Analysis of 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 nitrous oxide concentration and percent recovery. The values of nitrite and nitrous oxide will be estimates of their concentration as some of the nitrite will be lost to nitrous oxide in the Griess reaction. Standard curves will then be produced comparing the nitrite concentrations and absorbance.

Permits

This study has received biosafety approval and has been approved by the Human Subjects Committee as well.

Expected Results

It is expected that the nitrite concentration in blood plasma will increase from pre to post nitrate supplementation. The results of this study will confirm that ingestion of a nitrate supplement stimulates the nitrate-nitrite-nitric oxide pathway to convert nitrate to nitrite which can subsequently be absorbed into the bloodstream (Vanhatalo et al. 2018; Wylie et al. 2013).

Timeline

This project will begin immediately upon approval. Blood sampling has already been completed and experimentation will take place from mid-January to mid-February. Data analysis will take place from mid-February to early March. Starting in mid-January I will begin writing my report and have it submitted by late March, at least three weeks prior to the end of the semester. I will also present a poster at the annual TRU Undergraduate Research and Innovation Conference in late March – early April. Factors due to the COVID-19 pandemic are the only obstacles that I can foresee slowing the progress of this project.

Expenses

The cost of the reagents and supplies used in this project will be approximately $200. Expenses for the directed study will be split between Dr. Rakobowchuk's Research Acceleration Grant and JHV NSERC Discovery Grant.

Literature Sources

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