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Original article

Salivary nitrite production is elevated in individuals with a higher abundance of oral nitrate-reducing bacteria

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ABSTRACT

Nitric oxide (NO) can be generated endogenously via NO synthases or via the diet following the action of symbiotic nitrate-reducing bacteria in the oral cavity. Given the important role of NO in smooth muscle control there is an intriguing suggestion that cardiovascular homeostasis may be intertwined with the presence of these bacteria. Here, we measured the abundance of nitrate-reducing bacteria in the oral cavity of 25 healthy humans using 16S rRNA sequencing and observed, for 3.5 h, the physiological responses to dietary nitrate ingestion via measurement of blood pressure, and salivary and plasma NO metabolites. We identified 7 species of bacteria previously known to contribute to nitrate-reduction, the most prevalent of which were *Prevotella melaninogenica* and *Veillonella dispar*. Following dietary nitrate supplementation, blood pressure was reduced and salivary and plasma nitrate and nitrite increased substantially. We found that the abundance of nitrate-reducing bacteria was associated with the generation of salivary nitrite but not with any other measured variable. To examine the impact of bacterial abundance on pharmacokinetics we also categorised our participants into two groups; those with a higher abundance of nitrate reducing bacteria (> 50%), and those with a lower abundance (< 50%). Salivary nitrite production was lower in participants with lower abundance of bacteria and these individuals also exhibited slower salivary nitrite pharmacokinetics. We therefore show that the rate of nitrate to nitrite reduction in the oral cavity is associated with the abundance of nitrate-reducing bacteria. Nevertheless, higher abundance of these bacteria did not result in an exaggerated plasma nitrite response, the best known marker of NO bioavailability. These data from healthy young adults suggest that the abundance of oral nitrate-reducing bacteria does not influence the generation of NO through the diet, at least when the host has a functional minimum threshold of these microorganisms.

1. Introduction

NO is a multifunctional signalling molecule which is involved in various biological processes such as; host defence [1], regulation of mucosal blood flow and mucus generation [2], regulation of smooth muscle contraction [3], cerebral blood flow [4], glucose homeostasis [5], and mitochondrial function [6]. Ingestion of inorganic nitrate (NO_3^-) from sources such as green leafy vegetables and roots has been consistently shown to increase plasma and salivary [NO_3^-] [7] and augment NO bioavailability [8]. In this pathway, NO_3^- is rapidly absorbed in the upper gastrointestinal tract and enters the circulation [9] before it is subsequently concentrated in the saliva [10,11] and a proportion reduced to nitrite (NO_2^-). Salivary NO_2^- can be further reduced to nitric oxide (NO) in certain physiological conditions such as hypoxia or stored in the blood and tissues for use when endogenous production of NO via NO synthases (NOS) is limited [12]. As a consequence, ingestion of inorganic NO_3^- may elicit a myriad of positive biological effects likely mediated by an increased NO bioavailability. Some studies have demonstrated that ingestion of NO_3^-- rich beetroot juice can reduce blood pressure (BP) [13], enhance endothelial function [14], protect against ischaemic injury [15], and improve exercise performance [16] although these effects are not consistently observed [17–19].

The reduction of NO_3^- to NO_2^- in saliva is achieved through the

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action of certain microbes which reside in the oral cavity [20,21]. The whole human microbiome is characterised by body site-specific microbial ecosystems capable of exerting effects on their host through production of metabolites, immune responses, and gene expression [22]. Some microbes live in symbiosis with their host and can significantly contribute to health [23,24]. Conversely, low diversity of microbial species resulting in dysbiotic states, have been linked to a number of adverse health conditions including; metabolic syndrome, allergies, asthma, obesity, and cardiovascular disease amongst others [25]. The oral cavity is heavily colonised by microbes and is one site where a symbiotic relationship between humans and bacteria is clearly evident.

A series of studies have confirmed the importance of commensal bacteria to the mammalian enterosalivary cycle, and NO bioavailability. These studies show consistently that rinsing the oral cavity with chlorhexidine anti-bacterial mouthwash disrupts bacterial enzymatic activity and abolishes the BP lowering effects associated with dietary NO_3^- ingestion [26–28]. Hyde and colleagues [21] recently analysed oral microflora from a small sample of healthy human participants (n = 6) and identified 14 bacterial candidate species that are thought to contribute to NO_3^- reduction. The majority of operational taxonomic units (OTUs) with NO_3^- reducing capability originated from the genera *Granulicatella, Actinomyces*, *Veillonella*, *Prevotella*, *Neisseria*, and *Haemophilus*. Other studies have also associated OTUs from the genera *Rothia and Staphylococcus* with NO_3^- reduction [20,29]. Despite emerging evidence linking NO_3^- reducing bacteria with cardiovascular homeostasis no study has explored the relationship between the abundance of NO_3^- reducing bacteria in the oral cavity and the capacity to process dietary NO_3^- in vivo. This is important because the conversion of NO_3^- from the diet to NO_2^- is known to be profoundly variable [30] and the abundance of NO_3^- reducing bacteria may be a rate-limiting step in this process.

Therefore, our primary objective was to perform descriptive analysis of the abundance and diversity of oral NO_3^- reducing bacteria in a larger cohort than previously utilised [21]. The secondary objective was to determine the association between the abundance of known NO_3^- reducing bacteria with cardiovascular variables and NO biomarkers in blood and saliva. A further objective was to determine whether participants with a higher abundance of NO_3^- reducing bacteria had different salivary and plasma NO pharmacokinetics following ingestion of dietary NO_3^- compared to those with a lower abundance.

2. Methods

2.1. Participants

Twenty five healthy adults (age 27 ± 7 years, stature 172 ± 9 cm, body mass 75 ± 15 kg, 11 female) volunteered and provided written informed consent prior to participating in the study. Ethical approval was provided by the School of Science Ethics Committee at The University of the West of Scotland. All participants were in good cardiovascular and oral health, did not report any habitual use of antibacterial mouthwash, were free from non-prescription medicines known to interfere with stomach acid production, and were not taking any prescribed medication. Cardiovascular health status was confirmed by completion of a medical questionnaire and The World Health Organisation's oral health questionnaire was used to ascertain oral health status. All procedures were conducted in accordance with the Declaration of Helsinki 1974 and its later amendments.

2.2. Experimental design

Each participant attended the laboratory on one occasion for this cross-sectional study. Prior to the trial, participants were briefed on procedures and provided with an adapted version of the National Institutes of Health daily food list. The questionnaire was adapted to differentiate between high, medium, and low NO_3^- containing foods [31]. Participants were asked to record their diet for seven days prior to arrival at the laboratory and instructed to maintain a normal dietary routine. Participants arrived at the laboratory on the morning of the trial in a fasted and euhydrated state after consuming 500 ml of water. Prior to the trial, participants were instructed to avoid strenuous exercise for 24 h and caffeine for 12 h. On the morning of the trial participants were requested not to brush their teeth or tongue and not to use mouthwash or chew gum. Participants provided verbal assurance of their compliance with these instructions.

2.3. Procedures

On arrival at the laboratory, stature and body mass were recorded. Participants then lay supine for the remainder of the experiment. During the first 30 min a cannula was inserted into one of the forearm veins and a tongue scrape sample collected. No other physiological measurements were collected for 30 min to ensure plasma $[NO_2^-]$ had stabilised following the change in body posture [32]. Following this initial phase, baseline measurements of BP and heart rate (HR) were recorded and samples of blood and saliva were collected. Participants then ingested 2 × 70 ml of NO_3^--rich beetroot juice (~12.4 mmol NO_3^-) (Pro-Elite Shot, James White Drinks Ltd., Suffolk, England) and physiological measurements were collected at regular intervals for the next 3.5 h (Fig. 1).

2.4. Blood collection

Venous blood was collected in 4 ml aliquots in tubes containing ethylenediaminetetraacetic acid (BD vacutainer K2E 7.2 mg, Plymouth, U.K.). Plasma NO_2^- has been shown to peak, on average, at 2.5 h after ingestion of beetroot juice [33] so multiple blood samples were taken before and after this point. Samples of whole blood were immediately centrifuged for 10 min at 4000 rpm at 4 °C (Harrier 18/80, Henderson Biomedical. UK) immediately following collection. The plasma was then separated into two cryovials and immediately stored at −80 °C for later analysis of NO_3^- and NO_2^- content via ozone-based chemiluminescence. The cannula was flushed with 2 ml sterile 0.9% saline immediately following blood draws to keep the line patent.

2.5. Saliva collection

Samples of unstimulated saliva were collected via an oral swab (Saliva Bio Oral Swab (SOS) Salimetrics, Pennsylvania, USA) placed under the tongue for 3 min. Samples of saliva were collected from 0.5 h onwards as previous data has shown salivary $[NO_2^-]$ and $[NO_3^-]$ may peak earlier than 1 h [28]. Swabs were then transferred to a collection tube (Sartedt, Aktiengesellschaft & Co, Numbrecht, Germany) and centrifuged at 4000 rpm for 10 min at 4 °C (Harrier 18/80, Henderson Biomedical. UK). Samples were then separated into two cryovials and immediately stored at −80 °C for later analysis of NO_3^- and NO_2^-.

2.6. Measurement of salivary and plasma $[NO_3^-]$ and $[NO_2^-]$

For the analysis of plasma NO_2^-, tri-iodide reagent comprised of 2.5 ml glacial acetic acid, 0.5 ml of 18 Ω deionised water, and 25 mg sodium iodide, was placed in a glass purge vessel heated to 50 °C and connected to the NO analyser (Sievers NOA 280i, Analytix, UK). A standard curve was created by injecting 100 μL of NO_2^- solutions at concentrations up to 1000 nM. Plasma and saliva samples were thawed in a water bath at 37 °C and 100 μL of the thawed sample was injected immediately into the purge vessel, in duplicate. Saliva samples were diluted with deionised water at a ratio of 1:100 before injection. NO_2^- content was calculated via the area under the curve using Origin software (version 7.1).

For the analysis of NO_3^-, vanadium reagent consisting of 24 mg of