Analysis of Nitrate/Nitrite Concentration in Blood Plasma Pre and Post Nitrate Supplementation

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Introduction

Porphyromonas gingivalis is the keystone pathogen found in patients with periodontitis and produces toxic cysteine proteases called gingipains (Dominy et al. 2019). Levels of this bacterium have also been found in the brains, specifically the hippocampus, of patients with Alzheimer's disease suggesting that *P. gingivalis*, and its expressed gingipains, is involved in the pathophysiology that leads to cognitive decline in this disease (Dominy et al. 2019). Dominy et al. (2019) found that directly inhibiting gingipain expression decreases the colonization of the brain by *P. gingivalis*, therefore, reducing neurodegeneration in Alzheimer's disease.

Another mechanism that may provide similar effects to that of gingipain inhibitors is the administration of a dietary nitrate supplement. It is suggested that the oral microbiome is influenced by nitrate and the salivary composition can be altered via dietary nitrate supplementation (Vanhatalo et al. 2018). The nitrate-nitrite-nitric oxide pathway relies on bacteria within the oral microbiome to convert nitrate to nitrite, and this pathway is thought to be an important contributing factor to overall body health (Vanhatalo et al. 2018). Likewise, Rosier et al. (2020) suggested that nitrate is a health-associated molecule and could be used as a dietary intervention to promote eubiosis within the oral cavity. Their study confirmed that the abundance of *P. gingivalis* in the oral cavity significantly decreases post nitrate supplementation (Rosier et al. 2020).

Upon nitrate ingestion, up to 25% enters enterosalivary circulation where it becomes concentrated in the saliva and anaerobic bacteria in the oral cavity are responsible for the reduction of nitrate to nitrite (Wylie et al. 2013). When the nitrate enters the stomach, the acidic environment converts some of the nitrite into nitric oxide (NO) and the rest is absorbed to increase nitrite concentration in the circulating blood plasma (Wylie et al. 2013). NO is synthesized from l-arginine by isoforms of the NO synthase and is involved in several important biological functions including vascular tone regulation, immune response, and neurotransmission (Romitelli et al. 2007). Dietary nitrate supplementation has shown to increase nitrite concentration in blood plasma and serum and reduce resting blood pressure (Jones 2014).

It is difficult to determine NO content in blood plasma due to its short half-life in circulation, as well as its reactivity with oxygen species and biological molecules such as dioxygen, superoxide anion, and oxyhemoglobin to form products like nitrate and nitrite (Romitelli et al. 2007). Nitrate and nitrite are the most stable metabolites of NO; therefore, quantification of these inorganic molecules can allow for the indirect determination of NO content in blood (Romitelli et al. 2007).

Presently, Griess assays are most commonly used in clinical and experimental studies due to protocol simplicity, efficiency, and cost effectiveness (Romitelli et al. 2007; Ghasemi et al. 2007). Deproteination of the plasma and serum samples is a critical step in this assay in order to determine nitrate and nitrite concentrations (Romitelli et al. 2007; Ghasemi et al. 2007).
In a study conducted by Romitelli et al. (2007), methods for quantifying nitrite/nitrate levels in human plasma and serum were compared. One of the methods that was observed was the Griess reaction, and six different deproteination methods for this assay were looked at to assess and evaluate their efficiency (Romitelli et al. 2007). It was found that sample treatment with acetonitrile was the most efficient in precipitating proteins and eliminating their interference with the Griess reaction (Romitelli et al. 2007). Similarly, Ghasemi et al. (2007) evaluated several protein precipitation methods to determine NO concentration in serum samples. It was found that both acetonitrile and zinc sulfate proved to be the most effective at protein removal (Ghasemi et al. 2007).

Objective

This project is a part of a larger study currently being conducted by an honours student of Dr. Rakobowchuk and Dr. Bottos that analyzes the effect of nitrate supplementation on the abundance of *Porphyromonas gingivalis* in the oral microbiome. The main objective of this project is to analyze the nitrite concentrations in blood plasma prior to and after the administration of a nitrate supplement. The central question being asked is how the nitrite concentrations in blood plasma are being affected after the administration of a nitrate supplement. Zinc sulfate will be used to deproteinize the samples, and vanadium (III) chloride will mediate the reduction of nitrate to nitrite for detection by an acidic Griess assay.

Upon completion of this study, nitrite concentrations of the blood plasma samples pre and post nitrate supplementation will be provided for the larger study to allow for statistical analyses comparing other secondary measurements including blood pressure and flow-mediated dilation.

Materials and Methods

Experimental design

The experimental design consists of, first, the deproteination of blood plasma samples and, second, a Griess assay. There are a total of twenty blood plasma samples – ten samples are pre-intervention of the nitrate supplement and ten samples are post-intervention. The experiment will be run in triplicate. Experimentation will take place in the microbiology laboratory and the human physiology laboratory. The null hypothesis of this project is that there is no difference in nitrite concentration in the plasma samples pre and post nitrate supplementation.

Deproteination of samples

Zinc sulfate will be added to 400 µL of plasma. Samples will be vortexed for one minute and centrifuged at 10,000 x g for ten minutes at 4 °C. The supernatant will then be collected for use in the Griess reaction.

Griess Reaction

The supernatant collected from each sample will be aliquoted into respective wells of a 96-well microtiter plate. Supernatant will also be spiked with 200 µM $NaNO_2$ and added to the microtiter plate. For the reduction of nitrate to nitrite, vanadium (III) chloride (8 mg/mL) will be used. Sulfanilamide (2%) and N-(I-Naphthyl) ethylenediamine dihydrochloride (0.1 %) will be added respectively to each well. The plate will then be incubated in a microplate reader for thirty minutes at 37 °C, and absorbance values will be read at 539 nm.

Data Analysis

Data analysis will be carried out using a microplate reader to determine absorbance and nitrite concentration, as well as intra-assay measurements including percent recovery. All samples were spiked with a known amount of nitrate (32 µM) to provide a recovery value for this time-sensitive assay protocol. Standard curves will then be produced comparing the nitrite concentrations and absorbance.

Results

As demonstrated in Figure 1, there was no significant difference in plasma nitrate/nitrite concentration pre- and post-nitrate supplementation.

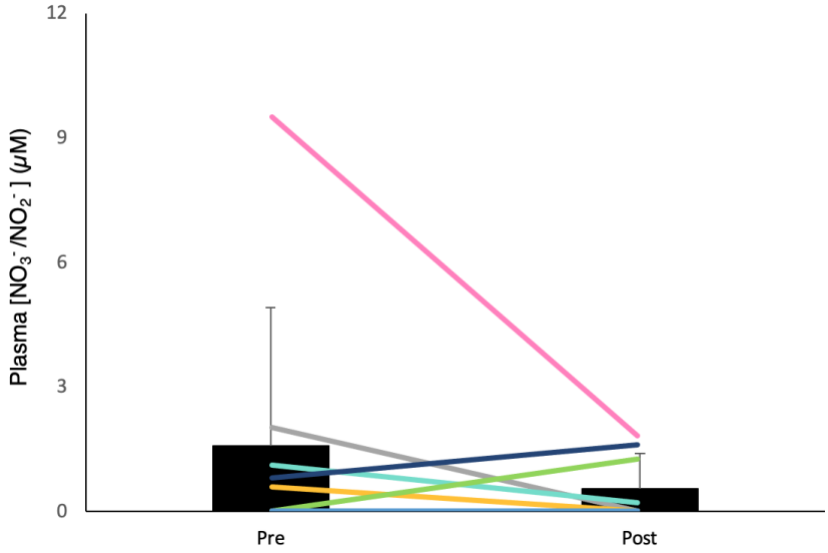

Figure 1. Plasma [NO_3^- /NO_2^-] for each of the participants pre- and post-nitrate supplementation as well as the average concentrations (n=10).

Figure 2 shows the standard curve of the Griess assay. Concentrations of plasma nitrate/nitrite were calculated using absorbance values and the standard curve.

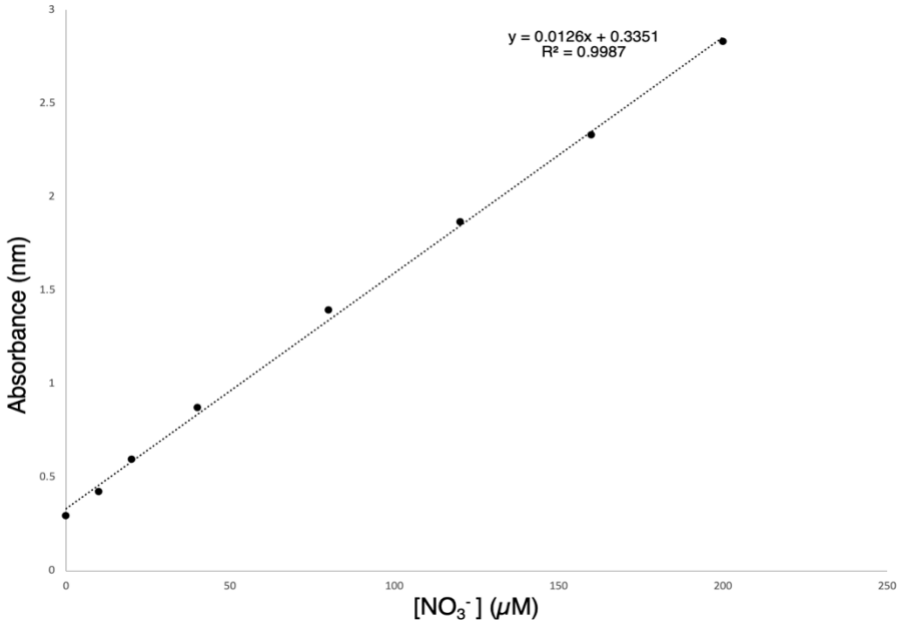

Figure 2. Standard curve of the Griess assay (n=8).

Percent recovery indicates proficiency with this time-sensitive assay although sensitivity within the range of concentrations measured in the blood samples is limited.

Table 1. Plasma nitrate/nitrite recoveries pre and post supplementation based on the standard curve of the Griess assay (Figure 2) as compared to plasma samples spiked with nitrate.

Participant	Percent Recovery of NO_3^- /NO_2^-
1	Pre: 91.37 %
	Post: 105.51 %
2	Pre: 96.58 %
	Post: 104.51 %
3	Pre: 97.35 %
	Post: 73.88 %
4	Pre: 88.79 %
	Post: 102.28 %
5	Pre: 93.11 %
	Post: 94.72 %
6	Pre: 86.19 %
	Post: 89.53 %
7	Pre: 82.84 %
	Post: 94.62 %
8	Pre: 55.31 %
	Post: 57.42 %
9	Pre: 82.44 %
	Post: 92.01 %
10	Pre: 96.70 %
	Post: 93.73 %

Discussion

In this study, zinc sulfate was used to deproteinize the blood plasma samples, and vanadium (III) chloride mediated the reduction of nitrate to nitrite for detection by an acidic Griess assay. It is thought that the oral microbiome is influenced by nitrate and the salivary composition can be altered via dietary nitrate supplementation (Vanhatalo et al. 2018). Nitrate/nitrite levels in the blood plasma were assessed pre- and post-nitrate supplementation. The results suggest that plasma nitrate/nitrite concentration were not significantly different before compared to after dietary supplementation ($p > 0.05$). Similar plasma nitrate/nitrite concentrations were seen between participants pre- and post-nitrate supplementation. Percent recovery values indicate proficiency with this time-sensitive assay although sensitivity within the range of concentrations measured in the blood samples is limited.

It has been previously demonstrated that the kinetics of nitrate/nitrite in the blood are rather fast in young, healthy adults (Wylie et al. 2013). A study conducted by Wylie et al. (2013) showed, at a similar dosage to the study presented here, a return of plasma nitrate concentration to baseline over time with the administration of 4.2 mmol of nitrate by twelve hours. This suggests that nitrate/nitrite concentration returned to baseline following acute administration (Wylie et al. 2013). Another possible reason for the lack of an effect of dietary supplementation on plasma nitrate/nitrite concentrations is the source of nitrate used. A potassium nitrate supplement was used in the current study whereas many other studies, including the study by Wylie et al. (2013) mentioned above, used beetroot juice as the nitrate source. There may be an additive effect of nitrate present in another compound within beetroot juice (Sundqvist et al. 2020).

Time course with potassium nitrate supplementation should be performed to determine exposure of the oral microbial community with nitrate/nitrite. Future studies should consider taking blood samples within one hour post nitrate supplementation as to obtain peak plasma nitrate response as demonstrated by Wylie et al. (2013). Additionally, salivary samples would provide more direct evidence of an altered oral environment capable of altering the microbial community.

Literature Sources

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