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The relationship between plasma and salivary NO_x

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ABSTRACT

Several studies have shown that fasting plasma nitrite (NO_2^-) is an indicator of endothelial nitric oxide synthase (NOS) activity while plasma nitrate (NO_3^-) or the sum of NO_2^- and NO_3^- (NO_x) does not reflect NOS function. Plasma NO_2^- can also be elevated through dietary NO_3^- where the NO_3^- is partially reduced to NO_2^- by oral bacteria and enters the plasma through the digestive system. NO_3^- is taken up from plasma by salivary glands and the cycle repeats itself. Thus, one may propose that salivary NO_2^- is an indicator of plasma NO_2^- and consequently of NO production. Many brands of nitric oxide (NO) saliva test strips have been developed that suggest that their product is indicative of circulatory NO availability. However, data supporting a relationship between salivary and plasma NO_2^- or NO bioavailability are lacking. Here we have measured basal salivary and plasma NO_2^- and NO_3^- to determine if any correlation exists between these in 13 adult volunteers. We found no significant correlation between basal salivary and plasma NO_2^-. Also no correlation exists between salivary NO_3^- and plasma NO_2^-. However, we did see a correlation between salivary NO_3^- and plasma NO_3^-, and between salivary NO_2^- and plasma NO_3^-. In a separate study, we compared the efficiency of salivary NO_3^- reduction with the efficacy of increasing plasma NO_3^- and NO_2^- after drinking beet juice, a high NO_3^--containing beverage, in 10 adult volunteers. No significant correlation was observed between the *ex vivo* salivary reduction of NO_3^- to NO_2^- and plasma increases in NO_3^- or NO_2^-. These results suggest that measures of salivary NO_3^-, NO_2^- or NO_x are not good indicators of endothelial function. In addition, the efficiency of saliva to reduce NO_3^- to NO_2^- *ex-vivo* does not demonstrate one's ability to increase plasma NO_2^- following consumption of dietary NO_3^-.

1. Introduction

Determining a diagnostic marker of endogenous NO bioavailability has been a major topic of research, one which would have clinical implications for monitoring cardiovascular disease, metabolic syndrome, and other conditions [1–4]. Endothelial dysfunction has been noted as a key event in early atherosclerosis. Due to defective synthesis, decreased levels of endothelium-derived NO characterize endothelial dysfunction [5]. In addition, increased scavenging of NO by oxygen radicals results in low NO bioavailability associated with endothelial dysfunction [6,7]. Individuals with endothelial dysfunction show a decrease in flow-mediated dilation (FMD) and an increase in intima media thickness (IMT), both representative of atherosclerosis [8–11]. Given the cost of procedures to measure FMD and IMT, it would be useful to establish a simple blood test to diagnose endothelial dysfunction or low NO bioavailability due to other conditions. The Kelm and Moncada labs have demonstrated that the majority of plasma NO_2^- is derived from constitutive NOS-activity [12,13]. Observations have shown that upon regional nitric oxide synthase (NOS) inhibition in forearm circulation, vascular resistance increases linearly as plasma NO_2^- levels decrease, thereby establishing plasma NO_2^- as a potential measure of endothelial function [12].

In some of the same studies, plasma NO_3^- was ruled out as an indicator of NOS function. The Moncada lab showed that only 16% of isotopic L-arginine infused into circulation was represented in plasma NO_3^- levels *versus* 90% of plasma NO_2^- [13]. The Kelm laboratory demonstrated no significant change in plasma NO_3^- in mammals with inhibition of NOS activity [12]. These data are somewhat expected since plasma NO_3^- has many NOS-independent factors which can drastically change the basal levels such as dietary NO_3^- intake, denitrifying liver enzymes, and renal function [14,15]. Interestingly, Hibbs and colleagues showed that inducible-nitric oxide

Abbreviations: NOS, nitric oxide synthase; FMD, flow-mediated dilation; iNOS, inducible nitric oxide synthase; IMT, intima media thickness.

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synthase (iNOS) is one of the main contributors to circulating NO_3^- due to the increase of NO production after the addition of the cytokine-IL2 [16]. However, the general consensus is that, under most conditions, plasma NO_3^- does not reflect NOS function or NO bioavailability. In addition, there are even some investigators who suggest that plasma NO_2^- does not accurately reflect eNOS function and NO bioavailability [17].

In recent years, dietary NO_3^- has become a known contributor to the pool of bioavailable NO [18]. It is known that dietary NO_3^- is reduced in the oral cavity by tongue flora, specifically by *Actinomyces* and *Veilonella* species [19,20]. Once ingested, NO_2^- is non-enzymatically reduced to NO in the gastric acidic milieu [21]. NO_3^- and the remaining NO_2^- are rapidly absorbed in the small intestine. Plasma NO_2^- can then be reduced to NO by various mechanisms [18,22]. Although most of the circulating NO_3^- is excreted in urine, approximately 25% is extracted by the salivary glands and recycled through the enterosalivary circulation [23]. Complementary to endogenous NO production, this cycle of dietary NO_3^- being converted to NO in physiology is referred to as the nitrate–nitrite–nitric oxide pathway [18].

Through this physiological pathway, it has been shown that dietary NO_3^- will increase plasma NO_3^- and NO_2^-. In addition, dietary NO_3^- has been shown to lower blood pressure with short and long term effects, be vasoprotective and reduce platelet aggregation, along with having acute effects on cerebral blood flow and an increase in exercise tolerance and performance [18,24–30]. Daily dietary NO_3^- ingestion also improves endothelial function and vascular stiffness in hypercholesterolemia [31–34].

As evidence suggests that basal plasma NO_2^- levels reflect NOS function and bioavailable NO, these measurements may have clinical utility. However, based on the nitrate–nitrite–NO cycle, one may also suggest that salivary NO_2^- could have the same utility, as recently pointed out [1]. Indeed, commercially available products exist that measure salivary NO_2^- and claim to report NO bioavailability. However, until now, no published studies have shown a positive correlation between basal plasma NO_2^- and salivary NO_2^- levels. Thus, in this work, we sought to investigate the basal levels of plasma and salivary NO_2^- and NO_3^-.

When studying increased plasma NO_2^- after a high NO_3^- load, Lundberg et al. observed attenuation after using an antibacterial mouthwash [35], suggesting that saliva's conversion from NO_3^- to NO_2^- greatly affects plasma NO_2^-. Consumption of high NO_3^--containing food or drinks increases plasma NO_3^-, NO_2^- and thus NO_x. However, many studies have observed a significant variation with the increase in plasma levels among individuals [13,24,25,28,35]. It appears that some individuals are poor or non-responders with respect to dietary NO_3^- interventions as measured by increases in plasma NO_2^- [24,27,36,37]. It would be useful to easily determine individuals' efficacy at converting oral NO_3^- to plasma NO_2^-. In this study we hypothesized that saliva would reflect the ability to convert NO_3^- to NO_2^- in the oral cavity and that this ability would correlate with an individual's dietary NO_3^- to plasma NO_2^- conversion efficacy. Thus we conducted a second study where we examined both *ex-vivo* conversion of NO_3^- to NO_2^- in saliva and *in vivo* conversion of dietary NO_3^- to plasma NO_2^-.

2. Methods

2.1. Study design

All human subjects use was approved by an internal review board following federal and institutional guidelines. For the basal level study, 13 volunteers (8 male and 5 female) participated between the age of 18 and 80 years old. Volunteers reported to the lab at 9:00 am on the day of their participation. Individuals did not eat or drink within two hours of their participation. If the volunteers had eaten any food the morning of the sampling, they were told to avoid any high NO_3^- foods (*e.g.* spinach, beets, lettuce, and other green leafy vegetables). In addition, volunteers did not use mouthwash but were permitted to brush their teeth. Upon arrival, blood was drawn from each volunteer from an antecubital vein and collected in a 4 mL lithium heparin vial. Simultaneously, volunteers expectorated 5 mL of saliva which was collected in a sterilized 50 mL Corning tube.

The beet juice study was ancillary to a larger study aimed at investigating potential additional benefits of beet juice combined with supervised exercise compared to supervised exercise alone. The larger study provided a great opportunity for the ancillary one discussed here to compare *in vivo* conversion of oral nitrate to plasma nitrite and *ex vivo* salivary conversion efficiency. Ten participants (5 male and 5 female) above the age of 55 were recruited. All recruits consumed a bottle of concentrated beet juice (Beet it Sport shot, 500 mg NO_3^-) a day for 6 weeks. On the first day of weeks 1, 3 and 6 participation, each recruit came in for sampling. As described for the study earlier, volunteers did not use mouthwash or eat any high NO_3^- foods but were permitted to brush their teeth. Blood was drawn before and 1 hour after beet juice consumption. Immediately before the blood draw, participants expectorated a 5 mL saliva sample into a 50 mL sterilized Corning tube. One plasma sample was excluded due to hemolysis during sample preparation. In addition, two anaerobic saliva samples were excluded due to having dried out during the deoxygenation procedure.

2.2. Measurements

Since salivary NO strips claim to be indicative of physiological NO, we sought to determine what these strips actually test in saliva. Two brands of NO test strips, Nitric Oxide Test Strips (Berkeley Test) and Nitric Oxide Indicator Strips (Neogenis; Austin, TX), were placed in solutions of $NaNO_3^-$ and $NaNO_2^-$. Since the strips have a colorimetric indicating tip, concentrations were varied in order to darken the strip with more reactant.

Similar measuring techniques were used for both basal and beet juice studies. Blood was taken from an antecubital vein and collected in a 4 mL lithium heparin vial. The tubes were immediately centrifuged at 11,500 *g* for 2 min. The supernatant plasma was removed and immediately frozen on dry ice in aliquots of ~0.4 mL of plasma and stored in a –80 °C freezer. Plasma NO_3^- and NO_2^- were determined and labeled "basal levels." For the beet juice studies, plasma NO_2^- levels were determined in the before- and 1 hr after-beet juice consumption timepoints. The difference was reported as the Δ plasma NO_2^-.

Plasma NO_3^- and NO_2^- levels were measured using an HPLC-based Eicom NOx Analyzer, model ENO-20 according to the instructions of the manufacturer. For all measurements, standard curves were obtained and used for quantitative measurements.

For the basal level study, each tube of saliva was centrifuged at 11,500 *g* for 5 minutes to exclude a large pellet of bacteria. The supernatant salivary matrix was removed and immediately frozen on dry ice in aliquots of ~1.0 mL of saliva until time of analysis. Freezing saliva is a method tested and employed by our lab which maintains the integrity of the sample after exclusion of the bacterial pellet [38,39]. Before instrumental analysis, saliva was thawed and mixed 1:1 by volume with methanol for deproteination (a method tested which maintains NO_3^- and NO_2^- but allows for a cleaner sample to eliminate possible syringe clogging of the HPLC system of the NO_x analyzer). Salivary NO_3^- and NO_2^- were determined and labeled "basal levels."

For the beet juice study, each tube of saliva (5 mL) was immediately split into two samples, one designated for aerobic testing and the other for anaerobic testing. Both samples were placed in a round bottom flask and left to incubate in a 37 °C water bath. The