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Dietary nitrate supplementation improves reaction time in type 2 diabetes: Development and application of a novel nitrate-depleted beetroot juice placebo

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ABSTRACT

Background: In this substudy of the *effect of dietary nitrate on blood pressure, endothelial function, and insulin sensitivity in type 2 diabetes*, we report the development of a novel nitrate depleted beetroot juice for use clinical trials and determine if dietary nitrate supplementation improved cognitive function in patients with type 2 diabetes mellitus.

Methods: Beetroot juice was treated with the anion exchange resin Purolite A520e. UV–vis-spectrophotometry, and a blind taste test were performed along with determination of sugar content, measurement of ascorbate and dehydroascorbate, the ionic composition of juice and Proton NMR. Subsequently, 27 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 beetroot juice (nitrate content 7.5 mmol per 250 ml) or placebo (nitrate depleted beetroot juice nitrate content 0.002 mmol per 250 ml). At the end of each 2 week supplementation period cognitive function was assessed using E-prime, E-Studio software with 5 separate tests being performed. The tests utilised in the present study have been adapted from the Cambridge Neuropsychological Test Automated Battery (CANTAB).

Results: The differences in the UV–vis spectra were comparable to the natural variation found in differing cultivars. There were no discernable differences in taste, sugar content, or Proton NMR. Ascorbate and dehydroascorbate were undetectable in either juice. After 2 weeks of beetroot juice simple reaction time was significantly quicker in the active arm at 327 ± 40 ms versus 341.8 ± 52.7 ms in the placebo arm, mean difference 13.9 ± 25.6 ms (95% CI 3.8–24.0 ms), $p = 0.009$. No other measures of cognitive function differed between treatment arms.

Conclusion: We have developed an effective placebo beetroot juice for use in trials of supplementation of dietary nitrate. Two weeks supplementation of the diet with 7.5 mmol of nitrate per day caused a significant improvement in simple reaction time in individuals with T2DM.

1. Introduction

Individuals with type 2 diabetes have measurable deficits in cognitive function when compared to age and sex matched populations [1]. The risk of developing dementia may be twice as high in individuals with diabetes compared with the background population [2]. This accelerated decline in cognitive function may have several potential mediators: hyperglycaemia, dyslipidaemia, genetic factors, and microangiopathy [3].

Improvements in glycaemic control have produced mixed results. One small trial in elderly subjects with type 2 diabetes, two weeks intensive management of glycaemic control resulted in a non-significant trend to improvement in reaction time and significant improvements in other cognitive measures [4]. Ryan et al. [5] found an improvement in working memory following significant reductions in fasting plasma glucose levels following a 24 week intervention with either glyburide or rosiglitazone but no differences in reaction time, spatial memory or rapid processing tests. In the largest study of the effect of glycaemic control on cognitive function a subset of the participants from the ACCORD trial underwent cognitive assessment and MRI scanning to determine total brain volume at baseline, at 20 months and 40 months [6]. While there was a significant reduction in the amount of total

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brain volume lost at 40 months in the intensive glycaemic control group compared with the standard glycaemic control group there was no difference between groups in cognitive performance.

Vascular dysfunction is thought to be an aetiopathogenic factor in Alzheimer's disease as well as vascular dementia [7]. The plasma nitrite concentration is becoming established as a marker of endothelial nitric oxide (NO) production and therefore indicative of vascular health [8]. Thus plasma nitrite may be a sensitive marker for risk of cognitive decline. The potential relationship between plasma nitrate and nitrite and cognitive function has been little studied. Two groups have found plasma NOx (total nitrate plus nitrite) was reduced in patients with dementia (Alzheimer's, vascular, and mixed) [9] or Alzheimer's dementia compared with healthy controls [10]. Another group [11] found a small but statistically significant increase in plasma NOx in patients with Alzheimer's dementia compared to healthy controls.

Endothelial function determined by brachial artery flow mediated dilation is impaired in individuals with Alzheimer's disease [7]. This impairment in endothelial function is present in younger individuals with vascular risk factors and is associated with poorer cognitive performance in the absence of overt clinically detectable cognitive impairment [12].

High levels of vegetable consumption and in particular green leafy vegetables appear to slow the rate of age related cognitive decline [13]. These beneficial effects are often attributed to a variety of anti-oxidants or vitamins. However in a group with cardiovascular disease or risk factors for cardiovascular disease supplementation of vitamin E, C, or beta-carotene did not alter the rate of slowing of cognitive function over time [14]. It has been postulated that it may be the vascular actions of inorganic nitrate in green leafy vegetables which mediate this protective effect [15,16].

Inorganic nitrate from the diet is involved in a complex cycle with nitrite and nitric oxide. Briefly, nitrate is rapidly and completely absorbed from the stomach and small intestine [17]. From the circulation, it is then concentrated in the salivary glands and secreted into the mouth. Bacteria residing in crypts on the dorsum of the tongue use nitrate rather than oxygen as an alternative electron acceptor [18]. In the process nitrate is reduced to nitrite, which is again swallowed. Some of this nitrite will further be reduced to nitric oxide (NO) in the acidic environment of the stomach with important localised effects [19]. Some nitrite will be absorbed into the circulation where, via a number of mechanisms, it is reduced to NO in the vasculature [20]. This may modulate vascular tone and a number of cell signalling pathways. NO generated in this way and from the nitric oxide synthase family of enzymes which utilise L-arginine and O_2 as substrates is rapidly oxidised to nitrate by oxyhaemoglobin. This nitrate is thought to act as a stable storage reservoir of NO's bioactivity [15].

Dietary nitrate supplementation has previously been shown to lower blood pressure [21,22], improve endothelial function [21], improve exercise tolerance [23], enhance muscle contractile efficiency and force production [24,25], modulate gastric blood flow [26], protect against ischaemia reperfusion injury [27], inhibit platelet aggregation [21], and potentially play a key role in host defence [28]. For review see [29]. There is some evidence that dietary nitrate supplementation may alter cerebral blood flow [30]. Presley et al.'s study [30] suggested that acute dietary nitrate supplementation increased blood flow in cerebral white matter, notably in the dorso-lateral prefrontal cortex and anterior cingulate cortex, areas associated with executive function. There was no change in overall cerebral blood flow. In the central nervous system neuronal activity is tightly coupled to local blood flow. This association is the cornerstone of investigational imaging techniques such as fMRI [31]. Nitric oxide, whether derived from eNOS or nNOS, is one of the key mediators of this relationship [32]. In a rat model, Piknova and colleagues [31] showed inorganic nitrite can restore neurovascular coupling where it has been disrupted by inhibitors of nNOS. Further evidence that dietary nitrate supplementation may be neuro-protective comes from a series of *in vitro* experiments by Gladwin's group [33] which have demonstrated that neuroglobin may function as a redox sensitive nitrite reductase which may protect neurons during oxidative stress.

We tested the hypothesis that supplementation of the diet with inorganic nitrate via sequential reduction to nitrite and NO would augment cognitive performance in subjects with T2DM.

2. Materials and methods

The data presented are from a sub-study of a trial examining the effect of dietary nitrate on blood pressure, endothelial function and insulin sensitivity in type 2 diabetes, where additional details on methods can be found [34].

This randomised double blind placebo controlled crossover trial was approved by the Devon and Torbay Research Ethics Committee (study No. 09/H0202/43). All studies were conducted in accordance with the Declaration of Helsinki.

Participants were identified from the Exeter 10000 (EXTEND) bio-resource. This is a large cohort of well characterised individuals who have consented to being contacted about biomedical research projects. Twenty-seven participants (9F:18M) with T2DM as defined by WHO, of at least five years duration were recruited.

Using a double blind, randomised placebo-control crossover design, participants were randomised to begin in either order beetroot juice (nitrate content 7.5 mmol per 250 ml) or placebo (nitrate depleted beetroot juice nitrate content 0.002 mmol per 250 ml). Subjects were instructed to consume one 250 ml bottle per day, for 14 days with cognitive function testing occurring at the end of the supplementation period. Subjects then entered a four week washout period before entering the opposing arm of the study. Throughout the study patients were asked to maintain their normal diet apart from the juices given and not to change any other lifestyle factors. Participants continued their usual antihypertensive medication and their usual hypoglycaemic medications.

2.1. Placebo production

The placebo juice was produced by passing beetroot juice through a column containing the anion exchange resin Purolite a520e which exchanges nitrate for chloride. As it is widely used in the treatment of water for human consumption, it was considered that the resin may offer a methodology suitable for the depletion of nitrate in beetroot juice.

Nitrate concentration was determined by ozone chemiluminescence as per for plasma samples.

2.1.1. UV–vis spectrophotometry

Relative absorbance in the UV–vis light spectrum was determined spectrophotometrically using a Varian Cary 300 spectrophotometer. Samples were diluted 1/10 for analysis.

2.1.2. Taste test

A convenience sample of 10 members of staff from our institute agreed to participate in a blind taste test. They were randomly assigned to consume both juices in either order with a one week gap to simulate the conditions of the larger trial. Subjects were asked to determine the order of the juices they received.

2.1.3. Sugar content

The predominant carbohydrate in beetroot juice is sucrose [35] (sucrose concentration was determined by Armanda Pinhoe, James