Effect of dietary nitrate on blood pressure, endothelial function, and insulin sensitivity in type 2 diabetes

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ABSTRACT

Diets rich in green, leafy vegetables have been shown to lower BP and reduce the risk of cardiovascular disease. Green, leafy vegetables and beetroots are particularly rich in inorganic nitrate. Dietary nitrate supplementation, via sequential reduction to nitrite and NO, has previously been shown to lower BP and improve endothelial function in healthy humans.
We sought to determine if supplementing dietary nitrate with beetroot juice, a rich source of nitrate, will lower BP, improve endothelial function and insulin sensitivity in individuals with type 2 diabetes (T2DM). Twenty-seven patients, age 67.2 +/-4.9 years, (18 male) were recruited for a double blind, randomised, placebo-controlled crossover trial. Participants were randomised to begin in either order a 2 week period of supplementation with 250 ml beetroot juice daily (active) or 250 ml nitrate-depleted beetroot juice (placebo). At the conclusion of each intervention period 24 hour ambulatory blood pressure monitoring, tests of macro and microvascular endothelial function and a hyperinsulinaemic isoglycaemic clamp were performed. After two weeks administration of beetroot juice mean ambulatory systolic BP was unchanged: 134.6 ± 8.4 mmHg *versus* 135.1 ± 7.8 mmHg (mean ± SD) placebo *vs.* active - mean difference of -0.5 mmHg (placebo-active), p=0.737 (95% CI -3.9 to 2.8). There were no changes in macrovascular or microvascular endothelial function or insulin sensitivity. Supplementation of the diet with 7.5 mmoles of nitrate per day for 2 weeks caused an increase in plasma nitrite and nitrate concentration, but did not lower BP, improve endothelial function or insulin sensitivity in individuals with T2DM.

Introduction

Type 2 diabetes mellitus (T2DM) is characterised by hyperglycaemia due to insulin resistance which over time leads to a myriad of micro and macrovascular complications. Individuals with type 2 diabetes mellitus (T2DM) are at much higher risk (2-4 times that of the background population) of developing coronary artery disease,[1] peripheral vascular disease [2] and cerebrovascular disease.[3] Mortality from cardiovascular disease may be up to four times higher in patients with T2DM.[4] The hypertension which typically accompanies T2DM appears to be the most significant contributor to this increased risk. [5]

Endothelium derived nitric oxide (NO) is a potent vasodilator which plays a pivotal role in the moment to moment control of vascular tone.[6] There is biochemical and physiological evidence suggesting that basal NO production and NO bioavailability are diminished in T2DM.[7-8]In physiological studies where vascular responses are mediated at least in part by NO, for example flow mediated vasodilatation (FMD), impaired responses are observed in subjects with T2DM.[9-10]Decreased NO bioavailability due to increased oxidative stress or quenching of NO by advanced glycation end-products may underlie the impairment in FMD.[11-13]This alteration to NO physiology may play a key role in the hypertension associated with T2DM and its attendant morbidity and mortality.

Glucose homeostasis is complex and regulated by multiple pathways. Skeletal muscle glucose uptake appears to have an NO dependent component.[14] This NO dependent component may

account for a greater proportion of skeletal muscle glucose uptake in subjects with T2DM compared to healthy controls.[15]

In health, insulin may promote vasodilation, notably in the microcirculation, in an NO-dependent manner. This effect appears to be diminished in subjects with T2DM.[16] Impairment in microvascular blood flow occurs early in the pathogenesis of T2DM with evidence of microvascular impairment apparent at time of diagnosis.[17] Much of this impairment may be due to the reduced bioavailability of NO.[18]

Increasing the bioavailability of NO to reduce vascular tone and thus BP, and improve insulin sensitivity is an attractive therapeutic target in T2DM.Diets rich in green leafy vegetables lower the risk of cardiovascular disease.[19-20]A recent meta-analysis has shown that diets rich in green leafy vegetables reduce the risk of developing T2DM.[21] These beneficial effects may be due to the high nitrate content of such a diet.[22-24]

It has previously been shown that dietary nitrate supplementation, via sequential reduction of nitrate to nitrite and nitric oxide, can lower blood pressure and improve endothelial function in healthy humans.[25-29]Inorganic nitrate from the diet may have numerous other beneficial effects including improving exercise tolerance, ameliorating ischaemia-reperfusion injury, inhibition of platelet aggregation, protection against gastrointestinal infection, and protection against gastric ulceration (for review see [30]). Furthermore, Lundberg and Weitzberg's group has shown that, in endothelial nitric oxide synthase (eNOS) knockout mice, supplementation of the diet with inorganic nitrate improved glucose tolerance and ameliorated other features of the metabolic

syndrome.[23] Whether nitrate from the diet has an insulin sensitising effect or may delay the development of insulin resistance in humans is not known.

We sought to determine whether supplementation of the diet with inorganic nitrate, using beetroot juice as a source, lowers blood pressure, improves both macro- and microvascular endothelial function and enhances insulin sensitivity in patients with T2DM.

Methods

Study design and participants

This randomised double-blind, placebo-controlled crossover trial was approved by the Devon and Torbay Research Ethics Committee (study no. 09/H0202/43). This study was conducted in keeping with the principles of the Declaration of Helsinki.

Subjects were recruited from the Exeter 10000 (EXTEND) bio-resource. This is a large cohort of well characterised individuals who have consented to being contacted about medical research projects. Twenty seven participants (9F:18M) with T2DM as defined by WHO, of at least five years duration, with a BP >125/85 mmHg or on one or more antihypertensive agents were recruited. Smokers and individuals who consume more than the UK Department of Health recommended amount of alcohol per week (21units for females, 28 units for males) were excluded.

Participants were randomised to begin in either order, a 2 week period of supplementation with 250 ml beetroot juice daily, or 250 ml nitrate-depleted beetroot juice (see below for details of placebo production and characteristics), followed by a 4 week washout period before entering the second arm of the study.

Subjects were instructed to consume the juice along with their evening meal in order to minimise any potential glycaemic excursion, typically between 18.00 and 20.00 hours. Throughout the study patients were asked to maintain their normal diet apart from the juices given and not to change any other lifestyle factors. They were asked to continue their usual physical activity levels. Diet and activity levels were not monitored in the study. Participants continued their usual antihypertensive medication and their usual hypoglycaemic medications including metformin. Hypoglycaemic agents were omitted on visits where subjects were fasted. Twenty four hour blood pressure monitoring was performed from 09.00 on day 13 of each supplementation arm. Macro and micro vascular function tests were conducted from 10.00 on day 14 on each supplementation arm following an overnight fast.

Fasting blood samples (09.30) for nitrate and nitrite were collected into lithium heparin collection tubes. Samples were centrifuged immediately in a pre-chilled centrifuge ($4^{o}C$), plasma immediately separated (1ml aliquots), flash frozen in liquid nitrogen before transfer to a -$80^{o}C$ freezer.

Placebo production

We developed a procedure for the production of placebo juice using a column containing a nitrate specific anion exchange resin, (Purolite A520e). Placebo juice was pasteurised, bottled and labelled in the same manner as beetroot juice normally sold as a beverage. The untreated juice used in the active arm of the trial provided 7.5 mmoles of nitrate per day and the placebo juice provided 0.002 mmoles of nitrate. To set this in context, a typical Western diet results in 1-2 mmoles of nitrate ingestion daily.[31]

Twenty four hour ambulatory blood pressure measurement

Each participant was fitted with a TM-2430 ambulatory blood pressure monitor (AD, Draycott, Gloucestershire, UK) (Validated by British Hypertension Society). The device was programmed to record BP every 15 minutes between the hours of 07.00 and 22.00 and every 30 minutes from 22.00 to 07.00. Participants were advised they could carry out their usual activities but to avoid strenuous exercise.

Macrovascular endothelial function

Brachial artery endothelium-dependent dilation (flow-mediated dilation: FMD) and endothelium-independent dilation were assessed noninvasively following established guidelines.[32]

A multi-frequency linear-array transducer set at 13 MHz (SSD-5500 SV, Aloka, Tokyo, Japan) was used to obtain a B-mode ultrasound image of the brachial artery. An appropriately sized cuff selected for the participant was placed around the forearm and inflated to 250 mmHg for 5 minutes using a rapid cuff inflation system (AI6, Hokanson, Bellevue, WA). Brachial artery diameter was recorded for 60 seconds at baseline. The diameter recording was restarted 30 seconds before cuff deflation and continued for 3 minutes post deflation. All brachial ultrasound images were recorded and analyzed offline using commercially available software (Vascular Imager / Brachial Analyzer, Medical Imaging Application, Coralville, IA). Sublingual nitroglycerin (0.4 mg) was then administered following a 10-minute rest to assess endothelium-independent dilation. Brachial artery images were recorded for further 10 minutes. In our laboratory the intra-individual CV for measurement of FMD was 14.3%.[33]

Microvascular endothelial function

Microvascular endothelial function and non-endothelial vasodilation were assessed by the application of acetylcholine and sodium nitroprusside to the skin by iontophoresis and the assessment of perfusion response using Laser Doppler Perfusion Imaging. The protocol used in this study has been described in detail by our group previously.[34] In our laboratory the intra-individual CV for the measurement of the Ach response was 12% and for the SNP response was 18.7%, determined from five subjects on five separate occasions.[34]

Hyperinsulinaemic isoglycaemic clamp

The hyperinsulinaemic euglycaemic clamp technique was first described by Defronzo and colleagues in 1979 as method of determining insulin sensitivity by ascertaining whole body glucose disposal during a continuous insulin infusion [35] and is recognized as the gold standard.[36]

Preliminary work suggested using a euglycaemic target of 5 mmol/L with a modest dose of insulin (1.5 mU/kg/min) would be ineffective in hyperglycaemic, insulin resistant subjects. As a result we used a hyperinsulinaemic isoglycaemic protocol, a modified version of the conventional hyperinsulinaemic euglycaemic method, with a higher dose of insulin 2.0 mU/kg/min.[37]

The isoglycaemic set point to be used in both arms of the study was determined by taking the average of the fasting capillary glucose samples obtained on the day prior to the study and the day of the clamp study. We aimed to maintain the participant's venous plasma glucose within ±0.25 mmol/L of this number.

A continuous infusion of Actrapid insulin (Novo Nordisk, Denmark) at a rate of 2.0 mU/kg/min delivered in 0.9% saline at a rate of 50 ml/h was commenced. The glucose infusion commenced at a rate of between 2-4 mg/kg/min. The glucose infusion was adjusted in 0.5 mg/kg/min or 1 mg/kg/min increments as required to achieve, and maintain isoglycaemia.

Participants' venous plasma glucose levels were stabilised over the first 60 minutes with adjustments of the glucose infusion rate as required to achieve, and maintain, isoglycaemia. During the subsequent 60 minutes values used to calculate M, based on the method described by Defronzo[35], were obtained from a minimum period of twenty minutes where the participant's venous plasma glucose was within the target range. Venous plasma glucose measured using a YSI 2300 stat plus (Yellow Springs Instruments, Ohio USA).

Plasma nitrate and nitrite analysis

To ensure there was no contamination from the sampling procedures, in preliminary experiments, 7.5 ml of deionised water was added to lithium heparin blood tubes and then pipetted into 1 ml aliquots. This procedure mimicked that used during the collection of blood for the preparation of plasma. The measured concentrations of nitrate and nitrite in the 1 ml aliquots were 0.63 ± 0.3 μmol/L and < 20 nmol/L respectively. Prior to analysis, samples were deproteinised using a modification of the technique described by Higuchi and Motomizo.[38] Plasma nitrate and nitrite concentration were determined using a Sievers nitric oxide analyser (Sievers NOA 280, Analytix Ltd, Durham, UK) using the methods described by Bateman et al.[39] The between-batch coefficient of variation for nitrate was 13% (n=3) and for nitrite was 8% for a control plasma sample (n=11).

Statistical Analysis

Data were tested for normality. Paired t-tests were applied to data where the differences in the parameter of interest between the active and placebo conditions were normally distributed. Wilcoxon signed rank test was used for non-parametric data. Correlations were tested using Spearman's rho for non-parametric data. The study was powered to detect a difference of 3.6 mmHg in the primary outcome measure systolic blood pressure determined by 24 hour ABPM between the two treatment arms with 80% power at the 5% level.[40]

Results

The beetroot juice was generally well tolerated. Discolouration of urine and stools was frequently reported but did not cause distress as all individuals had been informed about this possibility during the consent process. Participant-reported compliance was excellent and this was borne out in the plasma nitrate analysis. For the small number of subjects who monitored capillary glucose at home there were no apparent changes in values. For participant characteristics see Table 1. There was no evidence for a period or carryover effect for any of the measures reported.

Dietary nitrate elevated plasma nitrate and nitrite concentration in patients with T2DM

The median plasma nitrate and nitrite concentrations were increased following two weeks of nitrate-rich beetroot juice compared to placebo (Figure 1). The median plasma nitrate concentration rose from 31.0 µmol/L (median, interquartile range: 19.8-41.6) in the placebo arm to 150 µmol/L (122.7, 200.0) in the active arm ($p<0.001$). The median plasma nitrite concentration rose from 232 nmol/L (200,265) in the placebo arm to 390 nmol/L (312,537) in the active arm ($p<0.001$).

Dietary nitrate supplementation did not lower BP in patients with T2DM

Dietary nitrate supplementation did not lower systolic, diastolic or mean arterial pressure as determined by 24 h ABPM (Figure 2). The mean systolic BP, the primary outcome measure, was 134.6 ± 8.4 mm Hg in the placebo arm and 135.1 ± 7.8 mm Hg (mean ± SD) in the active arm, a mean rise of 0.5 mm Hg, p=0.737 (95% CI -3.9 to 2.8). The mean diastolic BP was 77.1 ± 7.0 mm Hg in the placebo arm and 75.2 ± 5.4 mm Hg in the active arm, a mean reduction of 1.9 mm Hg, p=0.106 (95% CI -0.4 to 4.1). The mean arterial pressure was 95.6 ± 6.1 mm Hg in the placebo arm and 94.6 ± 6 mm Hg on the active arm, a mean reduction of 1 mm Hg, p=0.375 (95% CI -1.3 to 3.4).

When the data were separated into waking and sleeping periods no differences were observed. The waking mean systolic BP was 138 ± 10.9 mm Hg in the placebo arm *versus* 138.2 ± 10 mm Hg in the active arm, p=0.93 (mean difference -0.2, 95% CI -4.8 to 4.4). During sleeping hours,

the mean systolic BP was 117.6 ± 12.7 mm Hg in the placebo arm *versus* 119.1 ± 10.2 mm Hg in the active arm, p=0.47 (mean difference -1.4, 95% CI -5.5 to 2.6).

Examination of the blood pressure data in the two to five hour period post-supplementation showed no differences between the groups. The mean systolic BP was 128.1 ± 6.4 mm Hg in the placebo arm *versus* 127.8 ± 5.7 mm Hg in the active arm, p=0.77 (mean difference 0.3; 95% CI -2.1 to 2.7). The mean diastolic BP was 71.8 ± 5.3 in the placebo arm *versus* 71.1 ± 3.7 mm Hg in the active arm, p=0.62 (mean difference 0.7; 95% CI -2.1 to 3.3).

There appears to be a trend towards a reduction in diastolic blood pressure, with a difference of 1.9 mm Hg, p=0.106 (95% CI -0.4 to 4.1) between placebo and active arms. The study had sufficient statistical power to detect a difference of 2.7 mmHg, meaning we cannot exclude the possibility of a real difference that is smaller than this. There was however no correlation (Spearman's r=0.1139, p=0.57) between change in diastolic blood pressure and change in plasma nitrite, suggesting there is unlikely to be a meaningful effect (Figure 3).

Dietary nitrate supplementation did not improve macrovascular endothelial function in patients with T2DM

Macrovascular endothelial function measured by FMD was unchanged with dietary nitrate supplementation (mean percentage change in brachial artery diameter 4.94 ± 2.87 % in the placebo arm *versus* 4.97 ± 2.59% in the active arm, p=0.936 (Figure 4)). The response to GTN

was unchanged by dietary nitrate supplementation (17.34 ± 6.77% *versus* 16.91 ± 5.63%, p=0.476 (data not shown)).

There was no correlation between FMD and plasma nitrite concentration or plasma nitrate concentration in the placebo arm. Nor was there a correlation between change in FMD and change in plasma nitrite from placebo to active arms.

Dietary nitrate supplementation did not improve microvascular endothelial function in patients with T2DM

Iontophoresis of acetylcholine (Ach; endothelial dependent vasodilation) increased skin perfusion as expected. However this response did not differ in the placebo phase compared to the active juice phase. Thus the peak Ach responses were 1.3 ± 0.3 AU in the placebo arm *versus* 1.3 ± 0.4 AU in the active arm, p=0.335. The area under the curve was 250.3 ± 102.9 in the placebo arm *versus* 267.9 ± 84.4 AU in the active arm, p=0.243 (Figure 4).

Iontophoresis of sodium nitroprusside (endothelial independent response) increased skin perfusion as expected. However this response did not differ in the placebo phase compared to the active juice phase. Thus the peak SNP responses were 1.1 ± 0.3 AU in the placebo arm *versus* 1.2 ± 0.3 AU in the active arm, p=0.59. The area under the curve was 172.4 ± 71.6 AU in the placebo arm *versus* 177.4 ± 54.1 AU in the active arm, p=0.613 (Figure 4).

Dietary nitrate supplementation did not improve insulin sensitivity in patients with T2DM

M is a measure of whole body glucose disposal. M was 5.83 ± 2.80 mg/kg/min in the placebo arm and 6.03 ± 2.56 mg/kg/min in the active arm. This gave a mean difference of -0.20 ± 0.87, p=0.344 (paired t-test) (Figure 5).

Discussion

The study presented here is the first study examining the effect of dietary nitrate on BP, endothelial function and insulin sensitivity in individuals with T2DM. Supplementation of the diet with 7.5 mmoles of nitrate in the form of beetroot juice did not lower blood pressure, or improve macro or microvascular endothelial function or improve insulin sensitivity in this group of men and women with Type 2 diabetes.

The work presented in this study has several key strengths. The use of 24 hour ABPM provides a robust, reliable method of determining blood pressure. This study involved the largest group of subjects to complete a study examining the effect of dietary nitrate on BP. This is also the first study examining the effect of dietary nitrate on BP using a true placebo.

Our findings that twenty four hour mean ambulatory blood pressure (systolic, diastolic, and mean arterial pressure) were unchanged with dietary nitrate supplementation, and that there was no difference between placebo and active arms during waking hours or during sleeping hours data contrast to some previous reports in the literature. These differences may be attributed to

characteristics in the groups of individuals studied, the techniques used to measure BP, and the time course of the BP effect. These points are considered in further detail below.

A major difference between our study and those previously published is the volunteer cohort studied. This is the first study using a group of participants with T2DM as opposed to healthy volunteers. As would be expected in T2DM our participants had clear evidence of the co-morbidity that typically accompanies T2DM. They were hypertensive with a BP (mean ± SD; systolic/diastolic) of 142.9 ± 13.9/81.1 ± 9.2 mm Hg at enrolment. The patients had an elevated BMI of 30.8±3.2 kg/m^2,and had evidence of both macrovascular and microvascular dysfunction endothelial dysfunction.[10] Diabetes results in numerous biochemical perturbations including hyperglycaemia, increased oxidative stress, accumulation of advanced glycation end products,[41] increased NF-κB activation,[42] and dyslipidaemia [43] all of which may attenuate NO-like activity and thus inhibit BP lowering by dietary nitrate supplementation. Furthermore every subject was on at least one medication which may affect vascular function and potentially the response to inorganic nitrate. These medications were continued throughout the study period. Prior to the present study, Kenjale *et al*'s pilot study,[44] in peripheral arterial disease, was the only trial examining the effect of dietary nitrate in subjects already taking vasoactive medications. There was no difference in systolic BP following nitrate supplementation in their study.

Onlytwo previous studies have been conducted in groups whose age is comparable with that of the group in the present study. Kenjale *et al* [44] examined the effect of dietary nitrate on exercise performance in subjects with peripheral artery disease. There was no effect on resting systolic BP in a group with a mean age of 67 ± 13 years following supplementation with 9 mmoles of

nitrate, using beetroot juice as a source of nitrate. This was, however, a small study with only eight participants, making it impossible to exclude a Type II error. Our own group has recently reported a significant fall in BP in a group of twelve healthy subjects with a mean age of 64 ± 2.7 years. Again this used a higher daily dose of 9.6 mmoles of nitrate, with the last supplementation episode 2.5 hours prior to BP measurement in laboratory conditions. [45] The unsupplemented plasma nitrite concentration in the diabetic cohort described in the present study – which had a median value and range of 232 nmol/L (200, 265) - was similar to that in the healthy subjects in Kelly et al's study (248 ± 182 nmol/l; mean ± SD). [45] The study by Sobko *et al* [46] examined the effect of a traditional Japanese diet. The subjects had a mean age of 36 ± 10 years and again no difference was seen in systolic BP. A small, but statistically significant, fall in diastolic pressure was observed. In contrast, where large falls in systolic pressure have been reported, the groups studied have been much younger. The subjects in Webb *et al*'s study [26] were 25.5 ± 4.5 years old; the subjects in Kapil *et al*'s study [27] were 22.5 ± 0.9 years old.

The stiffening of the vasculature and its diminished responsiveness to NO associated with increasing age [47] may have attenuated the effect of dietary nitrate supplementation on both the macrovascular and microvascular measures examined herein. This diminished vascular responsiveness to NO may be an important limiting factor to inorganic nitrate-based therapeutic strategies in older age groups. The effect of age on responsiveness to dietary nitrate supplementation may, however, be more complex than simply the age of the subject at the time of ingestion. Epidemiological data showing protective effects from diets which would be rich in nitrate may in part be influenced by dietary patterns established earlier in life at a time not necessarily captured in the studies themselves.[21]

The subjects in the present study had a BMI of 30.8 ± 3.2 kg/m^2. The participants in the study by Webb *et al*[26] had a BMI of 22.6 kg/m^2; in the study by Kapil *et al* [27] the mean BMI (± SEM) was 22.5±0.6 kg/m^2. In the DASH study, the mean BMI was 28.1 ± 4.0 for men and 29.0 ± 3.9 for women. There was no apparent difference in BP lowering effect in the DASH trial when subjects were stratified into obese/non-obese (women were defined as obese if their BMI was $\geq$ 27.3: men were defined as obese if their BMI was $\geq$ 27.8). [48] The subjects in the study by Kenjale *et al* - [44] who had no change in systolic BP - had a BMI of 28.6 ± 5.8 kg/m^2. Interestingly in the study by Sobko *et al,*[46] which showed a lowering of diastolic but not systolic BP in a Japanese cohort, the mean BMI was 18.5 kg/m^2. This may say more about the appropriateness of comparing BMIs between differing populations than the effect of adiposity on the blood pressure effect of dietary nitrate. In order to determine if there was a difference in the effect of nitrite in obese subjects, a trial comparing the effect of nitrate in age- and sex-matched lean *versus* obese subjects would be required. This is an area clearly deserving of investigation. Our study did not have sufficient numbers to perform a subgroup analysis.

Differences in the amount of nitrate given in this and previous studies and the effect this has on BP responses are worthy of discussion. The present study provided an additional 7.5 mmoles of dietary nitrate per day. Work from our group has shown statistically significant falls in systolic BP of 5 mm Hg when using daily doses down to 5.1 mmoles of dietary nitrate in younger healthy subjects.[49] Ahluwalia *et al* [27] examined the dose response effects of nitrate and reported that a 5.5 mmoles dose, using beetroot juice as a source, caused a peak fall in blood pressure of 5.4 ± 1.5 mm Hg at 3 hours post ingestion. These results support the likelihood that the 7.5 mmoles daily

dose of nitrate in the present study would reasonably have been expected to have a hypotensive effect.

Many of the studies reporting significant results with respect to blood pressure, or improvements in exercise tolerance with nitrate have used a low nitrate diet run-in[25 27]. In the present study there were no dietary modifications beyond the consumption of the active and placebo juices as we wished to explore the true effects of our patients adding a nitrate juice to their normal diet. Our subjects' usual nitrate consumption was not accounted for, neither was the consumption of dietary constituents likely to exert an upward effect on BP such as dietary sodium. This may have attenuated the difference in BP likely to be seen with supplementation. In the DASH study,[50] the group who supplemented their diet with fruit and vegetables had a more modest fall in blood pressure than those who, in addition to increasing their fruit and vegetable intake reduced total and saturated fat.

It could be hypothesised that by looking at the twenty four mean ambulatory blood pressure a significant effect on blood pressure might be missed at the time of peak nitrite levels which typically occur between 2 and 5 hours post-ingestion. [26-27] However when we examined the data corresponding to this period, and when the data was split into sleeping and waking periods, no blood pressure lowering effect could be observed.

The reported range of plasma nitrite concentrations in healthy human populations, as determined by the chemiluminescence method used in the current study, varies substantially. Lundberg's group recently reported a mean value of 35 ± 7 nmol/L, [51] whereas Ahluwalia's

group report mean values of 362 ± 30 and 536±50 nmol/L in males and females, respectively . [27] As noted previously, the plasma nitrite concentrations we obtained in this diabetic cohort were similar to the plasma nitrite concentrations in a group of healthy older adults.

We report an approximate doubling of the mean plasma nitrite concentration which, based on previous work, should have been sufficient to ascertain whether there was a blood pressure lowering effect. It should also be noted that by collecting blood samples approximately 16 hours following the last beetroot dose we did not observe the peak nitrite concentration that would be expected at 3 hours.

If, as has been suggested above, microvascular blood flow is an important determinant of insulin sensitivity, the absence of change in M (total body glucose disposal) during the hyperinsulinaemic isoglycaemic clamp may in part be anticipated in view of the observed lack of effect on microvascular function.

It is conceivable that dietary nitrate supplementation, and subsequent evolution of nitrite and nitric oxide, may lead to diminished endothelial nitric oxide synthase (eNOS) expression and activity. Zhen and colleagues [52] showed apparent suppression of eNOS activity in human coronary artery endothelial cells and rats by the NO donor S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP). This suggests NO exerts a negative feedback effect on eNOS expression and activity. While the mechanisms remain a matter for further exploration it may be possible that increased NO evolved by dietary nitrate supplementation might have a similar effect. However in a young healthy cohort the hypotensive effect of dietary nitrate supplementation has

been sustained following 15 days of dietary nitrate supplementation, suggesting this might not be the case, at least in health.[53]

We conclude that the blood pressure lowering effect of a short period of dietary nitrate supplementation seen in healthy young adults does not appear to be applicable to individuals with T2DM. This may reflect diminished vascular reactivity in T2DM, or with ageing, and particularly impaired responsiveness to NO. Anti-hypertensive therapeutic strategies targeted at NO supplementation under resting physiological conditions may therefore be less effective in subjects with T2DM. The effect of longer term dietary nitrate supplementation on vascular function and cardiovascular outcomes is unknown at present and remains worthy of further consideration based on the epidemiological evidence linking green leafy vegetable consumption to reduced cardiovascular risk.

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TABLE

	Mean	Std. Deviation
Age (years)	67.2	4.9
Duration of diabetes (years)	13.6	8.1
Mean office systolic blood pressure (mm Hg)	142.9	13.9
Mean office diastolic blood pressure (mm Hg)	81.1	9.2
BMI (Kg/m^2)	30.8	3.2
HbA1c (%)	7.6	1.1
Serum Creatinine (mmol/l)	88.2	27.9
Total number of antihypertensives	2.1	1.1
Retinopathy	5 (18.5%)	
Neuropathy	8 (29.6%)	
Nephropathy	1 (3.7%)	

Table 1. Characteristics of subjects in T2DM study.

FIGURES

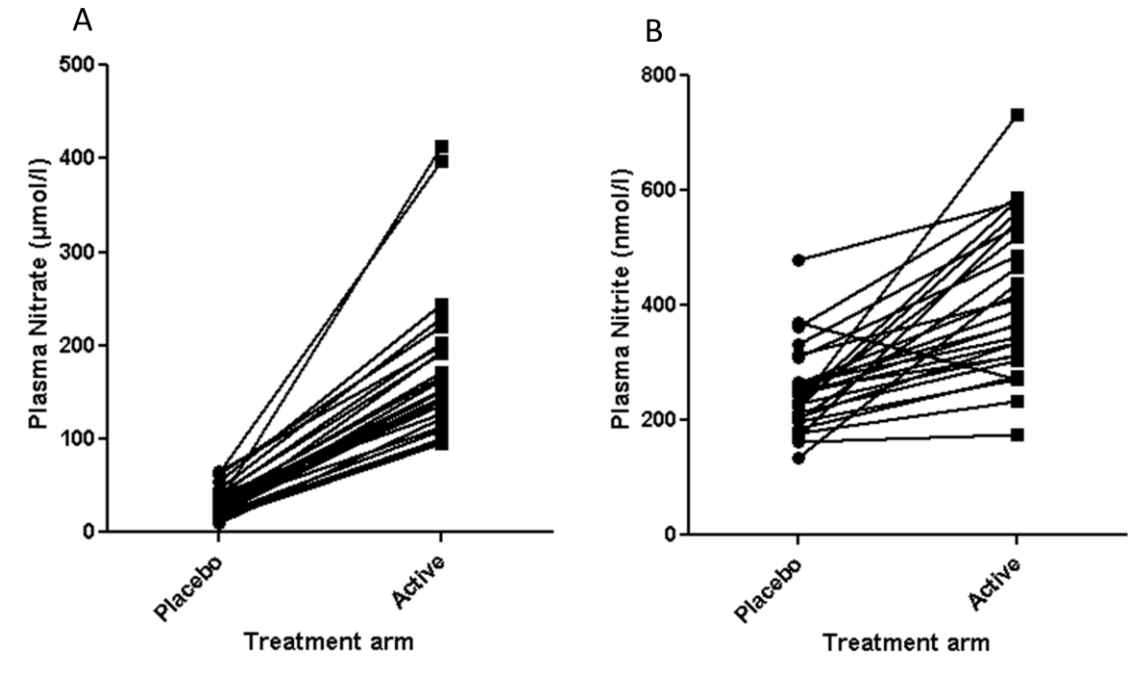

Figure 1: Plasma nitrite and nitrate concentrations in subjects with T2DM

A fasting blood sample was taken at 09.00 on the final day of testing at the end of each supplementation, for determination of plasma nitrate and nitrite concentration by an ozone-based chemiluminescence technique (see Methods section). The plasma nitrite concentration (A) rose from 232 nmol/L (median, IQR 200, 265) in the placebo arm to 390 nmol/L (312, 537), $p<0.001$, in the active arm. The plasma nitrate concentration (B) rose from 31 µmol/L (20, 42) in the placebo arm to 150 µmol/L (123, 200), $p<0.001$, in the active arm. All p values derive from a Wilcoxon signed rank test.

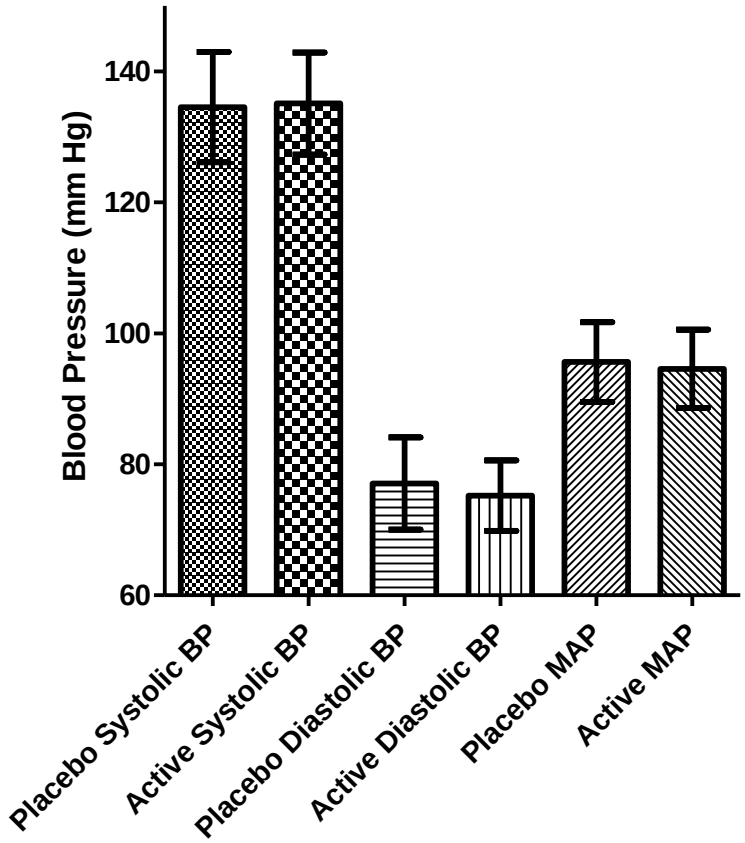

Figure 2: 24 hour ABPM results (mean ± SD)

Twenty four hour ambulatory BP was recorded on day 13 of each supplementation period with the placebo and active juices, using a TM-2430 ambulatory blood pressure monitor (AD, Draycott, Gloucestershire, UK) in subjects withT2DM. There were no differences in systolic, diastolic or mean arterial pressure (MAP).

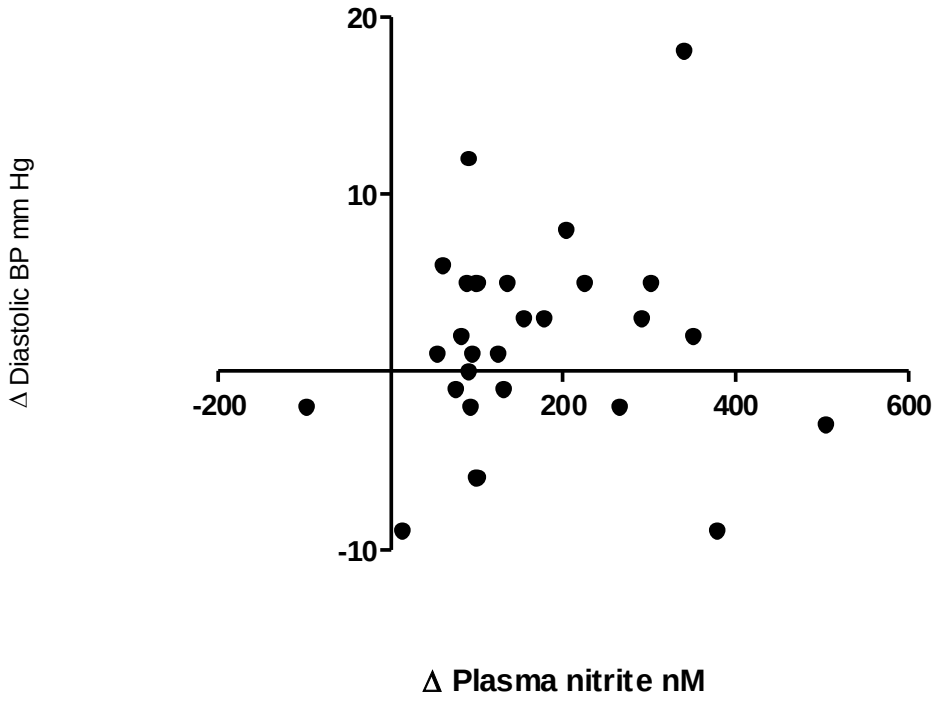

Figure 3: Relationship between change in plasma nitrite and diastolic blood pressure (active –placebo). There was no correlation between the change in diastolic blood pressure and the change in plasma nitrite concentration, Spearman's r=0.1139, p=0.57.

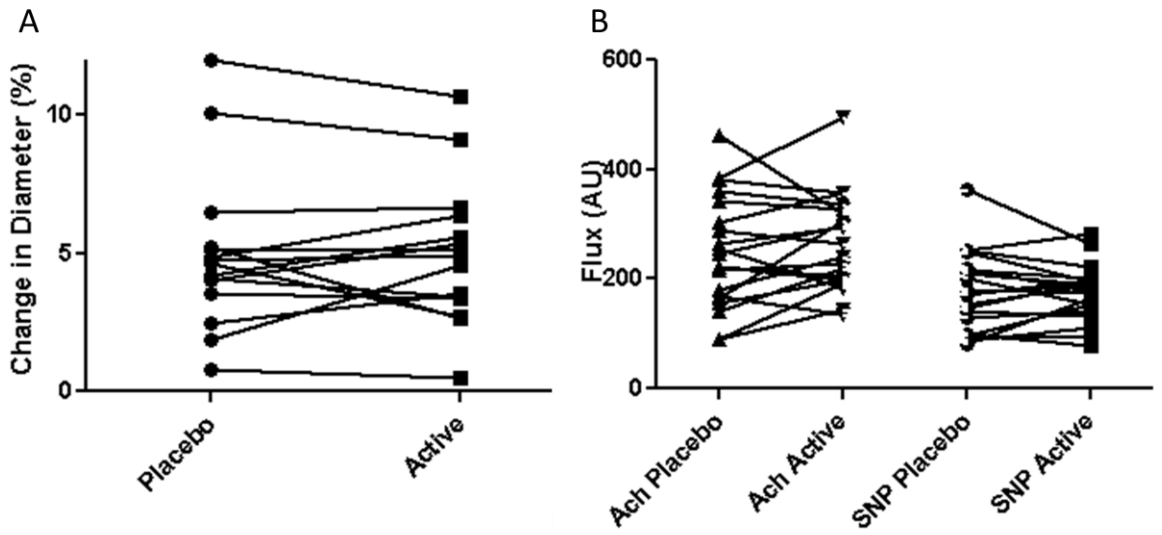

Figure 4: Macro and micro vascular endothelial function

Brachial artery flow mediated dilation (A) in subjects with T2DM after 14 days of placebo or active juice. Mean FMD (expressed as percentage change in vessel diameter) was 4.94 ± 2.87 % in the placebo arm and 4.97 ± 2.59% in the active arm, p=0.936. Endothelium dependent vasodilation measured as the flux in arbitrary perfusion units (AU) in response to iontophoresis of Ach (B) did not change between groups: there was a flux of 250.3 ± 102.9 AU in the placebo arm and a flux of 267.9 ± 84.4 AU in the active arm; p=0.243. Following iontophoresis of SNP, total area under the curve for endothelium independent vasodilation did not change between treatment arms: there was aflux of 172.4 ± 71.6 AU in the placebo arm and a flux of 177.4 ± 54.1 AU in the active arm; p=0.613. P values were obtained using a paired t-test.

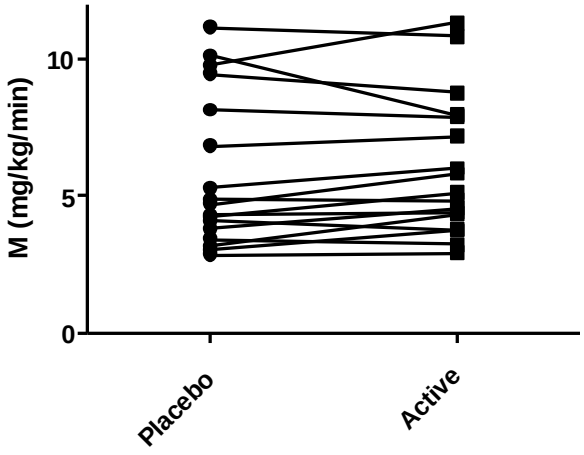

Figure 5: Hyperinsulinaemic isoglycaemic clamp

Insulin sensitivity (M) as determined by hyperinsulinaemic isoglycaemic clamp in subjects with T2DM following fourteen days of supplementation with placebo or active juices. M, expressed as the glucose infusion rate required to maintain isoglycaemia, was 5.83 ± 2.80 mg/kg/min in the placebo arm and 6.03 ± 2.56 mg/kg/min in the active arm. This gave a mean difference of -0.20 ± 0.87 mg/kg/min (p=0.344 by paired t-test).