Methods Supplement

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 described[1]. 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 previously[2]. We have previously demonstrated that this protocol results in brachial artery endothelial dysfunction but does not have an effect on vascular smooth muscle function[2]. 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 previously[3]. 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, 25ºC (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 method[4]. 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.

Reference List

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2. Loukogeorgakis SP, Panagiotidou AT, Broadhead MW, Donald A, Deanfield JE, MacAllister RJ. Remote ischemic preconditioning provides early and late protection against endothelial ischemia-reperfusion injury in humans: role of the autonomic nervous system. *J Am Coll Cardiol*. 2005;46:450-456.

3. Ignarro LJ, Fukuto JM, Griscavage JM, Rogers NE, Byrns RE. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. *Proc Natl Acad Sci U S A*. 1993;90:8103-8107.

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Table Supplement

Table S1. Demographic data of volunteers.

Characteristics	BP Study	Spitting study	FMD Study
Subjects (*n*)	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) mmHg	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 Supplement

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: $^{\dagger\dagger\dagger}P<0.001$, ANOVA of curve of nitrate vs. nitrite, * $^{**}P<0.01$ Dunnett's post test compared to baseline.

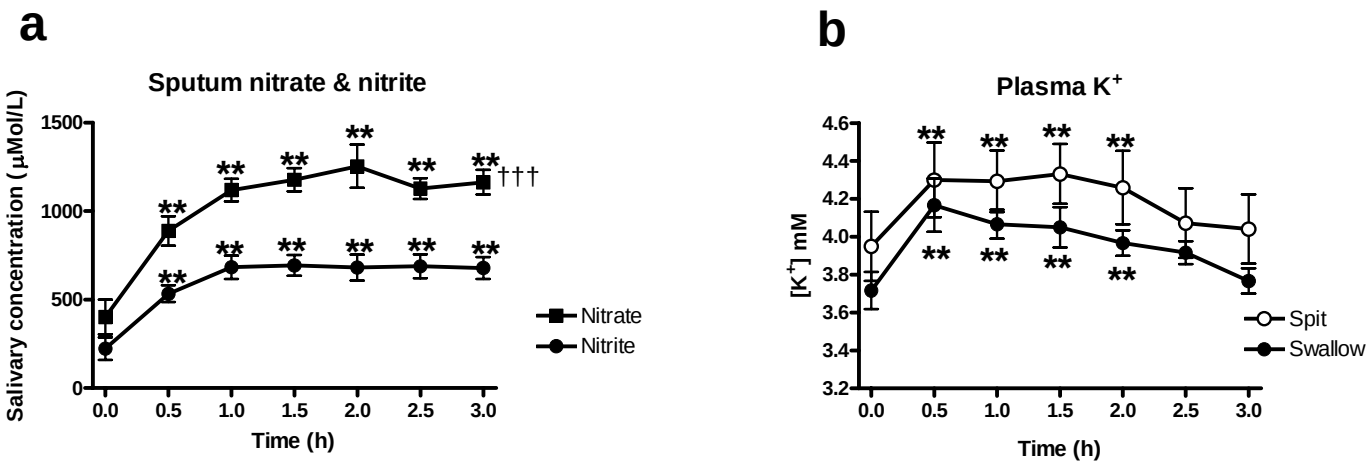

Figure S2

Effects on platelet aggregation of swallowing (□) and spitting out (■) saliva following beetroot juice ingestion, with (a, b) ADP (3-30 μM) and (c, d) collagen (3-30 μg/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.