 4 and 6 h post ingestion of the NO_3^--rich meal, with basal concentrations reached by 24 h.

Peak urinary [NO_2^-] occurred at 3 h post ingestion of concentrated beetroot juice, non-concentrated beetroot juice, beetroot flapjack and beetroot crystal conditions, with 26-, 25-, 6- and 12-fold increases noted above baseline, respectively. The results in the present study seemed to be higher than those previously reported (2-fold increase in urinary NO_2^- output) after ingestion of a high NO_3^- meal (Pannala et al., 2003). At 24 h, urinary [NO_3^-] and [NO_2^-] had returned to baseline values in the beetroot flapjack, non-concentrated beetroot juice and beetroot crystal conditions. However, urinary [NO_3^-] in the concentrated beetroot juice condition remained elevated above pre-supplementation values and the beetroot crystal condition 24 h post ingestion. The majority (60-75 %) of endogenously and exogenously derived NO_3^- is ultimately excreted in the urine (Hobbs, 2013; Wagner et al., 1983). Differences in the concentration of both urinary [NO_3^-] and [NO_2^-] were noted between the conditions and this is likely explained by the consistency of the vehicle and its absorption rate within the body, with the fluid conditions, particularly the concentrated fluid, resulting in higher excretion than the solid condition. However, it is important to note that total urine output was not measured in the present study, and therefore total NO_3^- and NO_2^- excretion is not known.

1.4.5 Blood Pressure and Heart Rate

Acute dietary NO_3^- supplementation reduced systemic BP at several time points when compared with the pre-supplementation baseline and CON. Specifically, concentrated beetroot juice resulted in a reduction in SBP (- 5 mmHg) at 3 h post ingestion when compared with baseline; however, there was no decrease in SBP in beetroot flapjack, non-concentrated beetroot juice and beetroot crystals when compared with pre-supplementation values. It is important to note, however, that the change in SBP across all conditions was negatively correlated with the increase in plasma [NO_2^-]. Wylie et al. (2013) also reported a 5 mmHg reduction in SBP after ingestion of a single bolus of a similar beetroot concentrate (containing 4.2 mmol NO_3^-). In contrast to our findings, Kapil et al. (2010) found that 3 h after the consumption of 250 mL of a non-concentrated beetroot juice (Beet It, James White Drinks, Ltd., Ipswich, UK, containing 5.5 mmol NO_3^-) SBP was reduced by ~ 5 mmHg. At 1 h post ingestion of concentrated beetroot juice and beetroot flapjack, DBP was reduced by 3 and 4 mmHg when compared with baseline, respectively. The magnitude of the reduction in DBP in both the concentrated beetroot juice and beetroot flapjack conditions are in line with the associated elevations in plasma [NO_2^-] at the same time point. Wylie et al. (2013) also reported that consumption of 2 (8.4 mmol NO_3^-) and 4 (16.8 mmol NO_3^-) concentrated beetroot shots reduced DBP by 3 and 4 mmHg at 4 h and 2 h, respectively, whereas consumption of 1 shot (4.2 mmol NO_3^-) did not alter DBP.

MAP was reduced in the concentrated beetroot juice condition only when compared with both the pre-supplementation baseline (1 h: 3 mmHg, 3 h: 4 mmHg) and CON (1 h: 1 mmHg, 3 h: 2 mmHg). Other studies have also reported similar reductions in MAP following consumption of a concentrated beetroot shot (Wylie et al., 2013). In contrast to our study, Webb et al. (2008) reported a reduction in MAP (3 h: 8 mmHg) when a larger volume of a non-concentrated NO_3^--rich beetroot juice was consumed. The discrepancy in results between the studies is likely due to the increased [NO_3^-] (22.5 mmol) consumed in the latter study (Webb et al., 2008) compared to the current study (5.76 mmol). In the present study, non-concentrated beetroot juice did not affect DBP across the 24 h time frame, findings that are consistent with Kapil et al. (2010). It may be suggested that despite the same NO_3^- dose being ingested, the larger volume of fluid consumed in the non-concentrated beetroot juice condition versus beetroot flapjack and concentrated beetroot juice, may have helped to sustain a relatively stable BP over the 24 h experimental period in this study (Jormeus et al., 2010). The beetroot crystal product did not reduce BP at any time point. This may be explained by the low dose of NO_3^- administered and consequently the smaller rise in markers of NO bioavailability and also by the fact that the earliest measurement of BP at 1 h did not coincide with the peak plasma [NO_2^-] in this condition. Another important consideration in the interpretation of the BP results in this study is that the subjects were normotensive (112/66 mmHg) such that large changes in BP following NO_3^- ingestion would not be expected. For a given NO_3^- dose, the reduction in BP is significantly correlated with the baseline BP (Ashworth et al., 2015).

Several studies have found no change in HR during rest and exercise following NO_3^- supplementation (Bailey et al., 2009; Ferguson et al., 2013a; 2013b). However, in the present study, there was a rise in HR across a number of conditions with the most noteworthy being the elevation in HR at 1 h post concentrated beetroot juice ingestion. This small rise in HR may be a compensatory mechanism to maintain cardiac output in the face of the reduced MAP (Klein, 2013).

1.4.6 Clinical Significance

Overall, concentrated beetroot juice was more effective in reducing BP when compared to beetroot flapjack, non-concentrated beetroot juice and beetroot crystals. It is noteworthy that a 5 mmHg reduction in SBP (as seen in this study), if maintained, may be estimated to decrease the risk of mortality by 7 % and, more specifically, reduce the risk of mortality by stroke and coronary heart disease by 14 and 9 %, respectively (Stamler, 1991). Even a sustained 2 mmHg reduction in SBP could result in a 10 and 7 % reduction in stroke mortality and cardiovascular disease mortality, respectively, (Lewington et al., 2002). The rise in the bioavailability of NO following NO_3^- ingestion is believed to be responsible for the reductions in systemic BP. Although NO_2^- itself may produce direct vasodilatory effects (Alzawahra et al., 2008), elevated levels of NO can encourage the binding of NO with guanylate cyclase (Ignarro, Harbison, Wood & Kadowitz, 1986) stimulating the release of cyclic guanosine monophosphate (cGMP). The consequent activation of several protein kinases by cGMP can reduce intracellular calcium concentration and consequently lead to smooth muscle relaxation (Lohmann et al., 1997). Therefore, it may be suggested that dietary NO_3^- supplementation, particularly in the form of a small, concentrated beetroot drink, may be useful therapeutically or prophylactically for maintaining a healthy BP.

1.4.7 Conclusion

In summary, dietary supplementation with NO_3^--rich concentrated beetroot juice, non-concentrated beetroot juice, beetroot flapjack and beetroot crystals successfully elevated NO-related metabolites when compared with pre-supplementation values and a water control. Beetroot flapjack and concentrated beetroot juice were the most effective vehicles for increasing plasma [NO_2^-] and reducing systemic BP, results which may be especially important for clinical populations. However, concentrated beetroot juice was the supplement that resulted in the greatest and most consistent reductions in both SBP and MAP. Based on these results, it may be suggested that the consumption of a small, concentrated volume of NO_3^--rich fluid, such as beetroot juice, may be recommended as a practical approach for maintaining or perhaps improving markers of cardiovascular health in young, healthy individuals.

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FIGURE LEGENDS

Figure 1. Salivary nitrate (**A**), salivary nitrite (**B**), plasma nitrate (**C**), plasma nitrite (**D**), urinary nitrate (**E**) and urinary nitrite (**F**) following consumption of a water control (**CON**;●), 5.76 mmol of a nitrate-rich beetroot concentrate (**BR**; ■), beetroot flapjack (**BF**; ○) and a non-concentrated beetroot drink (**BL**; ▲) and 1.40 mmol of nitrate containing beetroot crystals (**BC;** □). Data are presented as group mean ± SE. [a]Significantly different from pre-supplementation; [*]Significantly different from **CON**; [$]Significantly different from **BL;** [#]Significantly different from **BR;** [b]Significantly different from **BC** (all $P < 0.05$).

Figure 2. Nitric oxide indicator strip results following consumption of a water control (**CON**; ●), 5.76 mmol of a nitrate-rich beetroot concentrate (**BR;** ■), beetroot flapjack (**BF**; ○) and a non-concentrated beetroot drink (**BL**; ▲) and 1.40 mmol of nitrate containing beetroot crystals (**BC;** □). Data are presented as group mean ± SE. [a]Significantly different from pre-supplementation; [*]Significantly different from **CON**; [$]Significantly different from **BL**; [b]Significantly different from **BC** (all $P < 0.05$).

Figure 3. Change (Δ) relative to pre-supplementation baseline in **s**ystolic blood pressure (SBP; **A**) and mean arterial pressure (MAP; **B**) following consumption of a water control (**CON**; ●), 5.76 mmol of a nitrate-rich beetroot concentrate (**BR**; ■), beetroot flapjack (**BF**; ○) and a non-concentrated beetroot drink (**BL**; ▲) and 1.40 mmol of nitrate containing beetroot crystals (**BC;** □). Data are presented as group mean ± SE. [a]Significantly different from pre-supplementation; [*]Significantly different from **CON**;

$^{\$}$Significantly different from **BL**; $^{\#}$Significantly different from **BR;** ^{b}Significantly different from **BC** (all $P < 0.05$).

Figure 1.

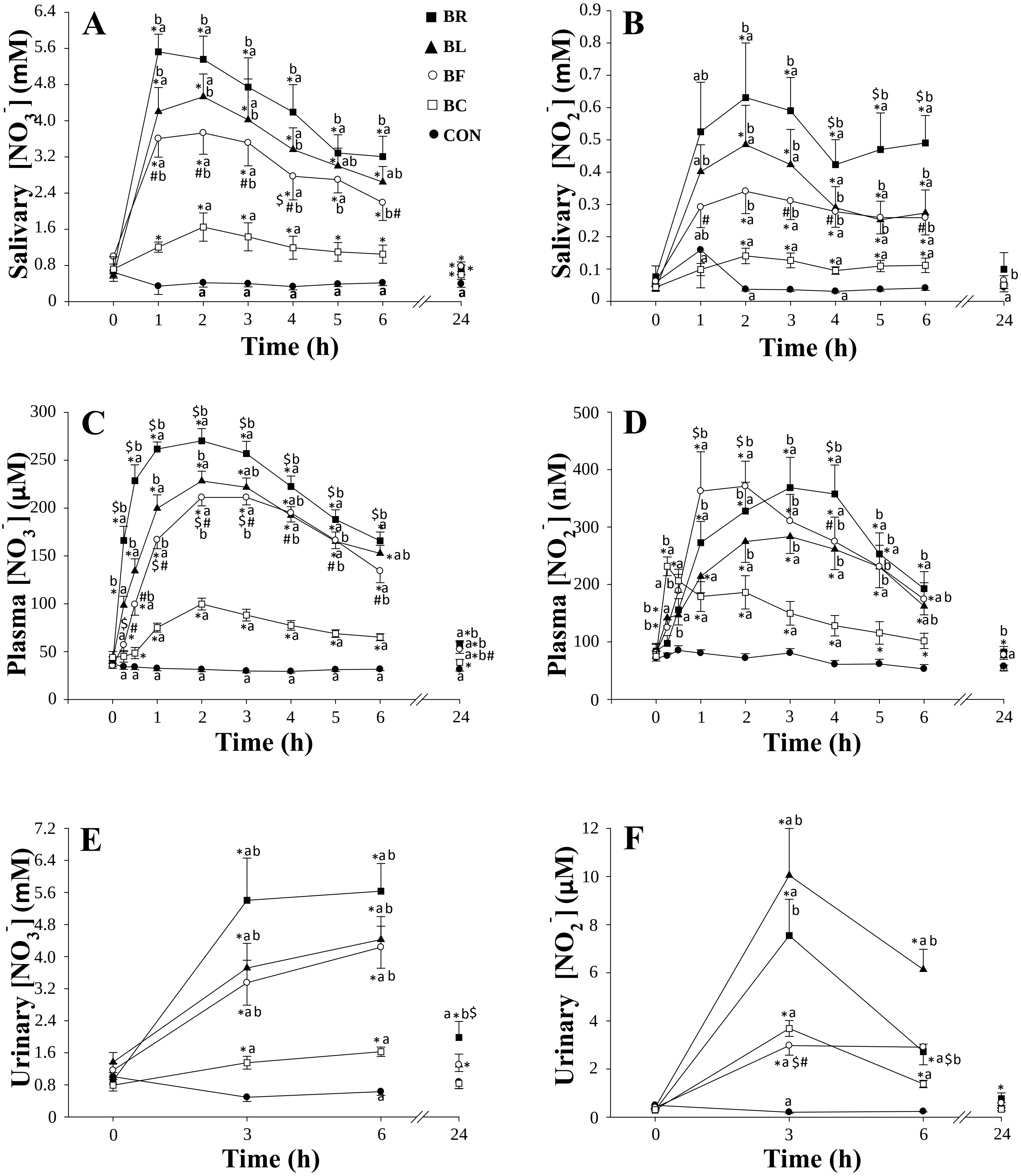

A
BR
BL
BF
BC
CON
Salivary [NO$_3^-$] (mM)
6.4
5.6
4.8
4.0
3.2
2.4
1.6
0.8
0
Time (h)
0
1
2
3
4
5
6
24
B
Salivary [NO$_2^-$] (mM)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Time (h)
C
Plasma [NO$_3^-$] (µM)
300
250
200
150
100
50
0
Time (h)
D
Plasma [NO$_2^-$] (nM)
500
400
300
200
100
0
Time (h)
E
Urinary [NO$_3^-$] (mM)
7.2
6.4
5.6
4.8
4.0
3.2
2.4
1.6
0.8
0
Time (h)
F
Urinary [NO$_2^-$] (µM)
12
10
8
6
4
2
0
Time (h)

Figure 2.

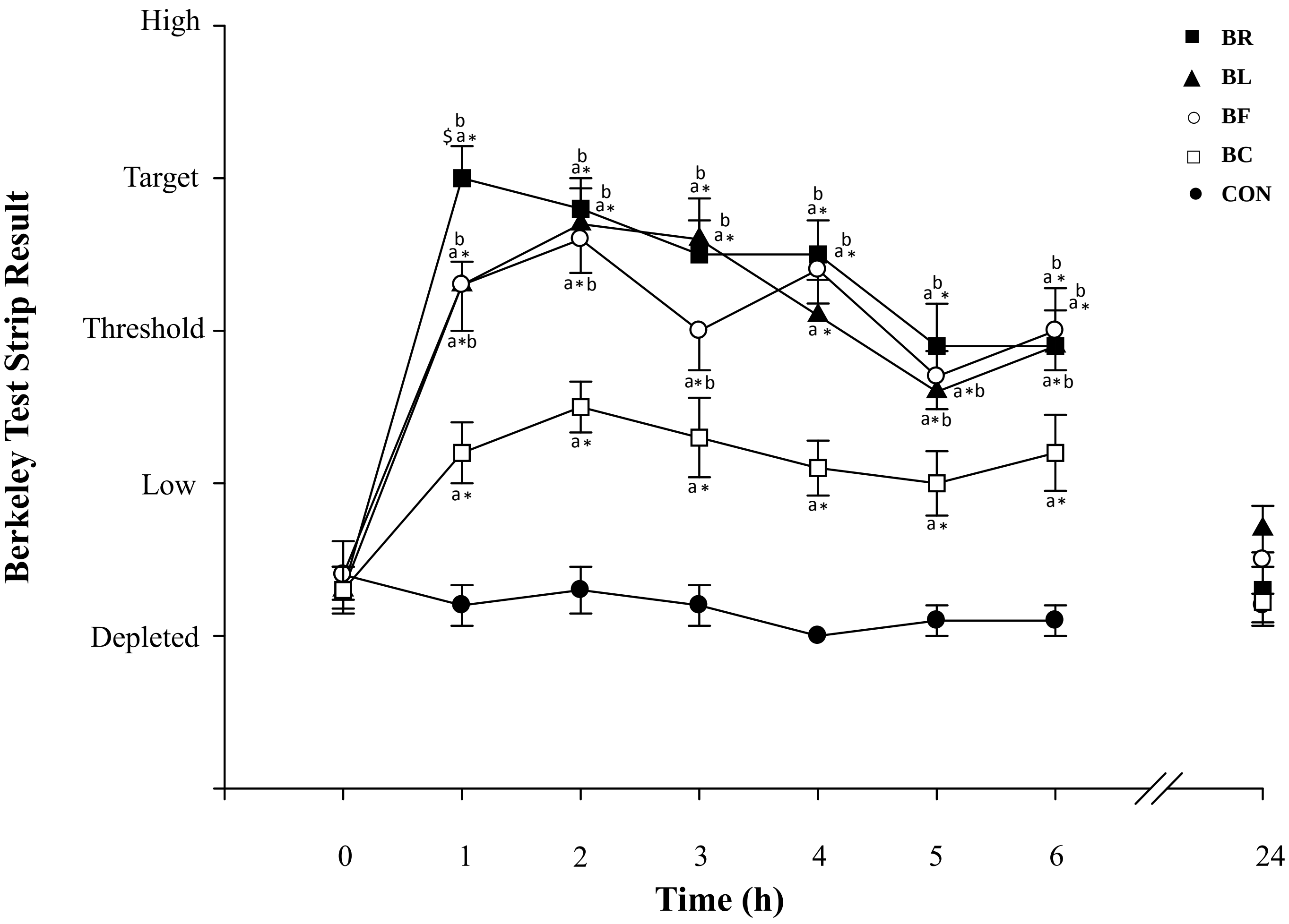
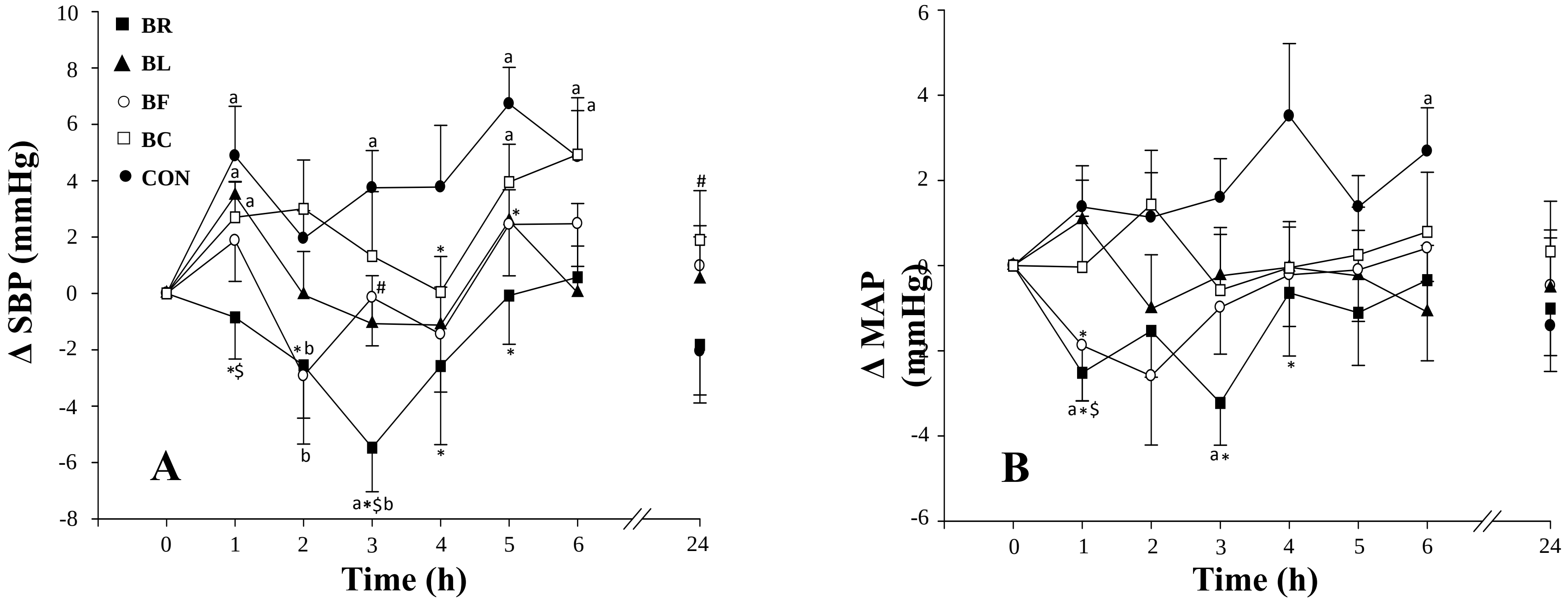

Berkeley Test Strip Result
High
Target
Threshold
Low
Depleted
Time (h)
0
1
2
3
4
5
6
24
■ BR
▲ BL
○ BF
□ BC
● CON
$
a*
b
a*b

Figure 3.


A
Δ SBP (mmHg)
Time (h)
■ BR
▲ BL
○ BF
□ BC
● CON
10
8
6
4
2
0
-2
-4
-6
-8
0
1
2
3
4
5
6
24
a
a
a
a
a
a
a
a
*$
*b
b
#
a*$b
*
*
*
*
#
B
Δ MAP (mmHg)
Time (h)
6
4
2
0
-2
-4
-6
a
*
a*$
a*
*

A randomised controlled trial exploring the effects of different beverages consumed alongside a nitrate-rich meal on systemic blood pressure

Original Article

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Chapter 7: A randomised controlled trial exploring the effects of different beverages consumed alongside a nitrate-rich meal on systemic blood pressure

Abstract

Background: Ingestion of nitrate- (NO_3^-) containing vegetables, alcohol and polyphenols, separately, can reduce blood pressure (BP). However, the pharmacokinetic response to the combined ingestion of NO_3^- and polyphenol rich or low alcoholic beverages is unknown. **Aim:** To investigate how the consumption of low and high polyphenolic alcoholic beverages combined with a NO_3^--rich meal can influence NO_3^- metabolism and systemic BP. **Methods**: In a randomised, crossover trial, 12 normotensive males (age 25 ± 5 years) ingested an acute dose of NO_3^- (~ 6.05 mmol) in the form of a green leafy salad, in combination with either a polyphenol-rich red wine (NIT-RW), a low polyphenol alcoholic beverage (vodka; NIT-A) or water (NIT-CON). Participants also consumed a low NO_3^- salad and water as a control (CON; ~ 0.69 mmol NO_3^-). BP and plasma, salivary and urinary [NO_3^-] and nitrite ([NO_2^-]) were determined before and up to 5 h post-ingestion. **Results:** Each NO_3^--rich condition elevated NO biomarkers when compared with CON ($P < 0.05$). The peak rise in plasma [NO_2^-] occurred 1 h after NIT-RW (292 ± 210 nM) and 2 h after NIT-A (318 ± 186 nM) and NIT-CON (367 ± 179 nM). Systolic BP was reduced 2 h post consumption of NIT-RW (-4 mmHg), NIT-A (-3 mmHg) and NIT-CON (-2 mmHg) compared with CON ($P < 0.05$). Diastolic BP and mean arterial pressure were also lower in NIT-RW and NIT-A compared with NIT-CON ($P < 0.05$). **Conclusion**: A NO_3^--rich meal, consumed with or without an alcoholic beverage, increases plasma [NO_2^-] and lowers systemic BP for 2-3 h post ingestion.

Keywords dietary nitrate, alcohol, red wine, blood pressure, pharmacokinetics, nitric oxide, Mediterranean diet

Chapter 7: A randomised controlled trial exploring the effects of different beverages consumed alongside a nitrate-rich meal on systemic blood pressure

Introduction

Hypertension is one of the leading and most avertable causes of morbidity and mortality. Much scientific and medical research has therefore focused on interventions to lower blood pressure (BP) and improve cardiovascular health and longevity (Appel et al., 1997; Joshipura et al., 1999, 2001). A vegetable-rich diet can elicit favourable effects on BP and these effects have, in part, been attributed to the high polyphenolic and nitrate (NO_3^-) content of these foods. Green leafy vegetables and beets are an important source of NO_3^- and a precursor for the production of the vasodilator, nitric oxide (NO; Hord, Tang and Bryan, 2001). Following absorption through the upper gastrointestinal tract, 25 % of the consumed NO_3^- passes into the enterosalivary circulation and accumulates in the oral cavity (Lundberg and Govoni, 2004). Here, commensal bacteria rapidly reduce NO_3^- to nitrite (NO_2^-; Duncan et al., 1995), ultimately leading to an increased systemic plasma NO_2^- concentration ($[NO_2^-]$; Dejam et al., 2004). NO and other nitrogen oxides, can be enzymatically and non-enzymatically produced from NO_2^- (Gladwin et al., 2005; Webb et al., 2004; Zweier et al., 1995). In environments with a low pH, like the stomach (Benjamin et al., 1994), swallowed salivary NO_2^- interacts with the gastric acid and is converted to nitrous acid (HNO_2) which can be further transformed into, for example, dinitrogen trioxide (N_2O_3), nitrogen dioxide (NO_2) and NO (McKnight et al., 1997). In the presence of ascorbic acid (Vitamin C), thiocyanate and polyphenols (which are abundant in fruit and vegetables), the production of NO is favoured over that of other nitrogen species (Gago et al., 2007; Peri et al., 2005; Weitzberg and Lundberg, 1998).

It is clear that the diet plays a fundamental role in the formation of NO and its derivatives, and the subsequent lowering of systemic BP (Gladwin, 2004). Indeed, the acute and chronic ingestion of dietary NO_3^- has been shown to increase plasma $[NO_2^-]$ and reduce BP in a range of different populations (Ashworth et al., 2015; Kenjale et al., 2011; Vanhatalo et al., 2010; Webb et al., 2008). Wylie et al. (2013) reported a dose-dependent increase in markers of NO and reductions in BP in young, healthy males after acute concentrated beetroot juice ingestion, with peak changes occurring 2-4 h post consumption. In another study, 7 days of high NO_3^- vegetable consumption resulted in a 4 mmHg decrease in systolic BP (SBP) in normotensive females (Ashworth et al., 2015).

The ingestion of red wine following sodium NO_3^- consumption has also been reported to promote NO formation, as measured in air expelled from the human stomach (Gago et al., 2007). In addition, anthocyanin and catechol fractions found in red wine have been reported to dose- and pH-dependently promote NO formation when mixed with NO_2^- *in vitro* (Gago et al., 2007). Moreover, a NO_2^--ethanol blend, under gastric conditions, can generate ethyl NO_2^-, a potent vasodilator, and relaxation of rat gastric fundus strips and femoral artery rings (Gago et al., 2008). It was recently reported that 50 g of lettuce followed by either 300 mL of red wine or 60 mL of whisky increased ethyl NO_2^- in the stomach, with higher concentrations noted in the whisky condition (Rocha et al., 2015). NO was also substantially elevated, but in the red wine condition only, which underlines the importance of polyphenols in the univalent reduction of NO_2^- to NO (Rocha et al., 2015). It is important to note here that these studies administered NO_3^- 15-45 min prior to ingestion of alcohol or polyphenols to increase their exposure to gastric NO_2^-.

However, this approach to increasing NO metabolites for subsequent cardiovascular benefit is not likely to be adopted in real life; a more realistic approach, such as the consumption of a NO_3^--rich meal alongside alcohol and/or polyphenols, as often occurs as part of the so-called Mediterranean diet (Giacosa et al., 2016; Shen et al., 2015), warrants investigation.

Overall, the consumption of green leafy foods and moderate red wine intake (as a whole or in the form of its constituents) can reduce systemic BP and risk of mortality (Appel et al., 1997; de Lorgeril et al., 1995; Foppa et al., 2002). While NO_3^- (Webb et al., 2008; Wylie et al., 2013) and polyphenol (Li, Xia and Förstermann, 2012; Migliori et al., 2015; Schroeter et al., 006) ingestion, both separately and in combination (Peri et al., 2005; Rocha et al., 2009, 2015), can elevate NO bioavailability, NO_3^- and NO_2^- pharmacokinetics and BP responses to a typical NO_3^--rich meal consumed alongside a low and high polyphenolic alcoholic beverage has not been determined in humans.

The purpose of this study was to establish the plasma, salivary and urinary [NO_3^-] and [NO_2^-] pharmacokinetic profiles and BP response to three NO_3^--rich salad meals in conjunction with either a polyphenol-rich red wine (NIT-RW), a low-polyphenol alcoholic beverage (NIT-A; vodka and lemonade) or a water control drink (NIT-CON) over a 5 h period. It was hypothesised that relative to pre-supplementation baseline and a low NO_3^- meal with a water control condition (CON), NIT-RW, NIT-A and NIT-CON would result in significant increases in plasma, salivary and urinary [NO_3^-] and [NO_2^-] and reductions in systemic BP. It was also hypothesised that NIT-RW would have a more pronounced effect upon BP compared with NIT-A and NIT-CON due to the polyphenol content.

Methods

Participants

Twelve healthy, normotensive males volunteered to participate in this study (See Table 1. for baseline characteristics). Subjects were recruited from the University student population from November 2016 until January 2017. None of the subjects smoked tobacco, consumed dietary supplements or used antibacterial mouthwash habitually. Prior to testing and after the requirements of the study and potential risks and benefits of participation were explained, written informed consent was obtained. The sample size was determined in G*Power by inputting previous data for changes in BP following red wine consumption and the calculation was approved by a statistician. This study was approved by the University of Exeter Research Ethics Committee and conformed to the ethical principles of the World Medical Association Declaration of Helsinki.

Participants were instructed to arrive at the laboratory in a fully rested and hydrated state, at least 3 h post prandial. In addition, participants were asked to avoid caffeine consumption and participation in strenuous exercise in the 24 h period prior to each visit to the laboratory. All participants were also asked to refrain from alcohol consumption (other than that administered by the researcher) and the use of antibacterial mouthwash and chewing gum for the duration of the study. Each participant recorded their habitual diet and exercise undertaken during the 24 h period prior to consumption of the first experimental condition and were asked to replicate these during the remaining three experimental conditions. Exclusion criteria were the presence of known cardiovascular disease and hypertension, allergy to alcohol, and the use of antihypertensive medication and antibiotics.

Experimental Design

Participants were asked to attend the laboratory on four separate occasions over a two week period. Prior to the first visit to the laboratory, each participant was randomly assigned, in a single-blind (i.e., the researcher was blinded to the order), simple crossover fashion to consume an acute dose of ~ 6.05 mmol of dietary NO_3^- in the form of a green leafy salad (50 g rocket, 88 g spinach and 160 g cucumber), in combination with either a polyphenol-rich red wine [NIT-RW; 175 mL Montepulciano d'Abruzzo, Tesco Stores Ltd, Welwyn Garden City, U. K; 12.5 % alcohol by volume (ABV)], a low polyphenol alcoholic beverage (NIT-A; 58 mL Red Label Smirnoff Vodka, The Smirnoff Co., London, U.K; 117 mL Tesco Sparkling Lemonade, Tesco Stores Ltd, Welwyn Garden City, U.K; 12.5 % ABV) or a control drink (NIT-CON; 175 mL deionised water). The NO_3^- content of the salad was determined by analysing multiple, homogenised samples of each component via chemiluminescence. We did not measure the polyphenol content of the red wine but a phenolic profile is provided by Sagratini et al. (2012). We calculated and provided similar total polyphenol content in the high and low NO_3^- salads based on previous data (Ashworth et al., 2015). The NIT-RW and NIT-A conditions were matched by total alcohol ingested and this was achieved by diluting the vodka prior to administration to 12.5 % ABV. In addition, participants consumed a salad low in NO_3^- (55 g cucumber, 68 g green beans and 200 g cherry tomatoes; 0.69 mmol NO_3^-) alongside the ingestion of a control drink (CON; 175 mL deionised water). Participants completed each of the four conditions in a random order (using a computer generated sequence), and consumed the allocated meal and drink within 25 min on each occasion. During each condition, saliva, blood and urine samples were collected and BP was recorded prior to and over a 5 h period following consumption of each meal. All tests were performed at the same time of day, usually 9 am (± 1 h), to minimise diurnal variation on the physiological variables under investigation and each visit to the laboratory was separated by a minimum of 48 h. The researcher performing the physiological measurements was not aware of the meal and beverage that had been consumed by the participants on each visit or the randomisation sequence.

Experimental Protocol

During each visit to the laboratory, an automated sphygmomanometer (Dinamap Pro; GE medical Systems, Tampa, FL, USA) was used to measure BP of the brachial artery, prior to meal consumption and at 1, 2, 3, 4 and 5 h post consumption of each meal. Following 10 min of seated rest in a quiet room, five BP and heart rate (HR) measurements were recorded and the mean of the final four measurements was used for data analysis.

Blood samples were obtained prior to ingestion of each meal and at 30 min, 1, 2, 3, 4 and 5 h following meal consumption. All blood samples (~ 6 mL) were drawn from a cannula (Insyte-WTM, Becton-Dickinson, Madrid, Spain) inserted into the antecubital vein and collected in lithium-heparin tubes (Vacutainer, Becton-Dickinson, NJ, USA). Blood samples were centrifuged for 10 min at 3000 *g* and 4 ºC within 2 min of collection and the plasma was subsequently extracted. Saliva samples (~ 1 mL) were collected by expectoration, without stimulation, at baseline and at 1, 2, 3, 4 and 5 h post consumption of each meal. Midstream urine samples were also collected and then aliquoted into 3 x 1.5 mL Eppendorf tubes prior to and at 3 and 5 h post consumption of each meal.

Plasma, saliva and urine samples were frozen at -80 ºC for later determination of [NO_3^-] and [NO_2^-] using a modified chemiluminescence technique (Wylie et al., 2013a). Following thawing and prior to analysis, plasma, saliva and urine samples were centrifuged for 10 min at 18 600 *g*. The supernatants were removed and diluted using deionized water by a factor of 100 (for salivary [NO_3^-] and [NO_2^-] and urinary [NO_2^-]) and 1000 (for urinary [NO_3^-]). Plasma samples were deproteinised using a cold ethanol precipitation technique: 1000 µL of ethanol (at 0 ºC) was added to 500 µL of plasma and vortexed for 30 s before being left to stand on ice for 30 min. Samples were centrifuged at 18 600 *g* for 5 minutes and the supernatant was removed and used for analysis of [NO_3^-] and [NO_2^-].

Throughout each 5-h laboratory visit, water consumption was standardised and participants remained seated between measurements to avoid influencing the physiological parameters or NO biomarkers under investigation (Liddle et al., 2018). All measurements (other than urine samples, which were provided in a private cubicle in close proximity to the laboratory) were collected in a seating position. The same protocol was repeated during each visit to the laboratory.

Statistical Methods

Differences in BP, HR and salivary, plasma, and urinary [NO_3^-] and [NO_2^-] were assessed using a 2-way (condition x time) repeated-measures ANOVA. Significant main effects and interaction effects were further explored using Fisher’s LSD. Relationships between variables were assessed via Pearson’s product-moment correlation coefficient. Plasma [NO_3^-] and [NO_2^-] incremental area under the curve (iAUC) from baseline until

5 h (0 – 5 h) was calculated for NIT-RW, NIT-A, NIT-CON and CON using the trapezium model (GraphPad Prism, GraphPad, San Diego, CA). Differences in iAUC between conditions were assessed using a 1-way ANOVA with significant main effects further explored using Fisher's LSD. Statistical analyses were performed using SPSS version 23.0 (Chicago, IL, USA). Data are presented as mean ± SD, unless otherwise stated. Statistical significance was accepted at $P < 0.05$.

Results

All participants reported that they adhered to the dietary and exercise requirements and restrictions of the study during the 24-h period prior to each laboratory testing session. The ingestion of the high and low NO_3^- meals and the beverages were well tolerated with no negative side effects.

The salivary, plasma, and urinary [NO_3^-] and [NO_2^-] prior to and post ingestion of all meals and beverages are presented in Fig. 1.

Salivary [NO_3^-] and [NO_2^-]

There were significant main effects for time and condition and interaction effects for salivary [NO_3^-] and [NO_2^-] ($P < 0.05$). The elevations in salivary [NO_3^-] and [NO_2^-] in NIT-RW, NIT-A and NIT-CON were higher than CON and pre-supplementation baseline from 1 - 5 h post ingestion ($P < 0.05$). The peak rise in salivary [NO_2^-] occurred at 2 h in NIT-A (0.75 ± 0.38 mM) and NIT-CON (1.00 ± 0.66 mM) and at 3 h in NIT-RW (1.18 ± 1.07 mM). The increase in salivary [NO_2^-] was significantly higher in NIT-CON compared to NIT-A at 2-4 h after meal consumption ($P < 0.05$). The peak increase in salivary [NO_2^-] relative to the peak increase in salivary [NO_3^-] (i.e. the $\Delta[NO_2^-]/\Delta[NO_3^-]$) ratio) was lower for NIT-A compared to NIT-CON ($P < 0.05$).

Plasma [NO_3^-] and [NO_2^-]

There were significant main effects for time and condition and interaction effects for plasma [NO_3^-] and [NO_2^-] ($P < 0.05$). Plasma [NO_3^-] was significantly elevated 30 min after consumption of NIT-RW, NIT-A and NIT-CON when compared with CON and pre-supplementation baseline and remained elevated until 5 h post consumption ($P < 0.05$). The peak rise in plasma [NO_3^-] above baseline occurred at 2 h in NIT-RW (226 ± 71 µM) and NIT-CON (220 ± 80 µM) and at 3 h in NIT-A (198 ± 38 µM). There were no differences in plasma [NO_3^-] at any time point between the three NO_3^--rich conditions ($P > 0.05$). Plasma [NO_3^-] iAUC 0 - 5 h was significantly greater in NIT-RW (49.1 ± 11.8 mM•min), NIT-A (43.3 ± 11.2 mM•min) and NIT-CON (51.2 ± 21.3 mM•min) compared to CON (2.5 ± 1.8 mM•min; all $P < 0.05$); however, there were no significant differences between the three NO_3^--rich conditions ($P > 0.05$).

Plasma [NO_2^-] was significantly increased above baseline in all NO_3^--rich conditions from 30 min until 5 h after ingestion of each meal and beverage ($P < 0.05$). The peak rise in plasma [NO_2^-] above baseline was similar in all NO_3^--rich conditions and occurred at 1 h in NIT-RW (292 ± 210 nM) and at 2 h in NIT-A (318 ± 186 nM) and NIT-CON (367 ± 179 nM). Plasma [NO_2^-] was higher in NIT-CON and NIT-A when compared with CON at 30 min post ingestion ($P < 0.05$). In addition, plasma [NO_2^-] was significantly increased in NIT-RW, NIT-A and NIT-CON compared with CON, from 1-5 h post ingestion ($P < 0.05$). At 4 h, plasma [NO_2^-] was higher in NIT-CON (294 ± 168 nM) when compared with NIT-A (205 ± 113 nM; $P < 0.05$). Plasma [NO_2^-] iAUC 0-5 h was significantly greater in NIT-RW (47.5 ± 25.0 μM•min), NIT-A (52.5 ± 40.3 μM•min) and NIT-CON (56.9 ± 43.9 μM•min) compared to CON (3.6 ± 3.3 μM•min; all $P < 0.05$); however, there were no differences between the three NO_3^--rich conditions ($P > 0.05$). There were no main or interaction effects on $\Delta[NO_2^-]/\Delta[NO_3^-]$ ratio between the three high NO_3^- conditions ($P > 0.05$).

Urinary [NO_3^-] and [NO_2^-]

There were significant main effects for time and condition for both urinary [NO_3^-] and [NO_2^-] and a significant interaction effect for urinary [NO_3^-] only ($P < 0.05$). Urinary [NO_3^-] and [NO_2^-] were elevated to a similar extent above baseline and CON at 3 and 5 h after consumption of NIT-RW, NIT-A and NIT-CON ($P < 0.05$), with peak increases occurring 3 h post ingestion of each condition (See Fig. 1.).

Blood Pressure and Heart Rate

The BP responses to ingestion of all meals are displayed in Fig. 2. There was a significant main effect for time ($P < 0.05$) and condition ($P < 0.05$) for SBP. SBP was reduced 2 h after consumption of NIT-CON (-2 mmHg; $P = 0.054$) and NIT-A (-3 mmHg; $P < 0.05$) when compared with baseline. At the same time point, SBP was lower in NIT-RW (111 ± 7 mmHg; $P < 0.05$), NIT-A (113 ± 5 mmHg; $P = 0.056$) and NIT-CON (112 ± 4 mmHg; $P < 0.05$) compared with CON (116 ± 6 mmHg). At 3 h, SBP was lower in NIT-RW by 4 mmHg compared with baseline (116 ± 7 mmHg;

$P < 0.05$). At 5 h, SBP was also significantly lower in NIT-RW (115 ± 8 mmHg; $P < 0.05$) and NIT-CON (115 ± 5 mmHg; $P < 0.05$) compared to CON (119 ± 7 mmHg).

There was a significant main effect for time for DBP ($P < 0.05$). Follow up analyses revealed that DBP was significantly reduced following consumption of NIT-RW (1 h: -3 mmHg; 2 h: -4 mmHg) and NIT-A (1 and 2 h: -4 mmHg; all $P < 0.05$) compared with pre-supplementation baseline. DBP was lower after ingestion of NIT-RW (1 h: 59 ± 5, 2 h: 58 ± 4 mmHg) and NIT-A (1 h: 59 ± 4, 2 h: 60 ± 4 mmHg) compared with CON (1 h: 62 ± 5, 2 h: 61 ± 6 mmHg; all $P < 0.05$). DBP was also lower in NIT-RW at 1 and 3 h (3 h: 60 ± 7 mmHg) post ingestion compared with NIT-CON (1 h: 62 ± 5, 3 h: 64 ± 6 mmHg; $P < 0.05$).

There was a significant main effect for time for MAP. Follow up analyses revealed that MAP was lower 1 h after NIT-A (77 ± 4 mmHg) compared with CON (80 ± 4 mmHg) and NIT-CON (80 ± 5 mmHg; all $P < 0.05$). In addition, MAP was reduced at 2 h post ingestion of NIT-RW (76 ± 4 mmHg) compared with baseline (80 ± 6 mmHg) and CON (80 ± 6 mmHg; $P < 0.05$). At the same time point, MAP was also significantly lower after NIT-A (77 ± 3 mmHg) compared with CON (80 ± 6 mmHg; $P < 0.05$). At 3 h, MAP was reduced after NIT-RW (77 ± 6 mmHg) compared with CON (81 ± 7 mmHg) and NIT-CON (80 ± 6 mmHg; all $P < 0.05$).

There was a significant main effect for time and condition and an interaction effect for HR (all $P < 0.05$). HR decreased from baseline (61 ± 10 $b{\cdot}min^{-1}$) to 4 h (54 ± 7 $b{\cdot}min^{-1}$) and 5 h (55 ± 8 $b{\cdot}min^{-1}$) post ingestion in CON ($P < 0.05$). HR was greater in NIT-RW (1 h: 69 ± 13 $b{\cdot}min^{-1}$) and NIT-A (1 h: 66 ± 13, 2 h: 68 ± 12 $b{\cdot}min^{-1}$) compared with pre-supplementation baseline (NIT-RW, 62 ± 12; NIT-A, 62 ± 12 $b{\cdot}min^{-1}$; $P < 0.05$). HR was higher in NIT-RW and NIT-A than CON at 1-3 h post ingestion ($P < 0.05$). In addition, HR was higher in NIT-RW compared with NIT-CON at 1-3 h post ingestion and higher than CON at 4 h post consumption ($P < 0.05$).

Discussion

This study is the first to investigate the pharmacodynamics and pharmacokinetics of salivary, plasma and urinary [NO_3^-] and [NO_2^-] and systemic BP to the ingestion of a NO_3^--rich meal consumed alongside low and high polyphenolic alcoholic beverages in humans. We investigated the influence of an acute dose of dietary NO_3^- (~ 6.05 mmol of NO_3^-), ingested in the form of a rocket and spinach salad, in combination with either 175 mL of red wine (NIT-RW), vodka and lemonade (NIT-A) or water (NIT-CON) on plasma, salivary and urinary [NO_3^-] and [NO_2^-] and on resting BP over a 5 h period. The key finding was that NIT-RW and NIT-A were effective in reducing BP over a 5 h period compared with baseline, CON and, at some time points, NIT-CON. Although the magnitude of SBP reduction was consistently more after consuming NIT-RW compared with NIT-A, there were no statistically significant differences between these conditions. This suggests that the alcohol content of the beverages may have modulated the changes in BP following ingestion of a high NO_3^- meal.

Salivary [NO_3^-] and [NO_2^-]

The three NO_3^--rich leafy salads in combination with red wine, vodka and lemonade, and water beverages elevated salivary [NO_3^-] and [NO_2^-] over a 5 h period compared with baseline and the low NO_3^- salad condition (CON). The pharmacokinetic profile and peak rise in salivary [NO_3^-], which occurred at 1 h following meal consumption, was similar in the three NO_3^--rich conditions. This may be due to the same, prolonged exposure time of the NO_3^--rich foods to the oral cavity and subsequent uptake into the systemic circulation and enterosalivary system.

It is well established that there is an association between the ingestion of dietary NO_3^- and an increase in salivary [NO_2^-] (Kapil et al., 2015; Pannala et al., 2003; Spiegelhalder, Eisenbrand and Preussman, 1976). In the present study, salivary [NO_2^-] was increased across the 5 h period, with maximum elevations occurring between 2 h (NIT-A; NIT-CON) and 3 h (NIT-RW) following meal ingestion. Interestingly, there was a reduced conversion of salivary NO_3^- to NO_2^- in the NIT-A (3 h: 22 %, 4 h: 25 %) condition compared with NIT-CON (3 h: 28 %, 4 h: 34 %). Previous research has shown that rinsing the mouth with a solution of ethanol similar to that ingested in the present study, can alter the bacterial composition of the oral microflora (Muto et al., 2000). McDonagh et al. (2015) reported that the rise in salivary [NO_2^-] was blunted after rinsing with a weak mouthwash prior to beetroot juice ingestion and speculated that the component adversely impacting the conversion of NO_3^- to NO_2^- in the mouth might have been alcohol.

In the present study, the rise in salivary [NO_2^-] was attenuated in NIT-A compared with NIT-RW, despite the beverages having the same alcohol content and the increase in salivary [NO_3^-] being similar between the two conditions. It may be speculated that the high polyphenol content in NIT-RW compared with NIT-A has a protective effect on the oral microflora upon exposure to alcohol or that it counteracts the negative effect of alcohol on NO_3^- to NO_2^- conversion in the oral cavity. Salivary [NO_2^-] has been reported to be highly variable between and within individuals and, in addition to the number and activity of bacterial nitrate reductases, appears to depend upon a number of factors, including salivary flow rate and mouth pH (Djekoun-Bensoltane et al., 2007; James et al., 2015). It is feasible that changes in these variables impacted upon the salivary [NO_2^-] measured in the present study.

Plasma [NO_3^-] and [NO_2^-]

To our knowledge, this study is the first to analyse NO_3^- and NO_2^- in plasma after consumption of a high NO_3^- meal alongside an assortment of possible accompanying beverages. NIT-RW, NIT-A and NIT-CON all increased plasma [NO_3^-] and [NO_2^-] above baseline values over the 5 h period. The absolute peak plasma [NO_3^-] was similar across all conditions, with peaks occurring at 2 h post ingestion in NIT-RW and NIT-CON and at 3 h post ingestion in NIT-A.

Similar increases in plasma [NO_3^-] have been reported after consumption of high NO_3^- foods (lettuce, rocket and spinach) without co-ingestion of alcohol (Jonvik et al., 2016; Pannala et al., 2003) and the overall pharmacokinetic response to NIT-RW, NIT-A and NIT-CON was similar to that reported previously after ingestion of beetroot juice (Webb et al., 2008; Wylie et al., 2013). It appears therefore that the ingestion of alcohol, either with or without polyphenols, alongside a NO_3^--rich meal, does not appreciably influence either the initial increase in plasma [NO_3^-] or the enterosalivary uptake of NO_3^- and the elevation of salivary [NO_3^-]. NIT-A, however, does result in a slightly delayed achievement of time to peak plasma [NO_3^-] compared with NIT-RW and NIT-CON (See Fig. 1).

Plasma [NO_2^-] increased significantly after NIT-RW, NIT-A and NIT-CON compared with pre-supplementation baseline. Interestingly, the peak values (190-270 % above baseline) measured in this study were higher than those typically reported (~ 50-140 %) after ingestion of a similar dose of NO_3^- (in the form of beetroot juice) without co-ingestion of alcohol (Lansley et al., 2011; Vanhatalo et al., 2010). Although some studies have reported similar increases in plasma [NO_2^-] (McMahon et al., 2017), the differences between some studies may be explained by the different routes of NO_3^- administration, different methods of plasma sample preparation prior to analysis, the presence and activity of NO_3^- reducing bacteria in the oral cavity and the exposure time of the NO_3^- source to these bacteria (Govoni et al., 2008; McDonagh et al., 2018; Webb et al., 2008).

Although there were no significant differences in the absolute rise in plasma [NO_2^-], the iAUC for plasma [NO_2^-] or the $\Delta[NO_2^-]/\Delta[NO_3^-]$ ratio between the high NO_3^- conditions, the peak values tended to be lower in NIT-RW and NIT-A compared with NIT-CON. It may be speculated that the two alcoholic beverages promoted formation of other nitrogen oxides in the stomach (Gago et al., 2007, 2008; Rocha et al., 2015). The rise in plasma [NO_2^-] was blunted in the NIT-RW condition across a number of time points, possibly due to the phenolic radicals in the red wine acting as gastric reducing agents and stimulating the formation of NO from the swallowed salivary NO_2^- in the stomach (Gago et al., 2007; Peri et al., 2005). Similarly, plasma [NO_2^-] was lower in NIT-A compared to NIT-CON and this was significant at 4 h post ingestion. The relatively lower plasma [NO_2^-] in NIT-A and NIT-RW compared to NIT-CON may be explained by the generation of ethyl NO_2^- as well as NO_2^-, after the consumption of an alcoholic beverage with a high NO_3^- meal (Gago et al., 2008; Rocha et al., 2015).

Unfortunately, we did not measure the production of NO markers in the stomach and therefore cannot confirm the mechanistic basis for the observed changes. It is important to note here that participants consumed the high and low NO_3^- meals at the same time as the allocated beverage in order to reflect the ingestion of a typical Mediterranean-style. We acknowledge, however, that this method may have reduced the likelihood of alcohol and/or the additional polyphenols from the red wine interacting with NO_2^- in the stomach and forming NO.

Overall, the kinetic response of plasma [NO_2^-] to the NO_3^--rich meals in combination with the three beverages was faster than that typically reported after ingestion of a vegetable juice in isolation (McDonagh et al., 2018). These findings may be due to the increased contact time of the NO_3^- source to the oral bacteria through mastication of the foods over a sustained period.

Urinary [NO_3^-] and [NO_2^-]

Our results suggest that the additional polyphenol and/or alcohol content of the beverages had limited influence on urinary [NO_3^-] and [NO_2^-] in comparison to NIT-CON (present study) and studies that have investigated both acute and chronic NO_3^- supplementation in the absence of alcohol using either salts (Bartholomew and Hill, 1984; Bescós et al., 2012) or food stuffs (Hobbs et al., 2012; Kapil et al., 2015; Oldreive et al., 2001; Radomski, Palmiri & Hearn, 1978).

Blood Pressure and Heart Rate

It has been suggested that a diet high in NO_3^- may represent a natural prophylactic for reducing the risk of hypertension (Gilchrist, Winyard and Benjamin, 2010; Joshipura et al., 2001 Webb et al., 2008). Consistent with our hypothesis and previous literature, the three NO_3^--rich conditions reduced BP compared with pre-supplementation baseline and CON. SBP was lower in NIT-RW (2 h: -5 mmHg; 5 h: -4 mmHg), NIT-A (2 h: -3 mmHg) and NIT-CON (2 and 5 h: -4 mmHg) compared with CON, with the largest reductions found in the NIT-RW condition. Similarly, NIT-RW consistently reduced DBP (by 2-4 mmHg) across a number of time points compared to NIT-CON, pre-supplementation baseline and CON. NIT-A also decreased DBP compared with pre-supplementation baseline and CON (by ~ 1-4 mmHg).

A novel finding in the present study was that the consumption of NO_3^- with an alcoholic beverage (NIT-RW and NIT-A) resulted in larger reductions in BP compared to NIT-CON, despite the tendency for plasma [NO_2^-] to be blunted in the former conditions compared to the latter. The more pronounced reduction in MAP in NIT-A compared to NIT-CON may be due to the production of another NO donor, ethyl NO_2^-, from the mix of ethanol and NO_2^- in the stomach (Rocha et al., 2015). The increased presence of dietary polyphenols consumed in NIT-RW can also contribute to reductions in BP through a greater reduction of some NO storage pools, such as NO_2^- and ethyl NO_2^-, but the results of the present study suggest that this is not likely to have occurred (Rocha et al., 2009, 2015). While plasma [NO_2^-] represents a good biomarker of increased NO bioavailability, dietary NO_3^- ingestion also increases the production of other reactive nitrogen intermediates including S-nitrosothiols (RSNO). Indeed, it has been reported in rats that the antihypertensive effect of oral NO_2^- administration is closely associated with plasma [RSNO] and can be dissociated from plasma [NO_2^-] (Pinheiro et al., 2016).

A limitation of the present study is that we did not measure plasma [RSNO] or exhaled NO as an indication of stomach NO production. Further examination of the effects of NO_3^-, alcohol and their co-ingestion on changes in BP relative to changes in plasma [NO_2^-] and other reactive nitrogen intermediates may be an important avenue for future work.

In general, NIT-RW and NIT-A were the most effective combinations for inducing reductions in BP, with the former eliciting slightly greater decreases in systemic BP across the 5 h time frame compared to the other conditions. It may therefore be suggested that alcohol contributed to the alterations in BP we observed. We did not include control (low NO_3^- meal) alcohol (vodka or red wine) or polyphenol (which could have been achieved through administration of alcohol-free red wine) conditions and so cannot confirm the isolated effects of red wine, ethanol or polyphenols on BP in this study. Although the changes in BP might appear to be relatively small, it is possible that such changes may be meaningful, if maintained, in terms of reducing stress on the endothelium. For example, a reduction of just 5 mmHg in SBP has been suggested to decrease the risk of death by cardiovascular related causes, such as a stroke and heart disease by 14 % and 9 %, respectively (Stamler, 1991). Even a sustained 2 mmHg reduction in SBP could result in a 10 % reduction in stroke mortality and a 7 % reduction in cardiovascular disease mortality (Lewington et al., 2002). Future work might usefully characterise longer-term BP responses to NO_3^-, ethanol and polyphenols separately, and together, using an ambulatory BP monitor.

The NO_3^--rich dietary conditions employed in the present study resulted in reduced BP despite the participants being young and ostensibly normotensive. It has been reported that BP reduction is negatively correlated with baseline BP (Kapil et al., 2010) and it may therefore be expected that hypertensive, or even pre-hypertensive, individuals may gain greater benefits from the ingestion of dietary NO_3^- alone or in combination with moderate consumption of high and low polyphenolic alcoholic beverages, than their normotensive counterparts. It is important to emphasise that, following an initial vasodilatory response, there may be a subsequent rebound in BP following acute consumption of alcohol in isolation, at least at higher doses than that employed in this study, and that chronic or heavy alcohol consumption can predispose to the development of hypertension (Barden et al., 2013; Beilen, Puddey and Burke, 1996). We therefore do not advocate increasing alcohol consumption but instead note that the occasional glass of wine, as practiced in the ‘Mediterranean diet’, could be beneficial, or at least not obviously harmful, in some circumstances (Giacosa et al., 2016; Shen et al., 2015).

NO_3^- supplementation has not typically been reported to alter resting HR (Bailey et al., 2009; Ferguson et al., 2013, 2013a). However, in the current study, HR was increased in the high NO_3^- conditions as systemic BP decreased. The most notable changes in HR were those present in the NIT-RW and NIT-A conditions compared with CON at 2 h post meal ingestion. These elevations in HR may be a means of maintaining cardiac output in the face of decreased MAP.

Conclusion

In summary, consumption of a NO_3^--rich meal in combination with red wine (NIT-RW), vodka and lemonade (NIT-A) and water (NIT-CON) elevated salivary, plasma and urinary [NO_3^-] and [NO_2^-] both above baseline and compared to a low NO_3^- condition

(CON). Although all three high NO_3^- meals and the accompanying beverages reduced systemic BP, NIT-A and NIT-RW were the most effective means of reducing BP, with a tendency for greater and more frequent reductions occurring after NIT-RW consumption. The ingestion of a green leafy salad may be a simple means of acutely lowering BP in young normotensive individuals and this effect is not compromised by the ingestion of an accompanying moderate alcoholic beverage.

ETHICAL STATEMENTS

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Availability of data and materials

Data and materials are available on request and at the authors' discretion

Authors' contributions

S.T.J.M contributed to study design, recruitment, data collection, statistical analyses and data interpretation. L.J.W was responsible for study design, statistical analyses and data interpretation. P.T.M contributed to data collection. A.V. and A.M.J. were responsible for study design, data interpretation and supervision. All authors contributed to the written manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

Consent for publication

All authors approved the submission of the manuscript to the Nutrition and Health Journal and consent to publication of this manuscript.

Ethical approval

This study was approved by the University of Exeter Research Ethics Committee (Approval number: 161026/B/04) and was conducted according to the World Medical Association Declaration of Helsinki.

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FIGURE LEGENDS

Figure 1. Salivary nitrate (**A**), salivary nitrite (**B**), plasma nitrate (**C**), plasma nitrite (**D**), urinary nitrate (**E**) and urinary nitrite (**F**) following consumption of a low-nitrate meal with a water control (**CON**; ●), a nitrate-rich meal (6.05 mmol) with red wine (**NIT-RW**; ▲), vodka and lemonade (**NIT-A**; ■) and a water control (**NIT-CON**; ○). Data are presented as group mean ± SE. ^{a}Significantly different from pre-supplementation; *significantly different from **CON**; $^{\$}$significantly different from **NIT-CON** (all $P < 0.05$).

Figure 2. Change (Δ) relative to pre-supplementation baseline in systolic blood pressure (SBP; **A**), diastolic blood pressure (DBP; **B**) and mean arterial pressure (MAP; **C**) following consumption of a low-nitrate meal with a water control (**CON**; ●), a nitrate-rich meal (6.05 mmol) with red wine (**NIT-RW**; ▲), vodka and lemonade (**NIT-A**; ■) and a water control (**NIT-CON**; ○). Data are presented as group mean ± SE. ^{a}Significantly different from pre-supplementation; *significantly different from **CON**; $^{\$}$significantly different from **NIT-CON** (all $P < 0.05$).

Table 1. Baseline characteristics

n	12
Age, years	25 ± 5
Male, n	12/12
Body mass, kg	79 ± 11
Height, m	1.78 ± 0.06
Systolic blood pressure, mmHg	116 ± 5
Diastolic blood pressure, mmHg	63 ± 6
Mean arterial pressure, mmHg	81 ± 5

Values are mean ± SD.

Figure 1.

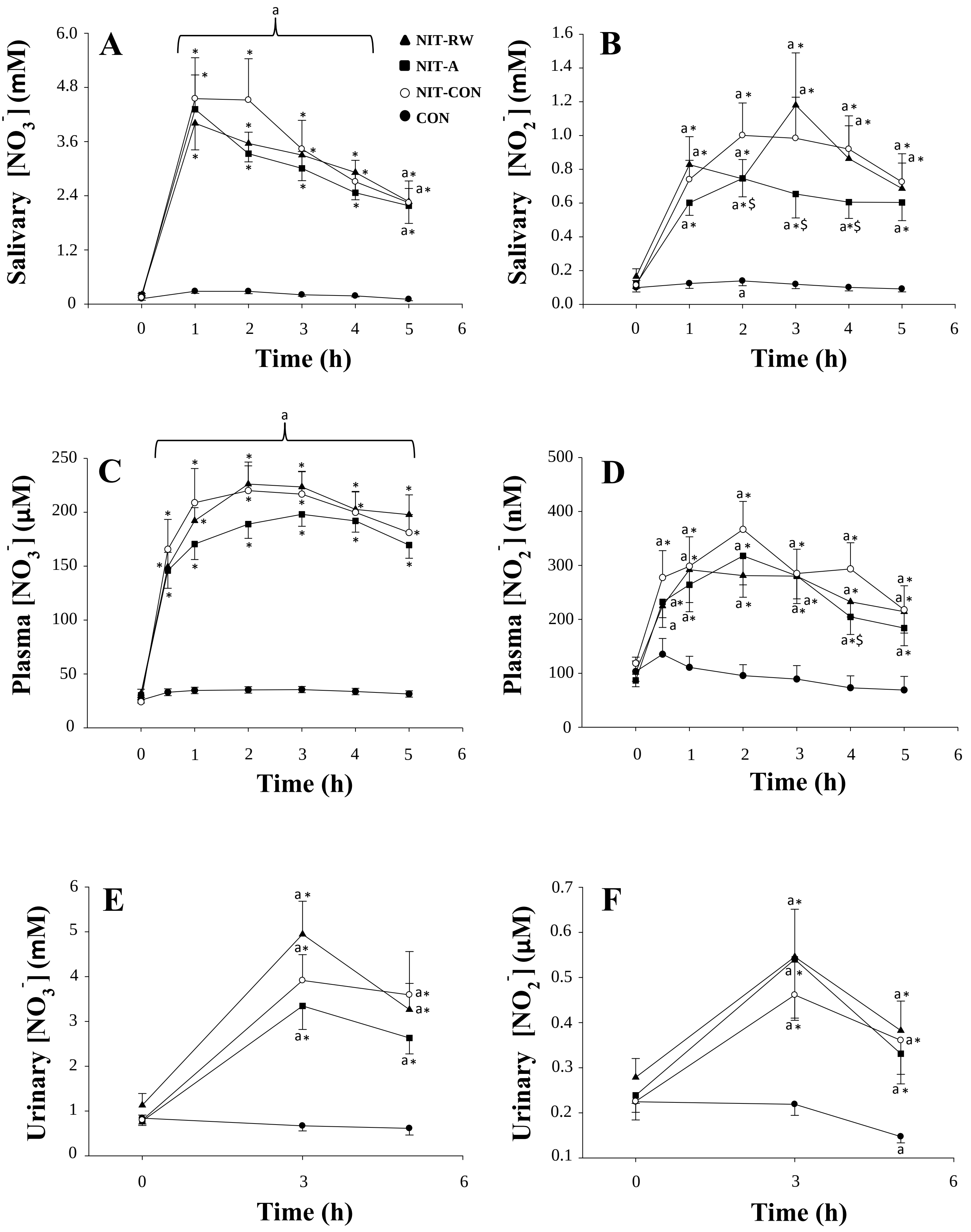
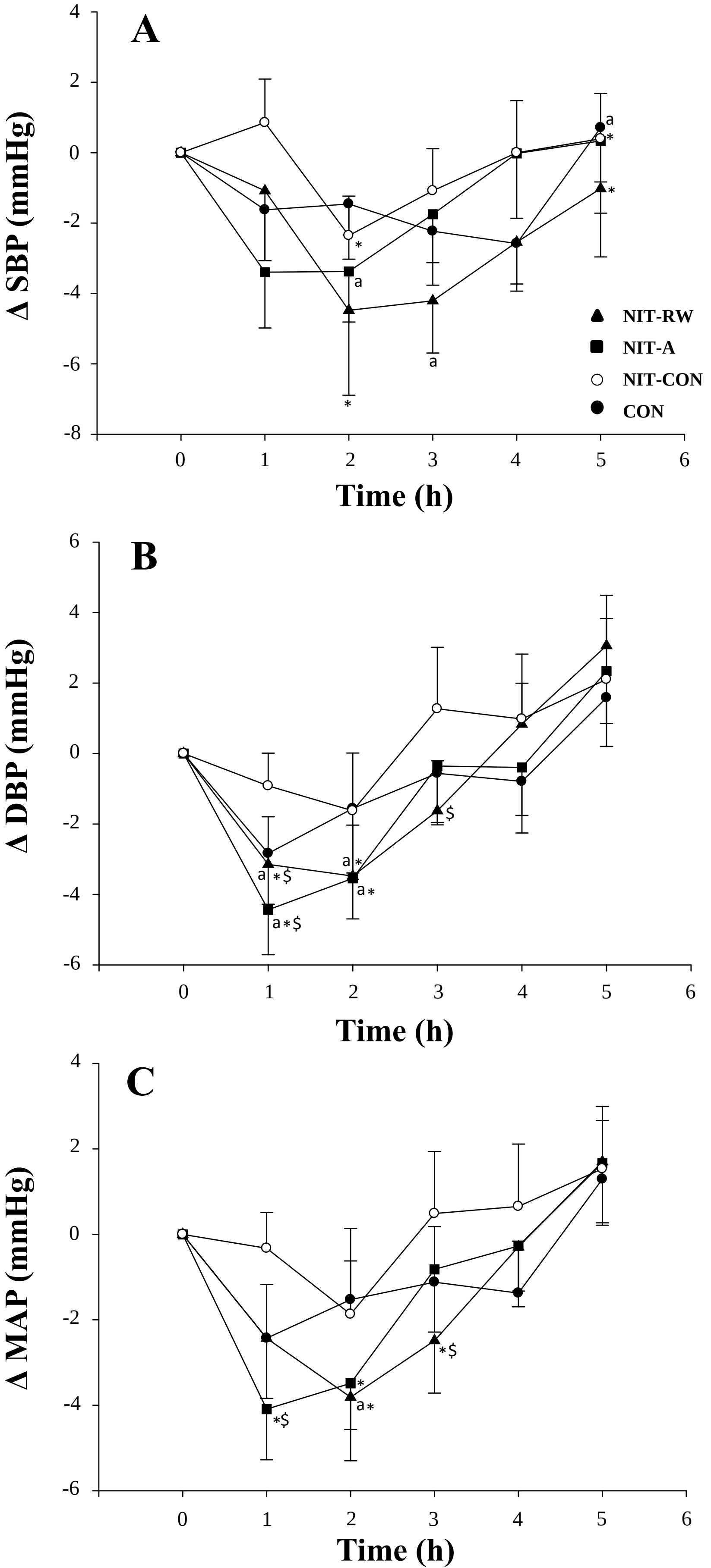

A
B
C
D
E
F
▲ NIT-RW
■ NIT-A
○ NIT-CON
● CON
Salivary [NO_3^-] (mM)
Salivary [NO_2^-] (mM)
Plasma [NO_3^-] (μM)
Plasma [NO_2^-] (nM)
Urinary [NO_3^-] (mM)
Urinary [NO_2^-] (μM)
Time (h)
0
1
2
3
4
5
6
1.2
2.4
3.6
4.8
6.0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
50
100
150
200
250
300
400
500
0.1
0.3
0.5
0.7
a
*
a*
a*$

Figure 2.


A
Δ SBP (mmHg)
4
2
0
-2
-4
-6
-8
0
1
2
3
4
5
6
Time (h)
NIT-RW
NIT-A
NIT-CON
CON
a
*
*
a
*
a
a
*
B
Δ DBP (mmHg)
6
4
2
0
-2
-4
-6
0
1
2
3
4
5
6
Time (h)
a *$
a*$
a*
a*
$
C
Δ MAP (mmHg)
4
2
0
-2
-4
-6
0
1
2
3
4
5
6
Time (h)
*$
*
a*
*$

Chapter 8: General Discussion

Traditionally, NO was considered to be a notorious air pollutant but has since been identified as a major physiological signalling molecule involved in the regulation of a number of biological processes, including BP (Rees, Palmer & Moncada, 1989; Webb et al., 2008), blood flow (Shen et al., 1994), metabolic control (Larsen et al., 2014) and skeletal muscle contractility (Hart & Dulhunty, 2000; Viner et al., 2000), to name a few. NO is known to be synthesised endogenously by the NOS family which promotes the oxidation of L-arginine (Stamler & Meissener, 2001) and by the NO_3^--NO_2^--NO route (Benjamin et al., 1994). A current and popular new avenue of research involves the use of dietary NO_3^- supplementation to increase NO bioavailability and to promote vascular health and exercise tolerance. However, recent reports indicate that many factors, some of which are related to daily lifestyle choices, can influence the physiological effects of dietary NO_3^- ingestion.

Research Questions Addressed

The primary aims of this thesis were to establish the role of dietary NO_3^- consumption in elevating markers of NO and improving systemic blood pressure in the presence of certain factors that might influence its effectiveness. Specifically, the questions posed included:

1) What are the effects of the prolonged use of different strength antibacterial mouthwashes, in combination with NO_3^- supplementation, on NO metabolites and resting and exercise BP?
2) What are the effects of short-term NO_3^- ingestion on exercise tolerance following voluntary blood donation?

3) What are the pharmacokinetic responses of salivary, plasma and urinary [NO_3^-] and [NO_2^-] and BP following consumption of different NO_3^- food forms?
4) What are the pharmacokinetic responses of salivary, plasma and urinary [NO_3^-] and [NO_2^-] and BP following consumption of a NO_3^--rich meal with an accompanying polyphenol-rich or -low alcoholic beverage?

Summary of main findings

NO_3^- and mouthwash

Chapter 4 investigated the influence of different strength mouthwashes on NO bioavailability and BP following chronic NO_3^- supplementation. Specifically, we explored how strong and weak antibacterial mouthwashes and a water control in combination with regular concentrated beetroot juice ingestion (6.2 mmol of NO_3^-, twice per day) over a 6 day period, impacted salivary and plasma [NO_3^-] and [NO_2^-] and resting and exercise BP. Despite a similar increase in plasma [NO_3^-] across the conditions, there was a decrease in the formation of salivary NO_2^- from NO_3^- in the oral cavity after rinsing with the strong mouthwash compared with the weak and control mouthwashes. In addition, a stepwise attenuation in plasma [NO_2^-] was noted with increasing strength of mouthwash used. Resting BP was not altered by any condition, possibly due to the low baseline BP in this cohort. However, SBP and MAP during low-intensity treadmill exercise were higher after using the strong mouthwash compared to baseline and the water control. Together, these results highlight the role of the oral microflora in elevating NO bioavailability and regulating BP, particularly during low-intensity exercise. These data provide important practical information for supplementation regimens. Specifically, limiting mouthwash use (unless it is recommended by a health professional to enhance oral hygiene) during periods of NO_3^- supplementation may help to maximise the increase in key NO markers and improvements in vascular health.

NO_3^- and blood donation

Approximately 3 % of the population aged 17-70 years donate blood each year in England. Whilst this does not sound like a large percentage, it equates to almost 2 million individuals (NHS Blood & Transplant, 2015). Chapter 5 explored the physiological response to exercise after short term supplementation with concentrated NO_3^--rich beetroot juice, following whole blood donation. Results showed that despite a similar reduction in haemoglobin concentration ([Hb]) between the groups, NO_3^--rich beetroot juice caused a 4 % reduction in $\dot{V}O_2$ during moderate-intensity exercise, and this improved efficiency, better preserved muscle oxygenation and attenuated the decrement in exercise tolerance to ramp incremental exercise, compared with the placebo condition.

NO_3^- supplementation may help to improve functional capacity and reduce fatigue in a typical person during recovery from a standard blood bank donation. These results have implications for offsetting the decline in exercise capacity in circumstances where [Hb] is lowered, such as in anaemia, following surgery or blood loss or in recreationally active members of the public wishing to donate blood without compromising exercise training or performance.

NO_3^- food forms

The remaining two experimental chapters of this thesis focused on the pharmacodynamic and pharmacokinetic response to acute NO_3^- ingestion in different food forms and different meal combinations.

Chapter 6 characterised the pharmacokinetic response to an equimolar (5.76 mmol of NO_3^- for concentrated beetroot juice, beetroot flapjack and non-concentrated beetroot juice; beetroot crystals contained a lower dose of 1.04 mmol of NO_3^- as per the manufacturer’s guidelines) dose of different NO_3^- vehicles. The solid, flapjack supplement resulted in a faster time (2 h) to reach peak plasma [NO_2^-] versus the concentrated beetroot juice shot and non-concentrated beverage (3 h). Interestingly, peak plasma [NO_2^-] occurred at 15 min in the beetroot crystal condition, which is likely due to the small dose of NO_2^- present in this supplement. Although all NO_3^-supplements used in this thesis elevated markers of NO bioavailability, the most effective food forms for increasing NO storage pools were the concentrated beetroot juice shot and the beetroot flapjack. In addition, the small, concentrated shot of beet juice, BR, was the most successful and consistent means of reducing SBP and MAP. However, it is noteworthy that the flapjack condition, BF, also reduced DBP.

These results provide important practical information for optimising supplementation regimens (with regard to type and timing of ingestion) for maximal cardiovascular and potential performance benefits in a young, healthy population. Specifically, consuming a small, concentrated volume of NO_3^--rich beetroot juice (BR) may be recommended as a way of elevating NO biomarkers and inducing reductions in systemic BP in normotensive individuals.

NO_3^- and alcohol

Chapter 7 established for the first time the [NO_3^-] and [NO_2^-] pharmacokinetics and BP response to a green leafy salad (containing 6.05 mmol of NO_3^-) consumed alongside an alcoholic beverage rich in polyphenols (red wine) or low in polyphenols (vodka and lemonade) and a water control. BP and salivary, plasma and urinary [NO_3^-] and [NO_2^-] were measured prior to supplementation and at regular intervals over the 5 h period post consumption of each meal and beverage combination. Results revealed that all three high NO_3^- conditions elevated salivary, plasma and urinary [NO_3^-] and [NO_2^-] above baseline and compared to a low NO_3^- condition. In addition, systemic BP was reduced after consumption of all NO_3^--rich meals and accompanying beverages. Although NIT-A and NIT-RW were the most effective means of lowering BP, there was a tendency for greater and more frequent reductions to occur after NIT-RW ingestion. These findings show that, in moderation, ingestion of an alcoholic beverage does not compromise the improvements in vascular health indices, such as BP, in a young, healthy cohort after a meal rich in NO_3^-.

Evidence of increased nitric oxide bioavailability

In all four Experimental Chapters, dietary NO_3^-, despite being ingested alongside a number of factors that might impact its effectiveness, such as mouthwash (Govoni et al., 2008), blood donation, the use of different food forms (Jonvik et al., 2016) and alcohol ingestion (Rocha et al., 2015), successfully elevated NO bioavailability as shown by increases in salivary, plasma and urinary [NO_3^-] and [NO_2^-]. These results are consistent with previous studies reporting elevations in markers of NO in a number of different bodily fluids following dietary NO_3^- ingestion only (Kapil et al., 2010; Pannala et al., 2003; Spiegelhalder et al., 1976; Wylie et al., 2013).

In Chapter 4, 6 days of concentrated beetroot juice supplementation elevated plasma [NO_2^-] by ~ 500 %. In Chapter 5, ingestion of 7 concentrated beetroot juice shots over the 48 h period post blood donation increased plasma [NO_2^-] by ~ 800 %. These values are higher than previously reported (50-150 %; Bailey et al., 2009; 2010; Bescós et al., 2010; Lansley et al., 2011; Larsen et al., 2007; 2010; Vanhatalo et al., 2010), which is likely due to the higher dose of NO_3^- (Chapter 4: 12.4 mmol per day; Chapter 5: ~ 43 mmol over 48 h) administered over the experimental period.

The pharmacokinetic response to the ingestion of an equimolar dose of NO_3^- (5.76 mmol) via three different food forms and the consumption of beetroot crystals (containing 1.4 mmol of NO_3^- as per the manufacturer's guidelines), on separate occasions, was determined in Chapter 6. The results of this study demonstrated that a small, acute dose of NO_3^- can elevate salivary, plasma and urinary markers of NO bioavailability. Specifically, beetroot flapjack and concentrated beetroot juice were the most effective means of increasing plasma [NO_2^-], and thus the potential for smooth muscle relaxation. Indeed, beetroot flapjack and concentrated beetroot juice resulted in reductions in systemic BP and these findings will be discussed in more detail in a later section.

Chapter 7 was the first study to reveal the salivary, plasma and urinary [NO_3^-] and [NO_2^-] pharmacokinetic response to a green leafy salad (containing 6.05 mmol of NO_3^-) in combination with an alcoholic beverage rich or low in polyphenol content, or a water control. All NO_3^--rich conditions increased markers of NO, with peak increases in plasma [NO_2^-] occurring 1 h after NO_3^- and red wine (NIT-RW) ingestion and 2 h post consumption of NO_3^- and vodka and lemonade (NIT-A) and NO_3^- and a water control (NIT-CON). BP was reduced after all high NO_3^- conditions; however, NIT-A and NIT-

RW were the most effective means of reducing systemic BP, with a tendency for larger magnitudes and more common reductions after NIT-RW ingestion.

Overall, it is reasonable to suggest that dietary NO_3^- ingestion, even when consumed in a single, acute bolus, particularly in the form of concentrated beetroot juice, non-concentrated beetroot juice, beetroot flapjack, beetroot crystals and a green leafy salad, can increase NO bioavailability, inferred from measured increases in salivary, plasma and urinary [NO_3^-] and [NO_2^-]. Although such measurements are consistently used as practical and sensitive markers of NO status, it must be acknowledged that elevations in circulating [NO_3^-] and [NO_2^-] are not a direct indication of increases in NO formation per se and the additional measurement of cGMP would have offered more insight into systemic [NO]. It is also important to note that swallowed salivary NO_2^- interacts with acid in the stomach and polyphenols, ascorbic acid and thiocyanate (to name a few) from the diet and can subsequently lead to the production of a number of different nitrogen species (other than NO_3^- and NO_2^-) that have not been measured in this thesis. Future work may include determination of an array of nitrogen species following NO_3^- ingestion. It may also be beneficial to confirm the minimum rise in, for example, plasma [NO_2^-] required to induce favourable physiological effects in healthy and diseased populations after consumption of inorganic NO_3^-.

Therapeutic effects of nitrate supplementation

The results from the experimental chapters in this thesis may have a number of therapeutic implications for both healthy and clinical populations.

Blood pressure

The lowering of systemic BP is one of the most valuable physiological benefits of dietary NO_3^- ingestion due to its potential prophylactic effects against hypertension. It has been consistently demonstrated that NO_3^- consumption can elevate NO

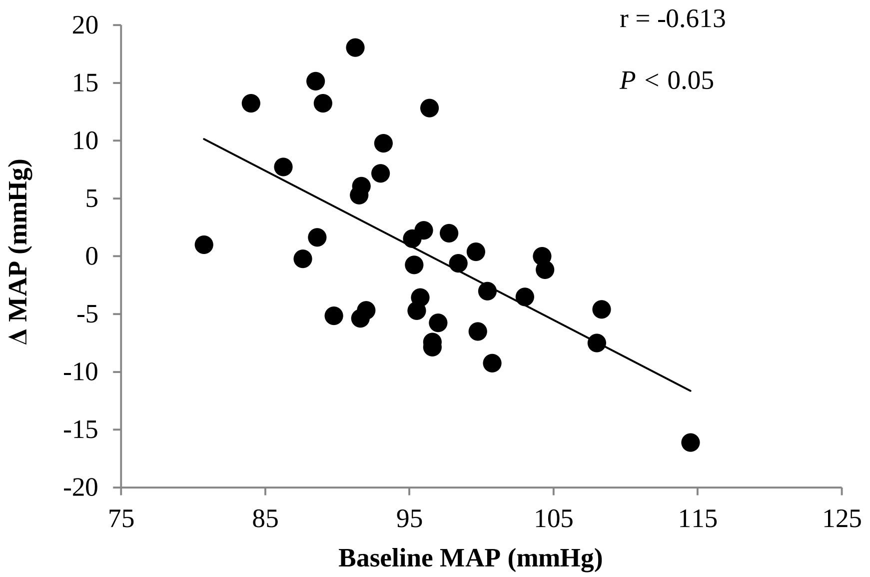


Figure 8.1 Negative Pearson product moment correlation between baseline MAP and the change in MAP following nitrate supplementation and prior mouth rinsing with STRONG and WEAK mouthwash or a water control (CON); this demonstrates that individuals with higher resting baseline BP have a greater reduction in MAP in response to dietary nitrate ingestion, regardless of mouthwash use.

bioavailability, as shown by increases in, for example, plasma [NO_2^-]. An increase in intravascular NO stimulates cGMP and it is the subsequent cascade of events that leads to smooth muscle relaxation and a reduction in BP (Archer et al., 1994; Lohmann et al., 1997).

The results from the current thesis support the notion that dietary NO_3^- is capable of reducing systemic BP. Data from Chapter 6 assessed resting, seated BP in a healthy cohort, and, consistent with previous studies (Jonvik et al., 2016; Kelly et al., 2013a; Muggeridge et al., 2014; Thompson et al., 2016; Wylie et al., 2013), demonstrated decreases in SBP (-5 mmHg) and MAP (-4 mmHg) following concentrated beetroot juice ingestion. Novel findings from this chapter also include a peak reduction in DBP (-4 mmHg) following a beetroot flapjack. In addition, Chapter 7 reported a reduction in SBP following a green leafy salad (-2 mmHg). However, more noteworthy decreases were found after the addition of a polyphenol-rich (-4 mmHg) or -low (-3 mmHg) alcoholic beverage. It has previously been suggested that a 5 mmHg decrease in SBP, as observed frequently in this thesis, is likely to reduce the risk of death by 7 %, and the risk of death by stroke and coronary heart disease by 14 % and 9 %, respectively (Stamler, 1991). In addition, it has been proposed that a 2 mmHg reduction in an adult's SBP could save > 14 000 lives in the United Kingdom each year (Critchley & Capewell, 2003). Turnbull (2003) also demonstrated that even a 1-2 mmHg decrease in BP is associated with reducing the risk of stroke and major cardiovascular events. It may therefore be suggested that, at present, this is the minimum clinically relevant reduction in BP required to improve cardiovascular health and reduce risk. However, further work, including long-term follow up of those ingesting dietary NO_3^- regularly, is needed to confirm this threshold, particularly in clinical populations. Overall, the findings in this thesis suggest that increased dietary NO_3^- ingestion, through commercially available supplements or increased vegetable consumption can be beneficial for vascular health (Van der Avoort et al., 2018).

However, resting BP was not always reduced following NO_3^- consumption, as shown in Chapters 4 and 5 following prolonged concentrated beetroot juice ingestion. It is likely that this is a consequence of a low baseline BP within the study populations (See Figure

8.1). Kapil et al. (2010) have suggested that there is a negative correlation between baseline BP and the lowering of BP in response to dietary NO_3^- ingestion, and our results support this notion (Figure 8.1). Overall, further work is required to determine the effects of different forms of NO_3^- supplementation, if consumed over a more prolonged period than explored in this thesis, on lowering systemic BP. It is important to note that the findings from Chapter 4 show that the use of strong, and even weak, mouthwash should be used with caution during periods of dietary NO_3^- supplementation, particularly when the aim is to maximise NO bioavailability and reduce systemic BP. However, the maintenance of good oral health is also important and daily routines to ensure this is achieved should not be overlooked.

Overall, these results show encouraging signs that dietary NO_3^-, consumed as a concentrated beetroot drink, beetroot flapjack or salad (with or without polyphenol rich or low alcoholic beverages - to be consumed in moderation) could contribute to future public health guidelines as an innovative, practical and potentially cost effective means of preventing and treating high BP and the associated risk of cardiovascular disease, reduced quality of life and mortality. However, further work is required to determine more prolonged effects of dietary NO_3^- ingestion, through supplementation or by increased vegetable consumption in the habitual diet, on cardiovascular health and risk of mortality.

Muscle oxygenation

In Chapter 5, indirect measures of muscle oxygenation were determined using near-infrared spectroscopy (NIRS) during moderate-intensity and ramp incremental exercise prior to and post blood donation. Following 7 shots of NO_3^--depleted beetroot juice over the 48 h after blood donation, tissue oxygenation index (TOI) was reduced and [HHb] tended to be higher when compared with pre donation values. This suggests that muscle O_2 availability was lower and more O_2 extraction at the muscle was required to attain the required $\dot{V}O_2$ during the submaximal exercise bout. In the NO_3^--rich condition, these changes were ameliorated, suggesting that muscle oxygenation was better preserved when compared with the placebo condition. Masschelein and colleagues (2012) reported similar responses to submaximal exercise in hypoxia after beetroot juice ingestion. In addition, Kelly et al. (2014) reported that the effect of NO_3^- ingestion on exercise efficiency and tolerance was greater in hypoxia than normoxia. The reduced arterial O_2 concentration and intracellular partial pressure of O_2 that results from breathing a hypoxic inspirate can lead to muscle tissue hypoxia and subsequently, increase muscle metabolic perturbation (Linnarsson et al., 1974). An increase in local blood flow, via hypoxia-induced vasodilation, can help to restore O_2 availability, through NO mediated processes (Casey et al., 2010). Therefore, supplementing the diet with NO_3^- increases the bioavailability of NO and may help to augment this process further, over and above that seen in normoxia.

Overall, it may be suggested that the high NO_3^- content of the aforementioned beverages helped to promote a better matching of muscle O_2 delivery to O_2 demand when O_2 availability is limited by reduced [Hb] or breathing a hypoxic inspirate.

Exercise efficiency

In Chapter 5, the O_2 cost of submaximal exercise was assessed during CWR cycle tests performed by a young, healthy cohort. The results demonstrated that 7 shots of concentrated beetroot juice (each containing 6.2 mmol of NO_3^-) over the 48 h period post blood donation lowered $\dot{V}O_2$ during baseline and moderate-intensity exercise, by 4 %. This study was the first to assess the effects of dietary NO_3^- on exercise efficiency after inducing a reduction in blood O_2-carrying capacity. The findings, however, are similar to previous studies that have reported that NO_3^- invoked a 4-8 % reduction in the O_2 cost of submaximal cycling exercise when O_2 availability was limited through breathing a hypoxic inspirate (Kelly et al., 2014; Masschelein et al., 2012; Muggeridge et al., 2014).

As stated in the literature review, the suggested mechanistic bases for a lower $\dot{V}O_2$ during moderate-intensity exercise include a reduction in the ATP cost of muscle force production (Bailey et al., 2009) which might be facilitated by enhanced Ca^{2+}-related contractility (Hernández et al., 2012), and/or an improvement in mitochondrial efficiency (Larsen et al., 2011; Vaughan et al., 2016). More recently, however, it has been suggested that an improvement in mitochondrial efficiency may not be present when NO_3^- is administered as beetroot juice (Whitfield et al., 2016). Specifically, 7 days of beetroot juice (26 mmol of NO_3^- per day) were shown to lower $\dot{V}O_2$ during submaximal exercise in the absence of changes in the P/O ratio and expression of ANT and UCP-3; therefore, it is likely that the reduced $\dot{V}O_2$ was due to a decrease in the ATP cost of muscle force production.

As well as changes in metabolic or muscle contractile efficiency, alterations in muscle O_2 delivery or intramuscular distribution may also occur after ingestion of dietary NO_3^- (Ferguson et al., 2013; 2013a). It is important to note that NO has been directly implicated in the regulation of mitochondrial O_2 consumption. Specifically, both NO and O_2 have a strong affinity for COX and compete for its binding site in the mitochondrial electron transport chain (Brown, 2001). Whilst it is likely that a combination of the elevation in NO bioavailability from dietary NO_3^- and a reduction in blood O_2-carrying capacity after blood donation (as seen in Chapter 5) increased the binding of NO to COX and modified the intramuscular distribution of O_2, resulting in impeded O_2 consumption at the mitochondrion (Brown & Cooper, 1994; Cleeter et al., 1994) and improved oxygenation of the muscle fibres situated further away from the capillaries (Hagen et al., 2003; Thomas et al., 2001; Victor et al., 2009), it may have also triggered a signalling cascade for a resultant downregulation in some mitochondrial proteins, such as ANT, and enhance respiratory chain efficiency (Larsen et al., 2011).

Exercise tolerance

Chapter 5 demonstrated that dietary NO_3^-, in the form of concentrated beetroot juice can attenuate the deleterious effects of whole blood withdrawal on incremental exercise tolerance (-2.7 %) compared with the NO_3^--depleted condition (-5.0 %). This finding is consistent with that of Masschelein et al. (2012), who reported that beetroot juice (0.07 mmol of NO_3^- per kg of body mass per day, for 6 days) partly negated (+ 5 %) the reduction in time to exhaustion achieved during incremental exercise in hypoxia when compared with the placebo condition. Others have also reported that NO_3^- consumption can enhance tolerance to CWR exercise (Kelly et al., 2014; Vanhatalo et al., 2011) and improve time trial performance (Muggeridge et al., 2014) under hypoxic conditions.

In Chapter 5, blood donation also decreased $\dot{V}O_{2peak}$ during incremental exercise and this occurred alongside an increase in muscle [HHb]. This may be suggestive of an increase in muscle O_2 extraction in an effort to counteract the reduced muscle O_2 delivery due to a lower [Hb] post blood donation (Roach et al., 1999; Schaffartzik et al., 1993). However, in the NO_3^- condition, $\dot{V}O_{2peak}$ and [HHb] were not altered.

Mechanisms for improvement in exercise tolerance following dietary NO_3^- ingestion and blood donation may include a combination of O_2 sparing as well as vasodilatory-dependent increases in blood flow and/or possible altered intramuscular O_2 distribution, which enables better preservation of muscle oxygenation (Ferguson et al., 2013; 2013a; Hernández et al., 2012; Vanhatalo et al., 2014).

These data suggest that NO_3^--rich BR may have therapeutic benefits for individuals with reduced O_2-carrying capacity, such as those with anaemia or those recovering from blood loss through surgery or combat. The improvements in functional capacity noted in Chapter 5 may also be transferable to other pathological conditions where O_2 availability may be limited, such as COPD, PAD, diabetes and heart failure with both reduced and preserved ejection fraction. Some research has already been undertaken regarding the potential benefits of dietary NO_3^- ingestion on exercise capacity in such populations, but mixed outcomes have been reported. No effect of beetroot juice ingestion has been reported for the distance covered in a six minute walk test in individuals with diabetes (Shepherd et al., 2015) and also those with COPD (Shepherd et al., 2015a). However, an extended time to exhaustion has been reported during different forms of exercise in individuals with COPD (Berry et al., 2015; Leong et al., 2015), PAD (Kenjale et al., 2011) and heart failure with preserved ejection fraction (Zamani et al., 2015; 2017). In another study, concentrated beetroot juice did not improve exercise tolerance in patients with heart failure with reduced ejection fraction (Hirai et al., 2017). More work is required to determine the effects of dietary NO_3^- on exercise capacity in clinical populations (McDonagh et al., 2018).

Translation of findings

In this thesis, the consumption of NO_3^- in a variety of different food forms resulted in beneficial effects on BP, muscle oxygenation and exercise efficiency and tolerance in young, healthy individuals. However, the use of mouthwash prior to NO_3^- ingestion markedly reduced elevations in NO biomarkers and the potential for BP to be lowered and, therefore, its use should be considered carefully if one's aim is to derive therapeutic or ergogenic benefits from NO_3^-. Although these findings agree with previous work (Bondonno et al., 2014; Kapil et al., 2013; Petersson et al., 2009), many other factors regarding the impact of mouthwash on the oral microbiome, NO bioavailability and the subsequent BP response remain unknown. Further work may involve determination of the species of NO_3^--reducing bacteria predominantly affected by mouth rinsing with strong and weak antibacterial agents, and whether such rinsing results in a reduction in NO_3^- reductase activity or total population of the bacteria residing in the oral cavity. It may also be beneficial to establish the duration of disruption of the oral microbiome following mouthwash and how the timing of NO_3^- ingestion (prior to or post mouthwash), number of mouth rinses per day and time between rinsing and NO_3^- ingestion impacts NO markers and cardiovascular indices.

In Chapter 5, concentrated beetroot juice shots were effective in lowering the O_2 cost of moderate-intensity exercise and offsetting the deleterious effects of blood donation on exercise tolerance. However, the NO_3^- dose administered over the 48 h period following blood withdrawal was high and unlikely to be achieved via typical vegetable intake. Future work should therefore attempt to characterise the physiological response to exercise in combination with a high NO_3^- diet, achieved through increased fruit and vegetable consumption, following whole blood donation and in those living with anaemia. A more ecologically valid approach to supplementing the diet with NO_3^- may enhance exercise tolerance, ability to perform ADLs and improve the quality of life of those living with reduced O_2 carrying capacity.

Although the findings in this thesis are important, the implications of a NO_3^--rich diet with or without polyphenol-rich or -low alcoholic beverages, may be much greater for older and clinical populations than young, healthy individuals. At present, the impact of dietary NO_3^- among older individuals and those with chronic disease are equivocal. A recent systematic review revealed that NO_3^- intake improved the physiological response to exercise, however, mixed findings were noted regarding benefits for cardiovascular and cerebrovascular health in persons over the age of 50 years (Stanaway et al., 2017). Similarly, NO_3^- ingestion has resulted in varied responses regarding elevations in plasma [NO_2^-], reductions in BP and improvements in exercise efficiency and tolerance in those with chronic conditions (McDonagh et al., 2018).

Elucidating the optimal NO_3^- dose and long-term effects of NO_3^- on parameters of cardiovascular health, cognitive function, tolerance to exercise and vascular risk may be beneficial for healthy and diseased individuals. Encouraging the consumption of NO_3^- via concentrated beetroot juice shots or flapjacks, or by devising a dietary strategy that may more acceptable to the general public, such as ingesting palatable fruit- and vegetable- rich meals, rather than potentially costly and often unpleasant tasting supplements, may improve uptake and subsequently, cardiovascular health and general well-being. Educating members of the public about the benefits of NO_3^- consumption and factors that might affect its effectiveness should also be undertaken. In addition, dissemination of key research findings, particularly from Chapters 4-7, via peer-reviewed publications, local newspapers, social media and charities, to target audiences, such as patients, the public, key stakeholders and policy makers may lead to changes in future cardiovascular and WHO guidelines.

Limitations

General

Overall, the experimental chapters in this thesis provide a novel insight into some factors that can affect the efficacy of dietary NO_3^- ingestion. However, a number of limitations must be acknowledged.

Although power calculations were performed, there were relatively small sample sizes in each study and this may have contributed to some of the non-significant main effects and interaction effects noted. In contrast to a more widely used statistical approach in which a non-significant interaction is not typically followed by comparisons of factor means, an alternative method was employed where post hoc tests were performed in the absence of significant interaction effects. Specifically, when main effects were noted for time and/or condition, follow-up tests were undertaken to determine which particular means differed (Wei et al., 2012). This approach was particularly useful in establishing whether dietary NO_3^- increased NO biomarkers or reduced BP when compared with baseline or other high or low NO_3^- conditions, and at which specific time point following ingestion such differences occurred.

It is also important to mention that inclusion of only young, healthy individuals in Chapters 4-7 may have induced selection bias regarding the efficacy of dietary NO_3^- on BP and exercise efficiency and performance. Future work may address the impact of

NO_3^- ingestion on cardiovascular biomarkers and exercise performance in a more general population, including those who are older and living with chronic disease.

Nitrate dose

In Chapters 4-7, all subjects were required to ingest a set dose of dietary NO_3^-, regardless of the size of the person, sex, baseline BP or baseline plasma [NO_2^-], all of which may contribute to the efficacy of exogenous NO_3^- ingestion (Kapil et al. 2010; Wilkerson et al., 2012). Although the fixed doses of NO_3^- given in each study (Chapters 4 and 5: 12.4 mmol per day for 6 days and 48 h, respectively; Chapter 6: 5.76 mmol, acutely; Chapter 7: 6.05 mmol, acutely) were based on previous research that had demonstrated beneficial effects on NO bioavailability, BP and exercise efficiency and performance after an acute dose of NO_3^- (~ 5.2 mmol; Vanhatalo et al., 2010; ~ 6-16.8 mmol; Wylie et al., 2013; 2016), it may be worth exploring the dose of natural NO_3^- sources relative to body mass, baseline characteristics and health status in the future. This would allow clarification on the dose of NO_3^- required to invoke changes in plasma [NO_2^-] and improvements in cardiovascular health and performance in different populations.

Safety, tolerance and efficacy of the interventions

The WHO guidelines (2002) stipulate an ADI of 3.7 mg of NO_3^- per kg of body mass. This guideline was introduced with the aim of reducing incidents of harmful side effects (such as methaemoglobinaemia and gastric cancer) previously thought to be associated with the consumption of NO_3^-. However, WHO (2010) have recently acknowledged that NO_3^- ingestion may be beneficial for health. In fact, a diet rich in vegetables is often promoted (e.g. the DASH diet) due to the subsequent improvements in cardiovascular indices, even though it exceeds the recommended NO_3^- intake threshold. In Chapters 4-7, the NO_3^- dose administered exceeded the ADI, but frequently resulted in cardio-protective (reductions in BP) and ergogenic (improvements in exercise tolerance and efficiency) effects. However, no measures of NO_3^- or NO_2^- toxicity were undertaken and therefore this is an avenue for future work. Specifically, it may be worth examining the effects of increasing NO_3^- doses and the long term effects of such doses on cardiovascular health and markers of oxidative and nitrosative stress in bodily fluids.

It is important to note that the concentrated beetroot juice (Chapters 4, 5 and 6), beetroot flapjack, non-concentrated beetroot juice, beetroot crystals (Chapter 6) and salad (Chapter 7) were well tolerated, with the latter two forms being the most popular choice of NO_3^- vehicle based on taste. The concentrated beetroot juice and non-concentrated beetroot juice did, however, sometimes result in the appearance of red urine and stools but did not cause any gastrointestinal discomfort.

Measurement of NO bioavailability restricted to [NO_3^-] and [NO_2^-]

The sole purpose of administering dietary NO_3^- was to encourage the potential for NO production. NO has a short half-life *in vivo* (0.1 s) and therefore is very difficult to quantify (Kelm & Schrader, 1990). However, NO-linked metabolites, such as NO_3^- and NO_2^-, which form large storage pools in the body (Cosby et al., 2003; Silver, 2011), are much more stable than NO and can be detected in a range of biological fluids. As a result, plasma (Lauer et al., 2001), salivary and urinary [NO_3^-] and [NO_2^-] were deemed as suitable and practical for determining NO bioavailability and therefore were measured in this thesis. Despite this, it must be acknowledged that additional NO storage forms or indicators of its presence could have been measured, including, *S*-nitrosothiols and *S*-nitrosohaemoglobin (Doctor & Stamler, 2011; Pinheiro et al., 2015) and cGMP (Archer et al., 1994; Murad et al., 2003). It may also have been beneficial to have measured NO in the air expelled from the stomach, particularly in Chapter 7 where the presence of polyphenols in red wine may have increased the conversion of NO_2^- to NO in the stomach (Gago et al., 2007). Future research may include the assessment of a combination of NO storage pools after NO_3^- ingestion which could help clarify their contribution to NO-mediated processes.

Exercise testing

In Chapters 6 and 7 the pharmacokinetic responses to different commercially available NO_3^- vehicles and a green leafy salad alongside an array of accompanying beverages was determined over a 24 and 5 h period, respectively. Due to the extensive nature of blood, saliva and urine sampling needed for the determination of the pharmacokinetic responses, there was insufficient time to include exercise protocols within the study designs and therefore the physiological responses were limited to BP measurements. A future line of work may be to determine exercise tolerance and the $\dot{V}O_2$ response to moderate-intensity exercise after consumption of different NO_3^- vehicles. This information would help to inform future supplementation strategies.

Future research questions

The current thesis contributes to the growing body of evidence which supports the role of NO_3^- supplementation as a therapeutic and ergogenic aid, particularly when certain factors that may influence its effectiveness are considered carefully prior to ingestion.

Overall, it may be suggested that dietary NO_3^- is beneficial for cardiovascular health when administered as a concentrated beetroot juice, flapjack (Chapter 6) or green, leafy salad perhaps in combination with an alcoholic beverage (in moderation; Chapter 7). It is also noteworthy that NO_3^- supplementation may improve exercise efficiency and tolerance post blood donation (Chapter 5). However, the cardio-protective effects of NO_3^- may only be present if habitual use of antibacterial mouthwash is restricted during periods of supplementation (Chapter 4). Whilst this thesis highlights the role of some factors on the effectiveness of NO_3^- ingestion as a natural aid for health and performance, further questions have come to light and may be worthy of future investigation.

Oral microbiome and lifestyle factors

It is well established that the oral microflora plays a crucial role in the NO_3^--NO_2^--NO pathway. The NO_3^- reducing bacteria in the mouth are capable of altering NO homeostasis and vascular function, as previously shown by the deleterious effects of antibacterial mouthwash on BP after NO_3^- ingestion (Chapter 4).

Whilst the microbial communities in the adult mouth are relatively stable, biological changes, such as ageing, pregnancy and the development of disease (such as diabetes; Chapple & Genco, 2013) can affect the balance of bacterial species within such communities (Marsh, Head & Devine, 2015). In addition, lifestyle choices, such as smoking, dietary intake, poor oral hygiene or use of antibiotics can also result in a dysbiotic shift and altered diversity of bacteria in the oral cavity (Kilian et al., 2016; Marsh, Head & Devine, 2014; Wu et al., 2016). However, the influence of many daily choices with or without NO_3^- ingestion on the oral microflora and subsequent BP response is not yet known. Future studies could be directed at exploring the effectiveness of NO_3^- ingestion on BP and cardiovascular disease risk, as well as the inter-individual differences in the oral flora, when consumed alongside chronic mouthwash use, smoking and dietary habits, particularly in clinical and athletic populations.

Clinical populations

The purpose of Chapter 5 was to identify the effects of concentrated NO_3^--rich beetroot juice on exercise capacity after voluntary whole blood withdrawal, which can simulate clinical conditions where blood O_2-carrying capacity and tissue O_2 delivery may be limited. Over the past few years, the influence of dietary NO_3^- in older persons (Kelly et al., 2013) and in individuals with diabetes (Gilchrist et al., 2014; Shepherd et al., 2015), COPD (Berry et al., 2015; Shepherd et al., 2015a), PAD (Kenjale et al., 2011) and heart failure with preserved (Zamani et al., 2015; 2017) and reduced (Coggan et al., 2018) ejection fraction has been investigated, but with varied outcomes. Future research could be directed toward the effects of NO_3^- ingestion in other clinical populations where promotion of the NO_3^--NO_2^--NO pathway may be particularly useful, such as in anaemia. The use of dietary NO_3^- as a therapeutic aid in the clinical domain may have larger implications than those seen in young, healthy persons and therefore, determining the timing and dosage of dietary NO_3^- needed to positively influence parameters of cardiovascular health in such populations may be a future channel of research.

Ascorbic acid and polyphenols

Chapter 7 highlights the potential role of alcohol, and more noticeably, although not significantly, the combination of alcohol and polyphenols (via red wine) in reducing systemic BP, alongside NO_3^- ingestion. Polyphenols, including quercetin and resveratrol have been linked to mitochondrial biogenesis, associated increases in aerobic capacity (Davis et al., 2009; Ganio et al., 2010; Lagouge et al., 2006) and reductions in BP (Liu et al., 2015). It has also been suggested that NO formation is enhanced in the presence of vitamin C (ascorbic acid; Carlsson et al., 2001). However, the influence of polyphenols and ascorbic acid in isolation and in conjunction with NO_3^- supplementation requires further research. The dose- and pharmacokinetic- response to NO_3^- with ascorbic acid and an array of polyphenols has yet to be determined, so too is the subsequent impact on BP and the physiological response to exercise.

Conclusion

The physiological response to the consumption of dietary NO_3^-, which is available in a multitude of different forms and ingested as part of a normal human existence and alongside a selection of factors that might impact its efficacy, is a fast-evolving area of research in exercise physiology and medicine today.

This thesis has highlighted cardiovascular benefits of dietary NO_3^- ingestion in young, healthy individuals. Overall, the results suggest that factors, such as the regular use of mouthwash, blood donation, choice of food form and beverage accompanying NO_3^--rich foods, can impact the effectiveness of dietary NO_3^- ingestion on cardiovascular parameters and therefore must be carefully considered when the aim is to derive physiological benefits. Specifically, dietary NO_3^-, in many different forms, can elevate markers of NO bioavailability and reduce systemic BP. Therefore, NO_3^- can be recommended as a means for improving cardiovascular health, but a number of factors must be taken into account during periods of supplementation to ensure maximum benefit is achieved.

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