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Relationship between urinary nitrate excretion and blood pressure in the InChianti cohort

Urinary Nitrate and BP in InChianti

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Abstract

Background Inorganic nitrate from the oxidation of endogenously synthesized nitric oxide or consumed in the diet can be reduced to nitric oxide via a complex entero-salivary circulation pathway. The relationship between total nitrate exposure by measured urinary nitrate excretion and blood pressure in a large population sample has not been assessed previously.

Methods Twenty four hour urinary nitrate excretion was measured by spectrophotometry in the 919 participants from the InChianti cohort at baseline and blood pressure measured with a mercury sphygmomanometer.

Results After adjusting for age and sex only, Diastolic blood pressure was 1.94 mm Hg lower in subjects with ≥2mmol urinary nitrate excretion compared with those excreting <1mmol nitrate in 24 hours: Systolic blood pressure was 3.41mm Hg (95%CI -3.48 to -0.39) lower in subjects for the same comparison.

Effect sizes in fully adjusted models (for age, sex, potassium intake, use of anti-hypertensive medications, diabetes, HS-CRP, or current smoking status) were marginally larger: systolic blood pressure in the ≥2mmol urinary nitrate excretion group was 3.87 (CI -7.06 to -0.69) mm HG lower than in the comparison <1 mmol excretion group.

Conclusions Modest differences in total nitrate exposure are associated with reductions in blood pressure which are at least equivalent to those seen from substantial (100mmol) reductions in sodium intake.

Key Words: Nitrate, diet, hypertension

Introduction

The continuous generation of nitric oxide (NO) from the oxidation of L-arginine by nitric oxide synthase (NOS) in the vascular endothelium plays a significant role in the control of vascular tone[1]. The vasodilatory action of NO is terminated by its rapid oxidation to nitrite and nitrate[2,3]. It has been shown that there is an entero-salivary pathway whereby nitrate can be reduced to nitrite by commensal bacteria on the mammalian tongue[4], with the nitrite produced swallowed in the saliva. This nitrite is absorbed into the circulation and can have a blood pressure lowering effect either directly or by further reduction to NO[5]. In addition to the NOS pathway nitrate may also be obtained from the diet, with green leafy vegetables and beetroot particularly rich in inorganic nitrate[6].

A typical western diet sees the consumption of 1-2mmol inorganic nitrate per day, predominantly from vegetables[7,8]. Humans produce approximately 1 mmol nitrate per day from the oxidation of endogenously synthesized NO[9]. It had been shown in hypertension and other associated conditions that endogenous synthesis of NO is diminished[10]. Trials of inorganic nitrate supplementation using beetroot juice[5], a range of nitrate rich vegetables[11], and pharmacological sodium or potassium nitrate[12] have shown a range of blood pressure effects from substantial reductions in systolic[5,12-14] and diastolic pressures[11], reductions in blood pressure variability[15] to no effect[16,17]. There has been considerable heterogeneity between studies in terms of populations studied and dose and duration of nitrate provided[18].

Beyond a possible blood pressure lowering effect inorganic nitrate supplementation may have multiple other beneficial effects including improvements in endothelial function[5], reductions

in the oxygen cost of exercise[13], improvements in cognitive function[19], protection against ischaemia reperfusion injury[20] along with potentially positive effects on metabolism[21,22].

Synthesis studies and some dietary intervention studies rely on the restriction of inorganic nitrate intake[10,17]. There have been no large scale population studies of the effect of total nitrate exposure, from both endogenous and dietary sources, on blood pressure. We examined the association between 24 hour urinary nitrate excretion, as a marker of both endogenous and dietary nitrate exposure, and blood pressure in the InChianti study.

Methods

Study population and design

We obtained baseline 24 hour urine samples from the InCHIANTI study, a prospective population-based study of older people, conducted by the Laboratory of Clinical Epidemiology of the Italian National Institute of Research and Care on Aging (INRCA), Florence, Italy. Ethical approval was granted by The INRCA Ethical Committee.

The InChianti study aimed to recruit the older residents in two towns of the Chianti area (Greve in Chianti and Bagno a Ripoli, Tuscany, Italy) plus younger controls, and achieved a 91.6% response rate at baseline. Data collection commenced in September 1998 and completed in March 2000. Sampling procedure and data collection method has been published previously[23].

Measures

Resting supine blood pressure was measured twice in both arms using a mercury sphygmomanometer. For the purposes of the present analyses we used the data from the limb with the highest recorded value. The mean of 2 values from this arm was used.

Total nitrate exposure was determined by measurement of 24 hour urinary nitrate excretion. Mean daily nitrate excretion was 1.33 mmol, with a standard deviation SD=1.1 and a range of 0 to 8.5 mmol. Subjects with 24 hour urine volume less than 400 ml (n=6) were excluded from analysis as such low volumes are unlikely to represent complete collections.

Urinary nitrate concentration was measured using the spectrophotometric plate method described by Miranda et al[24]. We have validated this method in urine samples against the gold standard ozone chemiluminescence method we have used previously (see on line data supplement). Each sample/standard (100 µl) was added to a 96 well plate, followed by 100 µl VCl3 (0.1 M VCl_3 in 1M HCl) and 100 µl Greiss reagent (sulphanilamide, 2% w/v in 5% HCl, and N-(1-naphthyl)ethylenediamine dihydrochloride (NEDD), 0.1 % w/v in water, mixed in equal volumes immediately before use) and incubated at 37 ^{O}C for 30 min. Absorbance was read using a plate reader at 540 nm. The VCl_3 reduces the nitrate in the sample to nitrite which then forms a coloured chromogen upon reaction with the Greiss reagent. Thus the assay does not differentiate between nitrite and nitrate. As the concentration of nitrate is approximately 1000 times that of nitrite in urine[25,26], a ratio that at the very least persists following nitrate supplementation[26], we have reported the results as urinary nitrate concentration.

Urine samples were diluted 1 in 10 so that measurements were within the linear range of the standard curve (1 – 500 µM). A urine blank was required for each sample, as samples varied

in their turbidity. Any samples where the duplicates varied by >10% were repeated and the coefficient of variation was 8.5 %. A small proportion (3%) of samples had poor replication of duplicates even on repeat duplicate analysis. These samples were filtered using 0.22 µm syringe filters before analysis, and this additional step provided good duplicates with < 10% variation.

Sample and Statistical analysis

The selection of subjects and variables for statistical modelling was based on our Directed Acyclic Graph of hypothesised causal influences linking nitrate excretion and measured blood pressure (see appendix A)[27]. Nitrate levels were measured in 24 hour urine samples from 1188 respondents. Data on 6 patients were excluded due to urine volumes less than 400ml, 215 for definite or probable congestive heart failure or missing data and 48 for a creatinine clearance (Cockcroft-Gault) <30 or missing data. The sample available for analysis was 421 men and 498 women (n=919 total) aged 21 to 95 years old.

Regression models were adjusted initially for age-group and gender, and then additionally for estimated daily potassium intake from a diet diary, high sensitivity C-Reactive protein, diabetes, current smoking, activity level in the last year and highest educational attainment. Further adjustment was made for 24 hour urinary sodium excretion. Models were also adjusted for antihypertensive medication, with separate terms for ACE inhibitors, diuretics, beta-blockers, peripheral vasodilators and other antihypertensives.

Analysis was carried out in Stata 13 using robust linear regression models.

Results

A total of 919 subjects were included in the analysis. Forty eight percent (n=441) of the sample had 24 hour nitrate excretion of <1 mmol, 33% (307) had 1 to <2 mmol and 19% ≥2 mmol (table 1). Overall 73.3% of the sample was aged 65 and over, and 45.8% were male. 25.6% were receiving at least one antihypertensive and 10.7% had definite or probable diabetes. There were significant differences in the age distribution by increasing category of nitrate excretion, with fewer of the older sample in the higher nitrate categories.

The mean diastolic blood pressure was 82.8 (SD 9.5) and mean systolic 143.1 (standard deviation 21.1).

In simple age sex adjusted models only (Table 2), Diastolic blood pressure was 1.94 mm Hg lower in subjects with ≥2mmol urinary nitrate excretion compared with those excreting <1mmol nitrate in 24 hours: Systolic blood pressure was 3.41mm Hg (95%CI -3.48 to -0.39) lower in subjects for the same comparison. Differences in blood pressures with the nitrate 1 to <2 mmol excretion group were intermediate, and statistically significant for diastolic blood pressure only. A tend estimate (per nitrate group change) in mean systolic blood pressure was significant (ceoff -1.82 SE 0.79, p=0.022).

Regression coefficients for fully adjusted models (for age, sex, potassium intake, the use of anti-hypertensive medications, diabetes, HS-CRP, or current smoking status) were marginally larger: for example, systolic blood pressures in the ≥2mmol urinary nitrate excretion group was 3.87 (CI -7.06 to -0.69) mm HG lower than in the comparison <1 mmol excretion group. Adjusting the model for 24 hour urinary sodium excretion did affect the outcome.

As nitrate excretion may diminish with declining GFR, an additional sensitivity analysis adjusting for renal function (Cockcroft-Gault estimate) was performed. The association between higher urinary nitrate excretion (≥2 mmol per day) and mean systolic blood pressure remained significant (coefficient -4.40 SE 1.58, p=0.005).

Discussion

The primary finding that systolic and diastolic blood pressure are 3.87 and 2.28 mm Hg lower in individuals with the highest nitrate exposure compared with the lowest provides further evidence to support strategies targeted at increasing NO bioavailabilty.

In an age and sex adjusted model, the InterSalt Cooperative Study Group found 3.5 mm Hg systolic and 1.5 mm Hg diastolic reductions in blood pressure when sodium intake was lowered by 100mmol/day[28]. After adjustment for potassium excretion, BMI, and alcohol intake reduces these estimates were more modest with reductions of to 2.2 mm Hg systolic and 0.1 mm Hg diastolic. In an additional sensitivity analysis of our data, adjusting for BMI (as a continuous variable) and daily alcohol intake (grouped), the association between higher urinary nitrates (≥2 mmol per day) and mean systolic blood pressure remained significant (coefficient -4.18 SE 1.57 p=0.008).If we assume a causal relationship, the very modest increment in nitrate intake required to achieve a urinary nitrate excretion may have at least equivalent effects on blood pressure to the more far reaching dietary changes which would be required to reduced sodium intake by 100mmol/day. To put these changes in to context, a

single serving of 80mg of a high nitrate vegetable such as rocket or spinach can provide 2-3 mmol in organic nitrate[29].

It has previously been shown that 60-65% of inorganic nitrate is excreted in the urine within 24 hours in young healthy subjects[30]. It is not known how the remainder is excreted nor if these rates and proportions change with increasing age or in the setting of various pathological conditions. It is however likely, that in the population studied, 24 hour urinary nitrate excretion represents a fair reflection of overall nitrate exposure.

This study cannot determine whether endogenous synthesis or dietary sources of nitrate made the greatest overall contribution to overall nitrate exposure. In Tessari et al's study basal NOx synthesis rates were not different between young, elderly, hypertensive or hypercholesteroleamic subjects with a maximum rate of 0.75 ± 0.4 mmol/day[31]. In earlier work by Forte et all, under conditions of low exogenous nitrate, 24 h urinary nitrate excretion was lower in hypertensive patients than in controls, mean 0.450 [SEM 37] *vs* 0.760 mmoles [77] /day[10]. It is likely based on estimated synthesis rates from these previously reported studies that those in the highest excretion category in the current study will have had the highest dietary exposure regardless of endogenous synthesis rates.

It would have been of interest to look for a relationship between dietary nitrate intake and urinary nitrate excretion. However the food item questionnaire was not designed to assess this. Consequently it does not cover sufficient high nitrate vegetables to permit such an analysis. Diets rich in fruit and vegetables are high in potassium. We adjusted the model for potassium intake in order to indirectly account for the effect a high fruit and vegetable intake *per se*, with no impact on the outcome.

Many of the previously published intervention studies demonstrating a blood pressure lowering effect from inorganic nitrate supplementation have looked at young healthy subjects[5,13]. There are some data to support a blood pressure lowering effect in older adults[14,32] however this is not consistent, particularly in groups with pathology[17,33,34]. The age range of the InChianti cohort begins at 65 and include individuals of greater than 90 years of age. It includes a large number of subjects with typical age-related co-morbidity.

Previous intervention studies have suggested a threshold of around 4mmol/day from dietary sources could lead to blood pressure lowering effects[12]. The present data suggests if there is a threshold effect, it may be at lower levels of dietary consumption. What is not clear is if a dose response exists above or below any such threshold. A meta-analysis of intervention studies using beetroot juice (dose range 5.1 to 45 mmol/day) or nitrate salts (dose range 2.5 to 24 mmol/day)found a -4.4 mm Hg (95% CI: -5.9, 2.8); $P < 0.001$, reduction in systolic blood pressure and a non-significant trend towards lower diastolic BP -1.1 mm Hg (95% CI:-2.2, 0.1); $P = 0.06$[18]. The findings in the present study are in keeping with the observed results derived from a range of nitrate doses.

Limitations –

In evaluating these results we note that sample studied is predominantly older and from the Chianti region of Italy, and generalisability to other groups needs to be established. It has previously been shown by Hill *et al* that urinary nitrate excretion varies significantly across geographical regions, likely reflecting differences in the dominant dietary habits of a given region[35].

The analysis is observational and cross-sectional, and therefore directions of causation cannot be established: longer term randomised trials will be needed to confirm the results reported. Many of the patients in the sample were on antihypertensive medications, and although numbers of medications at baseline were not associated with nitrate excretion category, some biasing effect may remain from medication. Despite these limitations, our use of a relatively large population based sample with 24 hour urine collections plus a structured approach to model exclusions and adjustments is likely to have provided robust results.

InChianti was primarily designed a tool to investigate health and mobility in the elderly. It was not designed with blood pressure studies in mind and while the methods for measuring blood pressure is not identical to recommendations from recognised hypertension societies we feel that the measures we have selected closely reflect guidelines for clinical practice[36].

This association of lower blood pressure with relatively modest increases in urinary nitrate excretion, which could easily be achieved with minor dietary modification, provides further support for interventions targeted at increasing dietary nitrate exposure at population level. Further work is needed to assess the impact of measured urinary nitrate exposure on health outcomes.

A robust, high throughput, assay for measurement of inorganic nitrate in biological fluids represents a major advance in the field. The previous ozone chemiluminescence method is time consuming and laborious. The spectrophotometric plate method developed by Miranda et al and validated by us for use in urine for the current study offers the prospect of large scale epidemiological studies in the field of inorganic nitrate research and the realistic possibility of an assay that could be used in clinical practice.

The degree of difference in blood pressure seen between the lowest to highest nitrate exposures in the InChianti cohort would likely be translated to substantial reductions in morbidity and mortality at the population level. Though the difference in blood pressure was relatively modest it was observed at modest differences in total nitrate exposure. Crucially the 1-2mmoles nitrate that would be required to elevate exposure from the lowest to the highest group can be obtained from small portions of green leafy vegetables or beetroot. Furthermore there are no known adverse effects of such an approach.

Disclosures: MG, NB and PGW received financial support from James White Drinks Ltd for the development of a nitrate-depleted form of beetroot juice. NB is a cofounder of Heartbeet Ltd, a non-profit making organization set up to promote the health benefits of dietary nitrate.

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Table 1: characteristics of the sample (number, %) by category of 24 hour nitrate excretion

	24 hour nitrate excretion (mmol)								
	<1		1 to <2		≥2		Total		p-value
	Number	%	Number	%	Number	%	Number	%	
Sample total	441		307		171		919		
Age group									<0.001
21 to 44	24	5.4	56	18.2	45	26.3	125	13.6	
45 to 64	42	9.5	51	16.6	27	15.8	120	13.1	
65 to 74	215	48.8	147	47.9	74	43.3	436	47.4	
75 to 84	128	29.0	40	13.0	20	11.7	188	20.5	
85+	32	7.3	13	4.2	5	2.9	50	5.4	
Male	185	42.0	154	50.2	82	48.0	421	45.8	0.070
Number of antihypertensive medications									0.647
None	321	72.8	234	76.2	129	75.4	684	74.4	
one	99	22.4	63	20.5	35	20.5	197	21.4	
Two plus	21	4.8	10	3.3	7	4.1	37	4.0	
Diabetes									0.653
Definite	47	10.7	26	8.5	16	9.4	89	9.7	
Probably	4	0.9	2	0.7	3	1.8	9	1.0	
Diastolic blood pressure, mmHg (means, sd)	84.3	9.1	81.9	9.5	80.5	9.9	82.8	9.5	
Systolic blood pressure , mmHg (means, sd)	147.1	20.3	141.0	21.2	136.6	20.5	143.1	21.1	

Table 2: Age sex and fully adjusted linear regression coefficients for systolic and diastolic blood pressure (measured in mm Hg) by 24 hour nitrate excretion groups (compared to <1 mmol)

	Age sex adjusted only			Fully adjusted models		
Nitrate excretion mmol/day*	Coefficient	95% CI	p-value	Coefficient	95% CI	p-value
Diastolic						
<1						
1 to <2	-1.35	(-2.69 to -0.01)	0.049	-1.51	(-2.87 to -0.16)	0.029
≥2	-1.94	(-3.48 to -0.39)	0.014	-2.28	(-3.83 to -0.72)	0.004
Systolic						
<1						
1 to <2	-1.29	(-4.09 to 1.52)	0.368	-1.4	(-4.27 to 1.47)	0.338
≥2	-3.41	(-6.56 to -0.26)	0.034	-3.87	(-7.06 to -0.69)	0.017

Note: *Sample numbers by nitrate excretion (mmol/day): <1 n=441, 1 to <2 n=307, ≥2 n=171