A 'green' approach to cardiovascular disease: recycling inorganic nitrate and nitrite

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Fruit and vegetable-rich diets reduce blood pressure[1] and risk of ischaemic stroke[2] and ischemic heart disease[3]. Whilst the cardioprotective effects of a fruit and vegetable-rich diet are unequivocal, the exact mechanisms of this effect remain uncertain. Recent evidence has highlighted the possibility that dietary nitrate, an inorganic anion found in large quantities in vegetables (particularly green leafy vegetables), may have a role to play. This beneficial activity lies in the processing in vivo of nitrate to nitrite and then nitrite to the pleiotropic molecule nitric oxide (NO). NO in turn exerts a range of beneficial effects on the cardiovascular system including lowering of blood pressure, suppression of inflammation and prevention/retardation of atherosclerosis. This range of bioactivity essentially endows NO with anti-hypertensive and anti-atherosclerotic activities that protects individuals from hypertension and associated cardiovascular disease[4]. The possibility that provision of nitrate may offer a mechanism for elevating beneficial NO that might, in particular, lower blood pressure is therefore an exciting prospect.

Despite the substantial advances made in anti-hypertensive pharmacotherapy, it is estimated that globally by 2025 there will be 1.5 billion people with hypertension[5]. The significance of this observation is fully appreciated when one considers that ~50% of all heart attacks and ~60-70% of all strokes occur as a direct consequence of raised blood pressure. Indeed, at a population level it has been estimated that as little as a 2mmHg increase in systolic blood pressure increases mortality due to ischaemic heart disease and stroke by 7% and 10% respectively[6]. Furthermore, of considerable concern is that, over the past 3 decades, the numbers of patients with uncontrolled essential hypertension is rising[7]. Thus, whilst pharmacotherapy plays an important role in the therapeutics of hypertension, there is a pressing need for identification of alternative but also preventative strategies that are both effective and easily achievable. This imperative has renewed interest in identifying approaches that take advantage of the beneficial effects of diets rich in fruit and vegetables[8], particularly since epidemiological[9], cohort[2,3] and trial data[1,10] demonstrate that increased consumption of a vegetable-rich diet protects against various forms of CVD, including hypertension[1]. In addition the focus, in part, stems from a perception that dietary interventions may be more acceptable and achievable for many patients. In particular, such an option may provide an alternative that could prove useful in the ~30-40% of treated hypertensives that remain with elevated blood pressures despite their pharmacotherpy[11]. Importantly, whilst some of these individuals likely have what is termed 'resistant hypertension' (recent estimates suggest ~9% in the US[12]), for many of these individuals it is thought that compliance plays a significant contribution[13].

Large-scale clinical trials of several different nutrients found in vegetables including antioxidant vitamins and folate have failed to show a beneficial cardiovascular effect[14-16]. As such attention has focused on other components of vegetables that may have a role, including inorganic nitrate[17,18]. Interestingly, the first published evidence of potential positive effects of inorganic nitrate on blood pressure date back to 1927 when Stieglitz published his observations that bismuth subnitrate lowered blood pressure in individuals with raised blood pressure[19]. Stieglitz published further observations confirming his original findings in specific cohorts[20], however the concept appeared to lose favour with publication of some dissenting observations[21]. In 2006, however, the concept was revived by Lundberg and Weitzberg and colleagues, who conducted a well-controlled clinical study i.e. double blind placebo control, that enabled clear cut assessment of potential bioactivity. This group demonstrated that supplementation of healthy volunteers with sodium nitrate (0.1mmol/kg/day) for 3 days resulted in a decrease in diastolic (DBP) of ~3mmHg[22].

More recently, we extended the above observations to determine whether dietary nitrate might offer similar bioactivity to nitrate salt supplementation. We used beetroot as our vehicle to deliver a dietary nitrate dose. Beetroot is a high nitrate-containing vegetable and, indeed, in our study a relatively high dose of 22.5 mmol was delivered, and a number of outcomes measured over the following 24 hours. A single dose of dietary nitrate lowered both SBP and DBP with the peak effect of 10/8 mmHg occurring approximately 2.5-3 hours following ingestion and blood pressure remaining lowered at the 24 hour time-point[23]. We then conducted a second study assessing the dose-response relationship as well as demonstrating that nitrate salt supplementation exerted an almost identical profile of bioactivity both in terms of the magnitude of the effect and dose-response relationship[24]. Importantly, we discovered that the threshold dose for blood pressure lowering in healthy volunteers was ~4 mmol which equates to just above the recommended daily amount of 4.2 mmol per day in a 70 kg individual (3.7mg/kg/day[25]). These effects of nitrate upon blood pressure are thought to be mediated by its sequential reduction to nitrite and then NO. NO in turn activates the enzyme soluble guanylate cyclase elevating cGMP levels, ultimately resulting in the relaxation of vascular smooth muscle, vasodilation and therefore decrease in blood pressure. Involvement of this pathway in the blood pressure lowering effects of dietary nitrate is supported by observations of nitrate-induced rises in circulating cGMP levels after either dietary or salt supplementation and by increases in vascular compliance, reflecting smooth muscle relaxation[26]. In addition to lowering blood pressure, we also demonstrated that both dietary nitrate ingestion or nitrate salt supplementation provided protection of the endothelium from ischaemia-reperfusion (IR)-induced endothelial damage as well as a suppression of platelet reactivity assessed ex vivo[23]. Together, such a profile of activity would suggest that dietary nitrate might prove useful in the therapeutics of cardiovascular disease.

The activity of orally ingested inorganic nitrate lies in its conversion to nitrite by facultative bacteria found on the dorsal surface of the tongue[27,28], the same pathways that have been proposed to underlie the potential detrimental effects of nitrate[29]. The swallowing of nitrite-rich saliva permits entry of nitrite into the circulation via the stomach and then, once within the circulation, nitrite can be converted to the potent vasodilator nitric oxide (NO)[24,30-32].Indeed, in our work with beetroot we

demonstrated that preventing this 'enterosalivary' circuit, by asking volunteers not to swallow their saliva for 3 hours following ingestion of beetroot, completely prevented the rise in circulating nitrite associated with nitrate ingestion as well as block of the decrease in blood pressure[23]. These findings are in accord with previous observations demonstrating that destruction of oral bacteria using an antibacterial mouthwash abolishes conversion of nitrate to nitrite in the oral cavity as well as blocks the consequent rise in circulating nitrite levels following sodium nitrate salt supplementation[33,34].

An important consideration is that the beneficial effects of inorganic nitrate result in modest rises in circulating nitrite i.e. no more than a doubling of circulating nitrite levels. Since baseline circulating nitrite is thought to lie somewhere in the region of 20-400nM (for review[35]), the biological effects of nitrate are evident with circulating nitrite concentrations, at the very most, in the low μM realm. Importantly, acutely such rises in circulating nitrite are not associated with any overt toxicity such as met-haemoglobinaemia[36]. Certainly, searches of the literature suggest no evidence that consumption of high nitrate containing vegetables is associated with met-haemoglobinaemia or any other negative aspects of activity that have been purported to be associated with inorganic nitrate ingestion either. Whether long term ingestion of a daily dietary nitrate load might result in any toxicity is uncertain, although to date no clear cut prospective evidence supporting the view that inorganic nitrate expresses a toxicity profile at the doses falling within the range tested in healthy volunteers exists[25].

Perhaps, our recent observations in hypertensive patients might go some way to allay concerns regarding potentially appropriate dosing regimes. We have recently demonstrated that despite 4mmol having little to no effect upon blood pressure in healthy volunteers, that in individuals with Grade 1 hypertension (i.e. SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg) a single dose just below this level (i.e.~3.5mmol) causes an average decrease in blood pressure of ~12/10 mmHg. Such a dose of nitrate clearly sits at the recommended daily consumption amounts and therefore poses perhaps less concern regarding potential, albeit unlikely, toxicity. As in healthy volunteers, the peak effect occurred at 2.5-3 h in parallel with the rise in circulating nitrite concentration, but also with significant blood pressure lowering still evident at 24h (~60% of the peak effect)[37]. This increased potency is in part due to an upregulation in the expression and activity of a key nitrite reductase in hypertensive patients, an enzyme called xanthine oxidoreductase[37]. We have hypothesised that this upregulation results in a greater conversion of circulating nitrite to NO within the blood vessel. We have just completed a double-blind placebo controlled study investigating the effect of this same once a day dose for 4 weeks in patients with hypertension. To date the dietary nitrate has been well-tolerated with no adverse effects reported. The results of this study will determine whether the effects of dietary nitrate on blood pressure can be sustained for longer periods of time (Clinicaltrials.gov identifier: NCT01405898).

Since, we published our initial observations with dietary nitrate there have been a number of studies that have confirmed the blood pressure lowering effects of an acute dietary nitrate load in various cohorts ranging from elite athletes to patients with peripheral vascular disease[38,39]. In addition, a particularly intriguing series of findings demonstrating that inorganic nitrate might also improve mitochondrial oxygen utilisation[38,40] raise the prospect that in addition to the potential beneficial

effects for hypertensives, individuals with conditions such as heart failure may benefit from improved dietary nitrate intake. Such observations provide encouraging support for the thesis that dietary nitrate expresses a range of attributes that need to be considered when assessing the risk:benefit ratio of dietary ingestion.

In sum, the demonstration that dietary nitrate expresses a range of activities that result in positive outcomes upon the cardiovascular system support the need for a reappraisal of risk:benefit ratio in determining acceptable levels of dietary intake.

Reference List

1. Appel LJ, Moore TJ, Obarzanek E, Vollmer WM, Svetkey LP, Sacks FM, Bray GA, Vogt TM, Cutler JA, Windhauser MM, Lin PH, Karanja N. A clinical trial of the effects of dietary patterns on blood pressure. DASH Collaborative Research Group. *N Engl J Med*. 1997;336:1117-1124.

2. Joshipura KJ, Ascherio A, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, Hennekens CH, Spiegelman D, Willett WC. Fruit and vegetable intake in relation to risk of ischemic stroke. *JAMA*. 1999;282:1233-1239.

3. Joshipura KJ, Hu FB, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, Colditz G, Ascherio A, Rosner B, Spiegelman D, Willett WC. The effect of fruit and vegetable intake on risk for coronary heart disease. *Ann Intern Med*. 2001;134:1106-1114.

4. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med*. 1993;329:2002-2012.

5. Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: analysis of worldwide data. *Lancet*. 2005;365:217-223.

6. Lewington S, Clarke R, Qizilbash N, Peto R, Collins RCPS. Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. *The Lancet*. 2002;360:1903-1913.

7. Chobanian AV. The Hypertension Paradox-- More Uncontrolled Disease despite Improved Therapy. *New England Journal of Medicine*. 2009;361:878-887.

8. Appel LJ, Brands MW, Daniels SR, Karanja N, Elmer PJ, Sacks FM. Dietary approaches to prevent and treat hypertension: a scientific statement from the American Heart Association. *Hypertension*. 2006;47:296-308.

9. Sacks FM, Rosner B, Kass EH. Blood pressure in vegetarians. *Am J Epidemiol*. 1974;100:390-398.

10. Rouse IL, Beilin LJ, Armstrong BK, Vandongen R. Blood-pressure-lowering effect of a vegetarian diet: controlled trial in normotensive subjects. *Lancet*. 1983;1:5-10.

11. Egan BM, Zhao Y, Axon RN. US Trends in Prevalence, Awareness, Treatment, and Control of Hypertension, 1988-2008. *JAMA: The Journal of the American Medical Association*. 2010;303:2043-2050.

12. Persell SD. Prevalence of Resistant Hypertension in the United States, 2003–2008. *Hypertension*. 2011;57:1076-1080.

13. Ahmed MI, Calhoun DA. Resistant Hypertension. *Hypertension*. 2011;57:1045-1046.

14. Vivekananthan DP, Penn MS, Sapp SK, Hsu A, Topol EJ. Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials. *Lancet*. 2003;361:2017-2023.

15. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. *JAMA*. 2007;297:842-857.

16. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. *Cochrane Database Syst Rev*. 2012;3:CD007176.

17. Lundberg JO, Feelisch M, Bjorne H, Jansson EA, Weitzberg E. Cardioprotective effects of vegetables: is nitrate the answer? *Nitric Oxide*. 2006;15:359-362.

18. Sobko T, Marcus C, Govoni M, Kamiya S. Dietary nitrate in Japanese traditional foods lowers diastolic blood pressure in healthy volunteers. *Nitric Oxide*. 2010;22:136-140.

19. Stieglitz EJ. Bismuth subnitrate in the therapy of hypertension. *J Pharmacol Exp Ther*. 1927;32:23-35.

20. Stieglitz EJ. BIsmuth subnitrate in the treatment of arterial hypertension. *Journal of the American Medical Association*. 1930;95:842-846.

21. Ayman D. BIsmuth subnitrate in the treatment of arteriolar (essential) hypertension. *Journal of the American Medical Association*. 1932;98:545-548.

22. Larsen FJ, Ekblom B, Sahlin K, Lundberg JO, Weitzberg E. Effects of dietary nitrate on blood pressure in healthy volunteers. *N Engl J Med*. 2006;355:2792-2793.

23. Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, Rashid R, Miall P, Deanfield J, Benjamin N, MacAllister R, Hobbs AJ, Ahluwalia A. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension*. 2008;51:784-790.

24. Kapil V, Milsom AB, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, Arghandawi S, Pearl V, Benjamin N, Loukogeorgakis S, MacAllister R, Hobbs AJ, Webb AJ, Ahluwalia A. Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. *Hypertension*. 2010;56:274-281.

25. FAO/WHO. Nitrate (and potential endogenous formation of N-nitroso compounds). In: WHO Food Additive series 50. 2003. World Health Organisation, Geneva.

26. Bahra M, Kapil V, Pearl V, Ghosh S, Ahluwalia A. Inorganic nitrate ingestion improves vascular compliance but does not alter flow-mediated dilatation in healthy volunteers. *Nitric Oxide*. 2012;26:197-202.

27. Spiegelhalder B, Eisenbrand G, Preussmann R. Influence of dietary nitrate on nitrite content of human saliva: possible relevance to in vivo formation of N-nitroso compounds. *Food Cosmet Toxicol*. 1976;14:545-548.

28. Tannenbaum SR, Weisman M, Fett D. The effect of nitrate intake on nitrite formation in human saliva. *Food Cosmet Toxicol*. 1976;14:549-552.

29. Tannenbaum SR, Correa P. Nitrate and gastric cancer risks. *Nature*. 1985;317:675-676.

30. Modin A, Bjorne H, Herulf M, Alving K, Weitzberg E, Lundberg JO. Nitrite-derived nitric oxide: a possible mediator of 'acidic-metabolic' vasodilation. *Acta Physiol Scand*. 2001;171:9-16.

31. Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon RO, III, Gladwin MT. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med*. 2003;9:1498-1505.

32. Dejam A, Hunter CJ, Tremonti C, Pluta RM, Hon YY, Grimes G, Partovi K, Pelletier MM, Oldfield EH, Cannon RO, III, Schechter AN, Gladwin MT. Nitrite Infusion in Humans and Nonhuman Primates. Endocrine Effects, Pharmacokinetics, and Tolerance Formation. *Circulation*. 2007;116:1821-1831.

33. Govoni M, Jansson EA, Weitzberg E, Lundberg JO. The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide*. 2008;19:333-337.

34. Kapil V, Haydar SM, Pearl V, Lundberg JO, Weitzberg E, Ahluwalia A. Physiological role for nitrate-reducing oral bacteria in blood pressure control. *Free Radic Biol Med*. 2013;55:93-100.

35. Kapil V, Webb AJ, Ahluwalia A. Inorganic nitrate and the cardiovascular system. *Heart*. 2010;96:1703-1709.

36. Pluta RM, Oldfield EH, Bakhtian KD, Fathi AR, Smith RK, DeVroom HL, Nahavandi M, Woo S, Figg WD, Lonser RR. Safety and Feasibility of Long-term Intravenous Sodium Nitrite Infusion in Healthy Volunteers. *PLoS ONE*. 2011;6:e14504.

37. Ghosh SM, Kapil V, Fuentes-Calvo I, Bubb KJ, Pearl V, Milsom AB, Khambata RS, Maleki-Toyserkani S, Yousuf M, Benjamin N, Webb AJ, Caulfield MJ, Hobbs AJ, Ahluwalia A. Enhanced vasodilator activity of nitrite in hypertension: critical role for erythrocytic xanthine oxidoreductase and translational potential. *Hypertension*. 2013;In press.

38. Bailey SJ, Winyard P, Vanhatalo A, Blackwell JR, DiMenna FJ, Wilkerson DP, Tarr J, Benjamin N, Jones AM. Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *Journal of Applied Physiology*. 2009;107:1144-1155.

39. Lansley KE, Winyard PG, Fulford J, Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Gilchrist M, Benjamin N, Jones AM. Dietary nitrate supplementation reduces the O2 cost of walking and running: a placebo-controlled study. *Journal of Applied Physiology*. 2011;110:591-600.

40. Larsen FJ, Schiffer TA, Borniquel S, Sahlin K, Ekblom B, Lundberg JO, Weitzberg E. Dietary Inorganic Nitrate Improves Mitochondrial Efficiency in Humans. *Cell Metabolism*. 2011;13:149-159.