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Acute Blood Pressure Lowering, Vasoprotective, and Antiplatelet Properties of Dietary Nitrate via Bioconversion to Nitrite

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Abstract—Diets rich in fruits and vegetables reduce blood pressure (BP) and the risk of adverse cardiovascular events. However, the mechanisms of this effect have not been elucidated. Certain vegetables possess a high nitrate content, and we hypothesized that this might represent a source of vasoprotective nitric oxide via bioactivation. In healthy volunteers, approximately 3 hours after ingestion of a dietary nitrate load (beetroot juice 500 mL), BP was substantially reduced ($\Delta_{\max} -10.4/8$ mm Hg); an effect that correlated with peak increases in plasma nitrite concentration. The dietary nitrate load also prevented endothelial dysfunction induced by an acute ischemic insult in the human forearm and significantly attenuated ex vivo platelet aggregation in response to collagen and ADP. Interruption of the enterosalivary conversion of nitrate to nitrite (facilitated by bacterial anaerobes situated on the surface of the tongue) prevented the rise in plasma nitrite, blocked the decrease in BP, and abolished the inhibitory effects on platelet aggregation, confirming that these vasoprotective effects were attributable to the activity of nitrite converted from the ingested nitrate. These findings suggest that dietary nitrate underlies the beneficial effects of a vegetable-rich diet and highlights the potential of a “natural” low cost approach for the treatment of cardiovascular disease. (*Hypertension*. 2008;51:784-790.)

Key Words: diet ■ nitric oxide ■ blood pressure ■ hypertension ■ ischemia/reperfusion
■ platelets ■ endothelium

Perhaps the largest public health initiative in the Western world has focused on improvement of diet, particularly in those with a high risk of cardiovascular disease. Trials have shown that diets rich in fruits and vegetables reduce blood pressure (BP; Dietary Approaches to Stop Hypertension; DASH, Vegetarian Diet and BP)^{1,2} and adverse cardiovascular events.³⁻⁷ These protective effects have previously been attributed to the high antioxidant vitamin content, yet large clinical trials have failed to provide evidence in support of this thesis.^{8,9} The greatest protection against coronary heart disease afforded by a change in diet is that associated with the consumption of green leafy vegetables (eg, spinach, lettuce).⁶ Such vegetables, also including beetroot, commonly have a high inorganic nitrate (NO_3^-) content.^{10,11} In humans, after absorption through the stomach wall, $\approx 25\%$ of consumed nitrate enters the enterosalivary circulation where it is reduced to nitrite (NO_2^-) by bacterial nitrate reductases from facultative anaerobes on the dorsal surface of the tongue.¹²⁻¹⁴

This nitrite is swallowed and in the acidic environment of the stomach is reduced to nitric oxide (NO) or re-enters the circulation as nitrite. Indeed, it has been hypothesized that dietary nitrate represents an intravascular source of the pleiotropic, vasoprotective molecule NO, which supplements conventional NO generation by NO synthases (NOS).¹⁵

Endothelium-derived NO is a potent dilator, governs systemic BP, and retards atherogenesis (NO inhibits inflammatory cell recruitment and platelet aggregation).¹⁶ Consequently, numerous cardiovascular pathologies (including prehypertension,¹⁷ hypertension,¹⁸ atherosclerosis,¹⁹ and stroke²⁰) are associated with endothelial dysfunction and diminished NO bioactivity. Recently, studies have demonstrated that nitrite confers marked protection against ischemia/reperfusion (I/R) injury in the myocardial, hepatic, renal, pulmonary, and cerebral vasculature.^{21,22} This cytoprotective effect has been attributed to reduction of nitrite to NO during ischemia or hypoxemia (conditions that inactivate endothelial NOS, the

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enzyme responsible for endothelial NO synthesis), facilitated by xanthine oxidoreductase (XOR),²³ deoxyhaemoglobin,²¹ deoxymyoglobin,²⁴ or via chemical acidification. Thus, in an environment where conventional NO synthesis is impaired, nitrite provides an alternative source of (vaso)protective NO. Furthermore, it has been proposed that nitrite plays an important physiological role. Indeed, nitrite causes dose-dependent vasodilatation in the brachial artery of normal volunteers, indicating that it may have an important role in maintaining normal cardiovascular homeostasis in addition to its cytoprotective role.²⁵

Studies in humans demonstrate a progressive rise over time in plasma nitrate²⁶ and nitrite¹⁴ concentrations after oral administration of sodium or potassium nitrate. We investigated the possibility that a similar increase in these anions can be achieved by consuming dietary nitrate through the consumption of beetroot juice and that this will acutely lower arterial BP, supplement endothelial function (measured by flow-mediated dilatation, FMD) during ischemia, and inhibit platelet aggregation as a result of bioconversion to NO.

Methods

Volunteers

The studies were granted full ethics approval by the Local Research Ethics Committee, and all subjects gave informed consent (see the online data supplement for inclusion criteria at <http://hyper.ahajournals.org>). The study was separated into 3 phases with distinct recruitment for each phase.

BP Study

An open-label crossover design was used with 14 healthy subjects randomized to drink 500 mL of either beetroot juice (Planet Organic) or water within 30 minutes. BP was measured every 15 minute for 1 hour pre- and 3 hours postbeetroot juice ingestion, then hourly to 6 hours, with a final reading at 24 hours. BPs were taken according to a standard protocol (see the data supplement), using an automated BP measuring machine (Omron 705CP) with the subject seated; 3 BP measurements were taken at each time point, and the mean of the 2nd and 3rd reading was used. Blood samples (5 mL each) were collected into citrate tubes for plasma nitrate and nitrite measurement at baseline and every 30 minutes for 2 hours, then hourly up to 6 hours, with a further measurement at 24 hours. Blood samples were centrifuged immediately at 2200g for 10 minutes at 4°C. The plasma was collected and stored at -80°C until measurement of nitrite and nitrate concentration. The second part of the study was conducted after a minimum of 7 days.

Interruption of Enterosalivary Circulation Study

The effects of spitting out all saliva during, and for 3 hours after, beetroot juice ingestion (500 mL) on BP and simultaneous changes in plasma nitrate and nitrite concentration were investigated in a further crossover study in 6 healthy volunteers, with normal swallowing of saliva as control. In this study blood was collected in lithium heparin tubes, for measurement of plasma potassium. For assessment of effects on platelet aggregation, blood was collected at baseline and at 2.5 hours into 3.8% trisodium citrate (9:1 v/v), pH 7.4, using a 19-gauge butterfly needle. Platelet-rich-plasma (PRP) was prepared and used for assessment of aggregation in response to collagen or adenosine di-phosphate (ADP) using an adaptation of the Born method²⁷ (data supplement).

FMD Study

In the second phase of this study endothelial function was assessed in 10 healthy subjects by measuring brachial artery diameter in the nondominant arm in response to the endothelium-dependent reactive

hyperaemia response before and after an ischemic insult as previously described^{28,29} (data supplement). In this open-label crossover study, healthy subjects were randomized to 500 mL of beetroot juice 2 hours before the I/R sequence or no treatment and returned for the second arm of the study after a minimum of 7 days.

Chemiluminescence

Samples were analyzed for nitrite and nitrate using chemiluminescence as described previously³⁰ (data supplement).

Data and Statistical Analysis

We analyzed data using the Graph Pad Prism Software. All data are expressed as mean ± SEM unless otherwise stated. Data were compared by repeated-measures ANOVA with Dunnett's post test for comparison with baseline and Bonferroni post test for comparison with the control group. In all cases, $P < 0.05$ was considered statistically significant.

Results

There were no significant differences in the general characteristics of the individuals recruited for the separate phases of the study (Table S1). Beetroot juice was generally well tolerated by the subjects. Beeturia (red urine) and red stools were common expected effects. The mean concentration of nitrate in the beetroot juice was 45.0 ± 2.6 mmol/L (2.79 g/L BP study) and 34.0 ± 0.1 mmol/L (2.11 g/L, spitting study), whereas nitrite was below the limits of detection (< 50 nmol/L).

A Dietary Nitrate Load Raises Circulating Nitrite and Nitrate Levels

No changes in plasma nitrate or nitrite concentration were found after ingestion of water. In contrast, after consumption of juice, there was a rapid rise (≈ 16 -fold) in nitrate concentration appearing after the first 30 minutes, peaking at 1.5 hours and remaining at this level up to 6 hours after ingestion ($P < 0.001$ compared to control). Nitrate levels showed a trend to remain elevated at 24 hours after beetroot juice compared to water ($P = 0.05$). Plasma nitrite also increased significantly (2-fold) after beetroot juice ingestion, an effect that reached a peak at 3 hours and remaining at this raised level up until 5 hours after juice ingestion. Levels had returned to near baseline by 24 hours (Figure 1). Plasma K^+ concentration increased rapidly after beetroot juice ingestion peaking by 1 hour but had returned to baseline levels by 3 hours (Figure S1).

Dietary Nitrate Lowers BP

There were no differences in BP between the 2 groups during the hour before ingestion of beetroot juice or water. However, BP began to decrease from 1 hour after ingestion of juice compared to the water control (Figure 2). The peak difference in systolic BP was achieved at 2.5 hours after ingestion with a drop of 10.4 ± 3.0 mm Hg ($P < 0.01$), whereas the peak differences in diastolic BP and MAP were seen at 3 hours after ingestion, with changes of 8.1 ± 2.1 mm Hg and 8.0 ± 2.1 mm Hg, respectively (both $P < 0.01$, Figure 2). At 24 hours, systolic BP was 4.4 mm Hg lower with beetroot juice than water, although not statistically significantly different ($P = 0.058$). However, systolic BP was significantly reduced by ≈ 6 mm Hg at 24 hours after beetroot juice ingestion compared to -1 hour (106.2 ± 2.8 and 112.4 ± 3.4 mm Hg, respectively, $P < 0.01$; Figure 2). There were no differences in diastolic BP at 24 hours. The mean heart rate was not

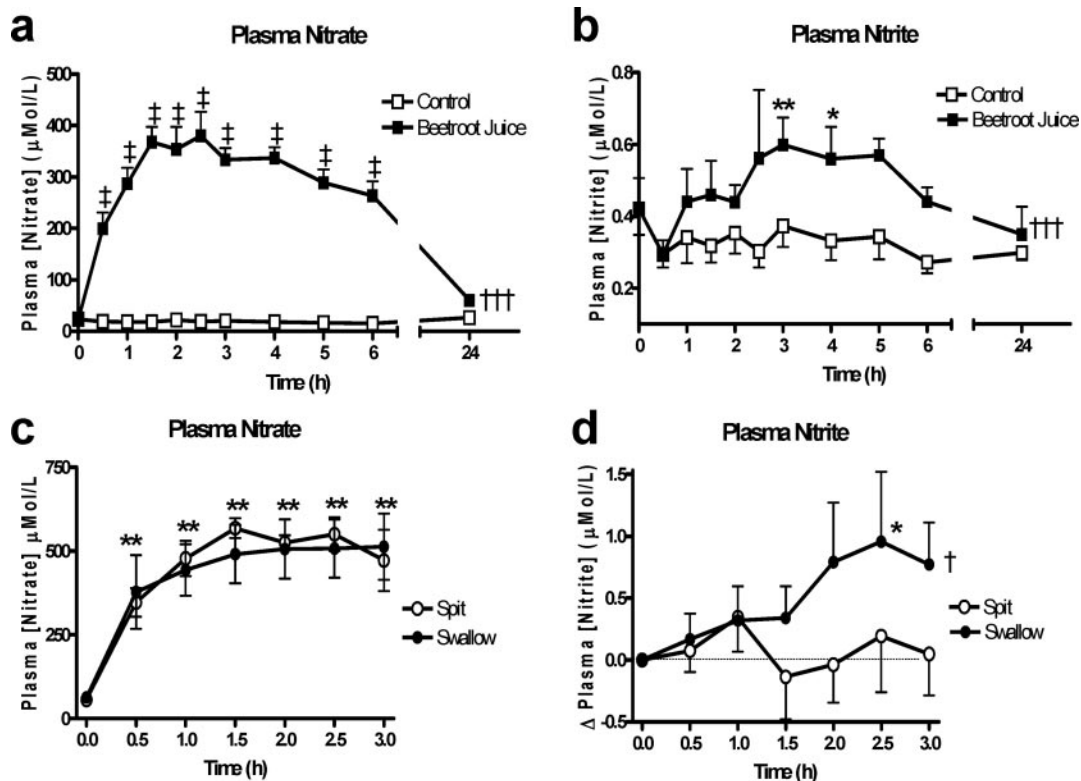


Figure 1. The effect of beetroot juice on the plasma concentrations of (a) nitrate and (b) nitrite and the effects of spitting vs swallowing of saliva on plasma concentrations of (c) nitrate and (d) nitrite. Data expressed as mean±SEM. Significance shown as: ANOVA of curve of †††*P*<0.001 beetroot juice vs control, or †*P*<0.05 spitting vs swallowing followed by ‡*P*<0.001 Bonferroni post test of beetroot juice vs control, **P*<0.05, ***P*<0.01 Dunnett's post test compared to baseline.

significantly altered over the 1- to 6-hour period after beetroot juice or water ingestion (70.2 ± 0.3 and 69.0 ± 0.5 bpm, respectively; Figure 2). The changes in BP were related to the plasma nitrite concentration as demonstrated by a

significant inverse correlation between the change in plasma nitrite concentration and the change in systolic BP from baseline (Pearson $r = -0.26$, $P = 0.008$; Figure 2). However, there was no significant correlation between change in

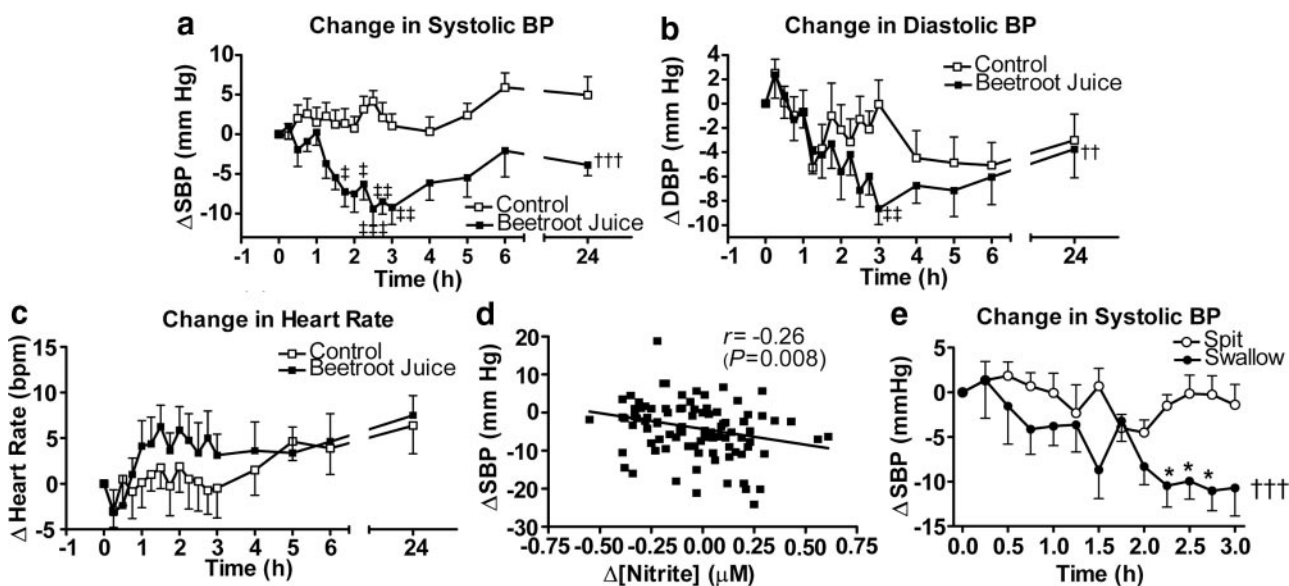


Figure 2. The effect of beetroot juice on the change from baseline in (a) systolic BP, (b) diastolic BP, (c) heart rate, (d) correlation of change in BP with change in plasma nitrite concentration, and (e) effect of spitting vs swallowing of saliva on changes in systolic BP after beetroot juice. Data expressed as mean±SEM. Significance shown as: †††*P*<0.001 ANOVA of curve of beetroot juice vs control, or spitting vs swallowing followed by ‡*P*<0.05, †††*P*<0.001 Bonferroni post test of beetroot juice vs control; **P*<0.05 Dunnett's post-test compared to baseline.

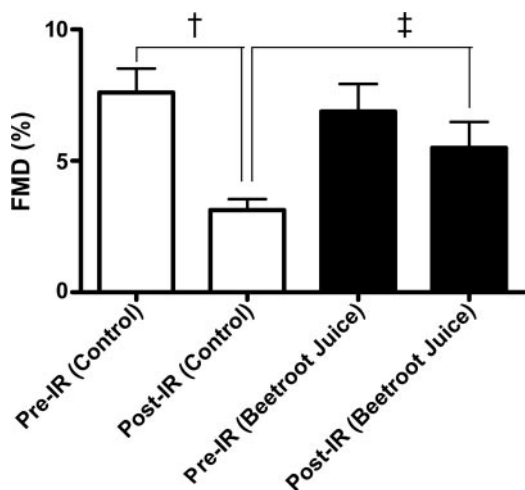


Figure 3. Flow mediated dilatation (FMD) in ischemia-reperfusion (I/R) in control and the effect of beetroot juice. Data expressed as mean \pm SEM. Significance shown as: † $P < 0.001$ compared to control pre-I/R, ‡ $P < 0.05$ compared to post-I/R with beetroot juice.

plasma nitrate concentration and change in systolic BP (Pearson $r = -0.17$, $P > 0.05$).

The Functional Effects of an Acute Dietary Nitrate Load Are Attributable to Nitrite

Interrupting the enterosalivary circulation by spitting out all saliva blocked the rise in plasma nitrite concentration at 2.5 hours (Figure 1; $P < 0.05$) but had no significant effect on plasma nitrate or plasma K^+ concentration (Figure S1). Spitting also blocked the reduction in SBP ($P < 0.05$ compared to swallowing; Figure 2). Platelet aggregation to ADP and collagen were inhibited 2.5 hours after beetroot ingestion (both $P < 0.001$), however no inhibition of platelet aggregation was seen with spitting (Figure S2).

Dietary Nitrate Protects Against Endothelial Dysfunction

I/R of the forearm significantly reduced the FMD response in control subjects resulting in an approximate $\approx 60\%$ suppression of the FMD response ($P < 0.001$; Figure 3). In contrast, although beetroot juice did not alter preischemic brachial artery dilatation, it protected against I/R-induced depression of this response as evidenced by complete prevention of ischemia-induced endothelial dysfunction ($P < 0.05$; Figure 3).

Discussion

Determining how vegetables confer protection against cardiovascular disease, and exploiting these to therapeutic advantage, is likely to have considerable health and economic implications. Although the protective effects of vegetable-rich diets have previously been attributed to the antioxidant vitamin content, recent large scale clinical trials have failed to provide evidence in support of this hypothesis.^{8,9} In this study we have shown that ingestion of dietary nitrate (beetroot juice) results in increased plasma nitrite concentration via bioconversion in vivo. This bioactive nitrite substantially decreases BP, inhibits platelet aggregation, and prevents endothelial dysfunction in healthy volunteers. These findings

suggest that dietary nitrate likely plays a major role in mediating the beneficial effects of a vegetable-rich diet.

After ingestion of beetroot juice plasma nitrate concentration increased rapidly (within 30 minutes), peaking at 1.5 hours. In contrast, the appearance of nitrite in the circulation was considerably slower, peaking at 2.5 to 3 hours. The vast majority of absorbed inorganic nitrate is ultimately excreted in the urine, but up to 25% of plasma nitrate is also excreted in the saliva.^{31,32} The exact mechanism for this concentrating effect is unknown, but the consequence is the provision of substrate for the nitrate reductases expressed by bacteria that colonize the dorsal surface of the tongue, resulting in the reduction of nitrate to nitrite. This nitrite is swallowed and in the acidic environment of the stomach is then reduced to NO or reenters the circulation as nitrite (Figure 4).³³ In the present study the beetroot juice consumed contained substantial amounts of nitrate but undetectable quantities of nitrite, supporting the thesis that the delayed appearance of nitrite is likely due to in vivo processing and that this enterosalivary circuit likely underlies the time-lag in the appearance of nitrite in the plasma observed after ingestion of beetroot juice. That this is the pathway used to elevate circulating nitrite concentration after a nitrate load is supported by the finding in the 2nd volunteer study where interruption of this circuit, by avoidance of swallowing of saliva¹⁴ for 3 hours subsequent to beetroot ingestion, blocked the rise in plasma nitrite but not nitrate concentration.

Beetroot juice ingestion lowered BP in healthy volunteers. There was a lag period of approximately 1 to 2 hours, after ingestion, with a peak drop in BP occurring after 3 to 4 hours. This time course of reduction in BP correlated with the appearance and peak levels of nitrite in the circulation; an effect that was absent in individuals within whom the enterosalivary circuit was disrupted by avoidance of swallowing. These observations, together with the fact that plasma nitrite, and not nitrate, concentration correlated with the decreases in BP implicates nitrite as the likely functional mediator of the beetroot juice-induced effects on BP.

Exactly how nitrite mediates this decrease in BP is uncertain, however recent evidence demonstrates that nitrite is a potent vasodilator in the human forearm,²⁵ and it is likely that such vasodilator activity underlies the BP effects evidenced here. This activity of nitrite has been attributed to its chemical reduction to the potent vasodilator NO. Until recently, it was assumed that the only route for NO synthesis in vivo was via NOS activity and that nitrite and nitrate were inert biological end-products of NO metabolism. However, Benjamin et al, and Lundberg et al, both independently demonstrated, in 1994, that nitrite derived from dietary nitrate was a substrate for NOS-independent production of NO in the acidic conditions of the human stomach.^{12,13} That this might be a mechanism that operates in the cardiovascular system has attracted considerable attention over the past 5 yr. In 1995 Zweier and coworkers demonstrated that during ischemic conditions in the heart sufficient acidosis develops permitting NO generation from endogenously stored nitrite.³⁴ More recently this potential for nitrite-derived NO production has been clearly demonstrated in both in vitro and in vivo animal models of I/R injury in various organs.^{23,35} While this

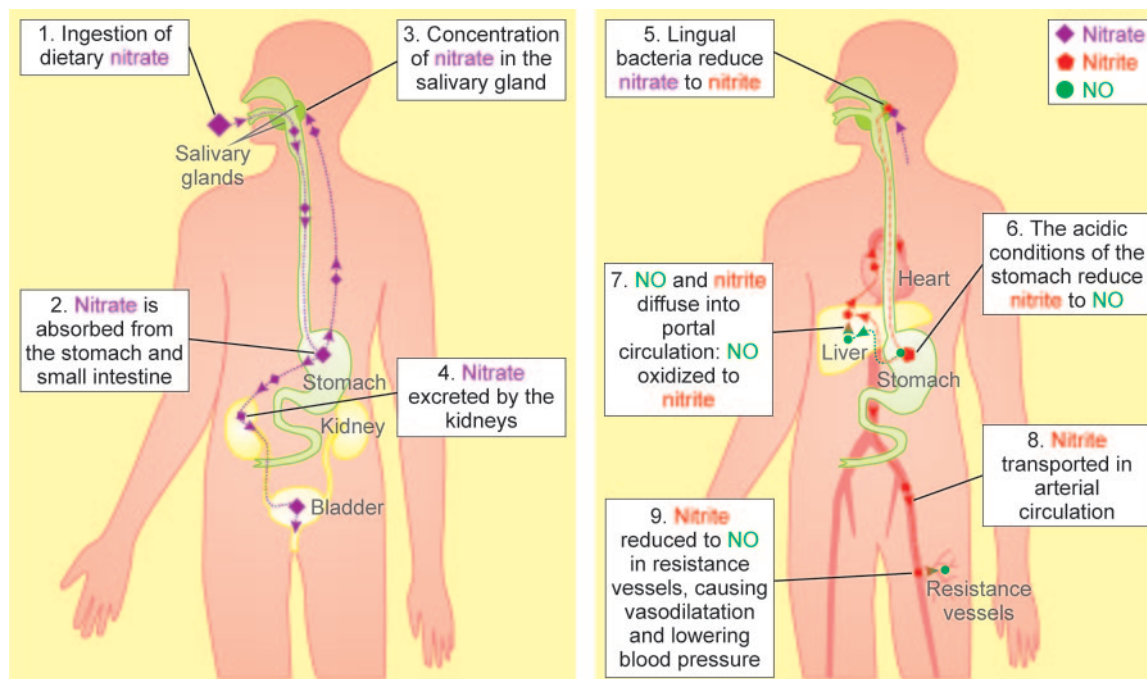


Figure 4. The fate of dietary nitrate, derived from consuming beetroot juice. Systemically absorbed nitrate is concentrated 10-fold in the salivary glands (left panel) and undergoes an enterosalivary circulation where it is reduced to nitrite by bacterial nitrate reductases on the dorsal surface of the tongue, and swallowed into the stomach providing a source of systemically available nitrite/NO. Right panel, Nitrite is transported in the arterial circulation to resistance vessels, where lower O_2 tension favors the reduction of nitrite to NO, causing vasodilatation, with consequent lowering of BP.

conversion is, in part, brought about by chemical acidification as in the stomach, there is an additional component that has been attributed to the reductant activity of at least, but principally, 2 distinct proteins; XOR and deoxyhemoglobin.²¹ In addition to occurring under ischemic conditions there is mounting evidence to support the thesis that nitrite reduction also occurs in physiological conditions within the blood vessel, resulting in alterations in vasoactivity and BP in both animal models and normal volunteers.^{25,36} It is likely that nitrate-derived nitrite generation, in the present study, provides an intravascular store of NO that results in arterial dilatation within the microcirculation to produce a decrease in peripheral resistance and hence a reduction in BP (Figure 4).

Support for the thesis, that nitrate was the component of the beetroot juice responsible for the effects seen, comes from a recent study where supplementation of dietary nitrate by administration of sodium nitrate (0.1 mmol/kg/d) to healthy volunteers over 3 days reduced diastolic (but not systolic) BP by 3.7 mm Hg compared to sodium chloride.³⁷ In addition, the loss of functionality of the beetroot juice in terms of BP by spitting of saliva further implicates dietary nitrate. It has, however, been proposed that the high K^+ content of fruit and vegetables could account for the BP lowering effects of such a diet.³⁸ In the present study K^+ concentration was measured in 6 different samples of beetroot juice and found to be 92.88 ± 0.68 mmol/L. Ingestion of the juice resulted in a rapid rise in plasma K^+ concentration, however levels had returned to baseline by 2.5 hours and this rise was not significantly altered by avoidance of swallowing suggesting that the effects of beetroot juice on BP were independent of K^+ levels.

The in vivo half life of nitrite (≈ 1.5 hour) in the present studies is much longer than the ex vivo half life of <2 minutes,³⁹ suggesting that nitrite is continuously produced from nitrate (which has a long half life of ≈ 8 hours) via the enterosalivary circulation. Nitrite is readily distributed throughout the body.^{40,41} More recently it has been demonstrated in rats that the absorption of nitrite across the abdominal cavity is rapid, and that it is then widely and rapidly distributed, reaching near steady-state concentrations in all tissues assessed within about 5 min.⁴² It is possible that this uptake of nitrite results in the provision of stores of nitrite that are then slowly released back into the circulation over time, and such a mechanism may underlie the sustained effects of nitrite on BP reduction in the present study.

Nitrite also confers marked protection against I/R and hypoxic injury, an effect that has been demonstrated in the myocardial, hepatic, renal, pulmonary, and cerebral vasculature.^{21,22} This activity of nitrite has been attributed to its reduction to NO during ischemia facilitated predominantly by XOR or deoxyhaemoglobin.²¹ In keeping with NO bioactivity, we also show that the beneficial effects of a dietary nitrate load are not limited to BP, but also include a reversal of the endothelial dysfunction associated with I/R injury in the brachial artery and inhibition of ex vivo platelet aggregation responses to the aggregating stimuli ADP and collagen. These cytoprotective effects of beetroot juice consumption likely relate to the elevation of systemic nitrite (which is converted to protective NO particularly during ischemia), because the responses were measured at a time point associated with the peak in plasma nitrite concentration and interruption of the enterosalivary circuit for nitrate reduction abolished these

effects. In addition, that dietary nitrate is protective against I/R injury is supported by the very recent findings of Bryan and colleagues⁴³ who elegantly demonstrated reduction of myocardial infarct damage after increases in dietary nitrite and nitrate intake in mice. Many preclinical studies support the concept that NO is protective in I/R⁴⁴ and NO is well known to inhibit platelet aggregation^{45,46}; however, there has been limited translation of protective effects of NO in human studies (eg, the ISIS-4 trial using the organic nitrate, isosorbide mononitrate⁴⁷). This may be because of timing of treatment or the difference between types of NO donor; specifically it is important to note that significant free radical production is associated with organic nitrate activity, and it is possible that this may be an important mechanism underlying the failure to demonstrate protection.

In 2002 the WHO reported that $\approx 11\%$ of all disease burden was a direct consequence of the deleterious effects of chronic hypertension. The economic burden is therefore immense, and cost-effective interventions would be of considerable value. Studies in patients and normal volunteers show that hypertension is associated with impaired endogenous NO synthesis, and we propose that provision of an alternative NOS-independent source of NO in the form of dietary nitrate, such as beetroot juice, would restore NO levels and improve hypertension. Present treatment guidelines target individuals with established hypertension and vascular disease, pathologies of considerable mechanistic complexity that are suitably matched with an ever increasingly complex treatment regime. However, recent calls suggest that treatment should occur before disease has evolved in individuals with “prehypertension”⁴⁸ or even in normotensives. Such a preventative strategy in relatively “well” individuals would require a minimally interventional approach, based on and optimized through an understanding of physiological processes. Our data support the thesis that dietary nitrate is likely to have been a major contributor to the BP lowering effects of the fruit- and vegetable-rich diets in previous studies,⁴⁹ and demonstrate that nitrate is likely to underlie the cardioprotective effect of vegetables. In addition, it is also of interest to consider that the BP response to a high fruit and vegetable diet was considerably greater in hypertensives compared to normotensives in the DASH study,¹ and therefore it is possible that the BP effect of dietary nitrate, evidenced in our study of normotensives, will be heightened in hypertensives. Therefore, we advocate consumption of a diet high in nitrate (ie, a “natural” strategy) to treat (pre-) hypertension and to protect individuals at risk of adverse vascular events.

Perspectives

In summary, an acute dietary nitrate load causes a marked reduction in BP in normotensives, reduces platelet activation, and protects against experimentally-induced endothelial I/R injury; effects that correlate with a rise in circulating levels of nitrite derived from dietary nitrate.

We hypothesize that this mechanism likely accounts for much of the cardioprotective effects of vegetables and suggests an important role of high dietary nitrate in delaying the development and treatment of prehypertension and hypertension.

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Disclosures

None.

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Online Supplement

Acute blood pressure lowering, vasoprotective and anti-platelet properties of dietary nitrate via bioconversion to nitrite

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Short title: Dietary nitrate and vasoprotection

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Volunteers

The inclusion criteria were healthy volunteers aged 18-45, screened by taking a medical history. The exclusion criteria were a history of any serious illnesses, including infectious diseases or systemic medication (other than the oral contraceptive pill) and smoking.

Volunteers were asked to refrain from caffeine-containing drinks or foods with a high nitrate content (green leafy vegetables, beetroot) for 12h prior to the study and were fasting on the morning of the study.

BP Measurement

BPs were taken according to a standard protocol using an automated BP measuring machine (Omron 705CP (Japan) with the subject seated; three BP measurements were taken at each time point and the mean of the 2nd and 3rd reading was used.

FMD study

In this study endothelial function was assessed in 10 healthy subjects by measuring brachial artery diameter in the *non-dominant* arm in response to reactive hyperaemia. This induces an endothelium-dependent increase in brachial artery diameter, as previously described¹. To determine the effect of I/R on endothelial function, FMD was assessed before ischemia, induced by inflating a BP cuff placed around the upper part of the arm to a pressure of 200 mmHg for 20 min, and following 20 min reperfusion as described previously². We have previously demonstrated that this protocol results in brachial artery endothelial dysfunction but does not have an effect on vascular smooth muscle function². Brachial artery diameter was measured in millimeters and dilation expressed as percentage increase from baseline diameter. The FMD flow stimulus during reactive hyperemia was expressed as the ratio of peak to baseline volume flow per minute.

A power calculation revealed that a sample size of 10 subjects would be needed to demonstrate that beetroot juice increases FMD following I/R injury by 30 %, based on a within subject standard deviation of 0.9, an α value of 0.05 and a β level of 0.8. The data was analyzed by an individual who was blinded to both the FMD sequence (pre- or post-I/R) and the intervention.

Chemiluminescence

Plasma samples were analysed for nitrite and nitrate using chemiluminescence as described previously³. Briefly, samples and standards containing nitrite and nitrate were first reduced to NO, which was then quantified using a NO analyser (NOA 280, Sievers). To determine total nitrite and nitrate concentrations, collectively termed 'NO_x', samples were added to 0.1 Mol/L vanadium (III) chloride in 1 M hydrochloric acid refluxing at 90°C under nitrogen. Nitrite concentrations were determined by addition of samples to 1.5 % potassium iodide in glacial acetic acid under nitrogen at room temperature. Concentrations of nitrate were calculated by subtraction of nitrite from NO_x values.

Platelet aggregation measurements

Platelet-rich-plasma (PRP) was obtained by centrifugation at 150 g for 15 min, 25°C (Beckman centrifuge). Platelet-Poor-Plasma (PPP) was prepared by centrifugation of PRP at 14,000 g for 2 min at room temperature. The effects on platelet aggregation were assessed using a 96-well plate adaptation of the Born method⁴. In brief, platelet aggregation of PRP was induced using ADP or collagen and absorbance measured at 595 nm every 15 s for 4 min at 37°C in a 96-well plate reader, with vigorous shaking for 10 s between readings. PRP alone was taken to be 0% light transmission (representing 0 % aggregation); PPP alone representing 100% light transmission (represents 100 % aggregation). The percentage change

in aggregation was calculated from the formula: $[(T_n - T_0)/(T_0 - \text{MeanPPP}) \times 100]^{x-1}$, where, T_n : Sample PRP value in each well at a particular time (n cycle); T_0 : Sample PRP value at time 0 represents 0 % aggregation; Mean PPP: Mean of PPP values represents 100 % aggregation.

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Table S1. Demographic data of volunteers.

Characteristics	BP Study	Spitting study	FMD Study
Subjects (<i>n</i>)	14	6	10
Male	9	5	4
Female	5	1	6
Age (years)	25.5 ± 4.5	31 ± 1.8	26.6 ± 7.4
Weight (kg)	64.7 ± 12.2	76.1 ± 4.2	62.2 ± 12.6
Height (cm)	169.4 ± 11.2	172.7 ± 5.3	170.9 ± 7.6
Baseline systolic BP (control or spitting limb) mmHg	108.8 ± 1.5	109.6 ± 4.0	ND
Baseline systolic BP (beetroot juice or swallowing limb)	108.0 ± 1.3	113.7 ± 4.7	ND
Baseline diastolic BP (control or spitting limb) mmHg	70.7 ± 1.1	70.2 +/- 2.8	ND
Baseline systolic BP (beetroot juice or swallowing limb) mmHg	70.3 ± 1.0	71.3 +/- 2.5	ND

Values quoted as mean ± SEM. Baseline BP readings are the means calculated for all measurements from time -1h to 0h prior to beetroot juice or water control ingestion.

Statistical analysis conducted using paired Students T-Test. ND=not determined.

Figure S1

The effect of beetroot juice on (a) sputum nitrate and nitrite concentration and (b) plasma potassium. Data expressed as mean±SEM (n=6). Significance shown as: ††† $P<0.001$, ANOVA of curve of nitrate vs. nitrite, * ** $P<0.01$ Dunnett's post test compared to baseline.

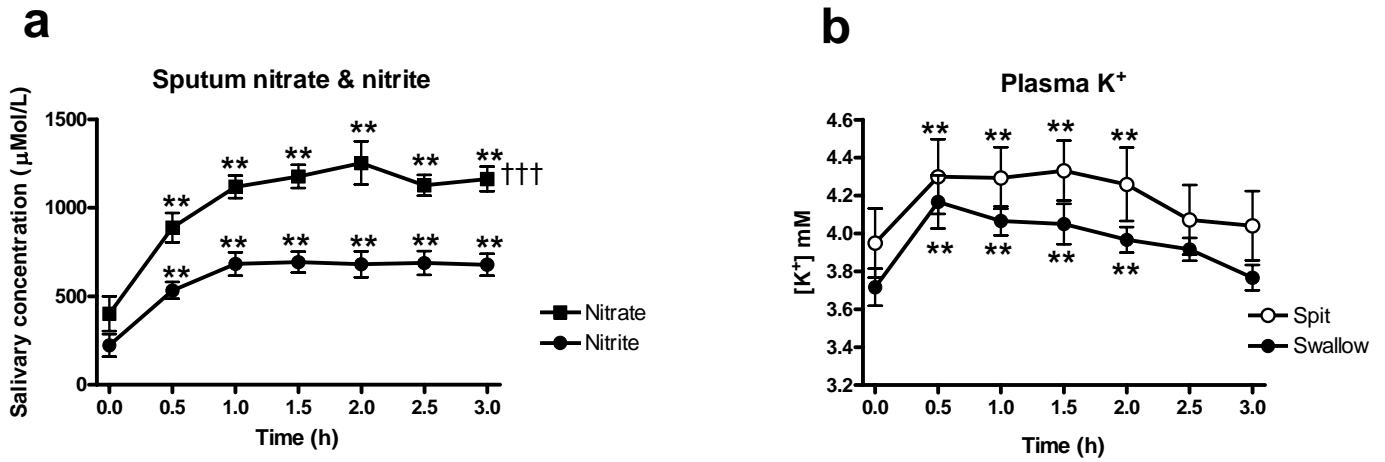


Figure S2

Effects on platelet aggregation of swallowing (\square) and spitting out (\blacksquare) saliva following beetroot juice ingestion, with (a, b) ADP (3-30 μM) and (c, d) collagen (3-30 $\mu\text{g/ml}$) as agonist. Significance shown as: $^{\dagger\dagger\dagger}P<0.001$, $^{\dagger\dagger\dagger\dagger}P<0.0001$ ANOVA of curve v control or pre-beetroot juice, $^{\ddagger\ddagger}P<0.01$ $^{\ddagger\ddagger\ddagger\ddagger}P<0.0001$ Bonferroni post test v control or pre-beetroot juice.

