The Effects of Antiseptic Mouthwash Use and Sodium Intake on Systemic Blood Pressure Regulation and Salivary Nitrate Levels: A Randomized Controlled Crossover Trial

by

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ABSTRACT

Background. Despite extensive research in the literature aimed at understanding the role of hypertension as a major risk factor for numerous leading causes of death in the United States, rates of this disease continue to rise. Recent findings suggest that antiseptic mouthwash use may increase blood pressure through elimination of oral bacteria that facilitate the enterosalivary nitrate-nitrite-nitric oxide pathway.

Objective. The purpose of this randomized, controlled, crossover trial was to examine the effects of antiseptic mouthwash use and sodium intake on blood pressure and salivary nitrate levels in prehypertensive adults.

Methods. Healthy adults (n=10; 47.3±12.5) with mildly elevated blood pressure (average baseline blood pressure of 114.9/75.2 mmHg) were recruited and were randomly assigned to a control condition, antiseptic mouthwash use, or antiseptic mouthwash use + consumption of three pickles per day (~6000 mg/day of sodium) for a total of 7 days. Given the crossover design of this study, participants adhered to a 1-week washout period between each condition and all participants received all three treatments. Findings were considered significant at a p-value of <0.05 and a repeated measures ANOVA test was used to compare change data of each condition.

Results. Changes in systolic and diastolic blood pressure were not statistically significant ($p=0.469$ and $p=0.859$, respectively). Changes in salivary nitrite levels were not statistically significant ($p=0.493$). Although there appeared to be fluctuations in sodium intake between interventions, differences in sodium intake were not statistically significant when pickles were not accounted for ($p=0.057$).

Conclusion. Antiseptic mouthwash use did not appear to induce significant changes in systolic or diastolic blood pressure in this population.

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CHAPTER 1

INTRODUCTION

According to the Centers for Disease Control and Prevention (CDC), currently one in three adults in the United States has hypertension (Fryar, Ostchega, Hales, Zhang, & Kruszon-Moran, 2017). Hypertension, or high blood pressure, is a major risk factor for both heart disease and stroke, which are leading causes of death in the U.S. and across the globe. Despite consistent efforts of health care providers to offer lifestyle recommendations and aggressive interventions with prescription medications, only about half (54%) of the 75 million people with hypertension in the U.S. have managed their high blood pressure (Fryar et al., 2017). Frequent blood pressure monitoring, both in the home and in the doctor's office, along with adherence to hypertension guidelines by the patient and physician are essential in the proper management of high blood pressure. Unfortunately, hypertension remains prevalent among U.S. adults and recent data has shown an increase in high blood pressure during childhood and adolescence (Fryar et al., 2017). Identifying behaviors that contribute to the onset of hypertension may provide insight into simple strategies that individuals can adopt as methods of blood pressure management.

It is well recognized in the scientific community that diet plays an important role in the prevention or promotion of hypertension and other chronic disease states. There is a general consensus in the literature that dietary sodium consumption is a significant contributor to the pathogenesis of hypertension, as well as a major risk factor for a number of other cardiovascular pathologies (Rust & Ekmekcioglu, 2016). It is estimated that dietary sodium consumption in the standard American diet ranges from 9 to 12 grams

per day, as compared to the World Health Organization's (WHO) recommendation to limit consumption to 5 grams per day and the CDC's recommendation to limit consumption to less than 2300 mg per day (Pilic, Pedlar, & Mayrommatis, 2016). Sodium homeostasis is regulated by the renin-angiotensin-aldosterone system (RAAS), a physiological mechanism of regulating sodium and water excretion or retention, causing an increase or decrease in blood pressure (Pilic et al., 2016). High sodium intake activates the RAAS, stimulating sodium and water retention and an increase in blood pressure (Pilic et al., 2016).

There are also dietary sources of nutrients that contribute to a decrease in blood pressure. Recently, there has been an influx of data that supports the consumption of leafy green vegetables, specifically those with a high concentration of inorganic nitrate (NO_3^-) such as lettuce and beetroot, as beneficial to cardiovascular health (Mcdonagh, Wylie, Winyard, Vanhatalo, & Johes, 2015). Reduction of dietary NO_3^- to nitrite (NO_2^-) is facilitated by facultative anaerobic bacteria within the saliva and is further reduced to nitric oxide (NO) after swallowing via a variety of mechanisms (Hord, Tan, & Bryan, 2009). Cardiovascular protecting effects are likely due to the vasodilating and blood flow regulating properties of NO once it has entered systemic circulation. This can lead to reductions in both systolic (SBP) and diastolic (DBP) blood pressure (Hord et al., 2009). However, evidence supporting the role of the oral microbiome in reducing inorganic NO_3^- to NO_2^- and NO indicates that the presence or absence of such bacteria may play a significant role in systemic blood pressure regulation (Bryan, Tribble, & Angelov, 2017). The elimination of oral bacteria through the use of antiseptic mouthwash may be contributing to increased blood pressure in susceptible individuals.

Although epidemiological and correlational data exists in the literature to support the notion that antiseptic mouthwash use increases blood pressure, few trials have explored the link between mouthwash use on blood pressure and salivary nitrate levels. To date, a randomized clinical trial that explores this link in prehypertensive adults, while simultaneously placing participants on a sodium diet regimen, has not been conducted. More research is needed to establish the effects of both diet and mouthwash use on blood pressure regulation. It is possible that discoveries regarding the relationship between the oral microbiome and cardiovascular health may provide a new therapeutic target for blood pressure management and chronic disease prevention.

Purpose of Study

The purpose of this randomized, controlled, crossover study was to examine the effects of antiseptic mouthwash use and sodium intake on blood pressure and salivary nitrate levels in a population of prehypertensive adults (>120/80 mmHg) from the Phoenix area.

Research Aim and Hypotheses

- Primary Aim: To determine the effect of antiseptic mouthwash use and sodium intake on blood pressure and salivary nitrate levels in prehypertensive adults from the Phoenix area.
- Primary Hypotheses:
    - Antiseptic mouthwash use combined with a high sodium diet will increase blood pressure to a greater degree than mouthwash use alone in prehypertensive adults in the Phoenix area.

- Antiseptic mouthwash use alone and combined with a high sodium diet will decrease salivary nitrate levels compared to baseline in prehypertensive adults in the Phoenix area.

Definition of Terms

- Hypertension: A state of abnormally high blood pressure, currently defined as a blood pressure higher than 130 (systolic) over 80 (diastolic) millimeters of mercury (mmHg) (American Heart Association [AHA], 2017).
- Prehypertension: A state of elevated blood pressure ranging from 120-129 (systolic) over greater than or equal to 80 (diastolic) millimeters of mercury (mmHg) and not currently on medications for blood pressure management (AHA, 2017).
- Nitrate (NO_3^-) / Nitrite (NO_2^-)/ Nitric Oxide (NO): Inorganic anions/signaling molecules involved in nitric oxide metabolism that influence a number of different physiological responses
- Vasodilation: The dilation of blood vessels, which results in decreased blood pressure
- Oral Microbiome: The collection of microorganisms that are found in the human oral cavity, which includes the lips, mucosa lining of the lips and cheeks, teeth, gums, mobile tongue, floor of the mouth below the tongue, and the hard palate or roof of the mouth

Delimitations

Participants who volunteered in this study were required to be healthy, non-smoking adults over 30 years of age who have blood pressure greater than 120/80 mmHg

but are not currently taking blood pressure management medications. Also, those included had no recent antibiotic use, were not currently taking any other systemic medication other than a contraceptive pill and had no history or recent treatment of oral conditions such as gingivitis, periodontitis, or halitosis.

Limitations

- Subjects may not adhere to the prescribed mouthwash use or the dietary sodium intervention.
- Self-reported dietary sodium intake may be inaccurate due to participant recording error.

CHAPTER 2

REVIEW OF LITERATURE

Hypertension has become one of the most important public health challenges in the United States (U.S.). According to the Centers for Disease Control and Prevention (CDC), 75 million Americans currently have high blood pressure (Fryar et al., 2017). This accounts for almost 30% of the population and approximately 1 in 3 adults. Additionally, hypertension has been strongly associated with the onset of various cardiovascular diseases and is one of the most crucial risk factors in the development of coronary artery disease, cerebrovascular disease, congestive heart failure, chronic kidney disease, and peripheral vascular disease (Institute of Medicine Committee on Public Health Priorities, 2010). As trends increase every year and the management of hypertension through pharmaceuticals continues to burden the lives of Americans, scientists have begun to discover a connection between the oral microbiome and blood pressure regulation. The oral microbiome and its role in nitric oxide homeostasis through the nitrate-nitrite-nitric oxide pathway has been linked to the management of hypertension (Bryan et al., 2017). The presence of such bacteria may play a role in the maintenance of normal blood pressure levels and serve as a potential therapeutic target for hypertension management (Bryan et al., 2017). More recently, it has been postulated that the elimination of oral bacteria through the use of antiseptic mouthwash products may be causing an increase in blood pressure (Bryan et al., 2017). Thus, the purpose of this four-week randomized, controlled, crossover trial is to examine the effects of using mouthwash on blood pressure and salivary nitrate levels. In order to support this study, topics that need to be explored include the cardiovascular mechanisms of hypertension,

the effects of dietary nitrates and nitric oxide on cardiovascular health, and the role of the oral microbiome in cardiovascular health management.

HYPERTENSION

Prevalence. According to the American Heart Association Guidelines published in 2017, hypertension is defined as having a systolic blood pressure equal to or greater than 130 mmHg and a diastolic blood pressure equal to or greater than 80 mmHg (AHA, 2017). In 2014, the U.S. Department of Health and Human Services published data from the National Health and Nutrition Examination Survey (NHANES) that explored the prevalence and control of hypertension among adults in the United States. According to this survey, the total prevalence of hypertension among adults was 29% and continued to increase with age up to 64.9% in ages 60 and over (Fryar et al., 2017). There is substantial evidence in the literature supporting racial/ethnic, geographic, and socioeconomic disparities in the prevalence of hypertension in the U.S. adult population. Hypertension is lower among non-Hispanic white (28.0%), non-Hispanic Asian (24.9%), and Hispanic (25.9%) adults than in Hispanic black (41.2%) adults (Fryar et al., 2017). Over the last decade, the overall prevalence of hypertension has remained relatively unchanged, however, controlled hypertension has increased significantly to 53.0%, with adults aged 60 and over more likely to have controlled hypertension than adults aged 18 to 39 (Fryar et al., 2017). Additionally, the prevalence of controlled hypertension was lower among non-Hispanic black (48.5%), non-Hispanic Asian (43.5%), and Hispanic (47.4%) adults and highest in non-Hispanic white (55.7%) adults (Fryar et al., 2017). Because hypertension significantly increases the risk of developing cardiovascular disease, a number of initiatives have been conceived to increase public awareness of the

importance of blood pressure control. One such initiative is Healthy People 2020, which aims to attain longer lives free of diseases that are preventable and often lead to premature death (Fryar et al., 2017).

Healthy People 2020 Goals. Healthy People 2020 established hypertension goals for adults in the U.S. in an attempt to reduce the impact of hypertension on the risk for cardiovascular disease development. These goals include decreasing prevalence to 26.9%, raising hypertension treatment to 69.5%, and raising hypertension control to 61.2% (Egan, Li, Hutchison, & Ferdinand, 2014). Unfortunately, the feasibility of achieving these goals is questionable as the Healthy People 2010 goal of reducing hypertension prevalence to 16% was not achieved and higher hypertension control is not anticipated in the coming years (Egan et al., 2014). This is likely due to fewer resources and support for quality improvement in rural clinics and federally qualified health centers, as insurance and frequency of healthcare are among the strongest modifiable variables associated with untreated and uncontrolled hypertension (Egan et al., 2014).

Etiology. Hypertension is often classified into one of two categories: essential high blood pressure or secondary high blood pressure. Essential hypertension refers to high blood pressure with no apparent cause and secondary hypertension refers to high blood pressure related to another health problem. Strong evidence suggests that hypertension is most often a result of an underlying condition, specific risk factors, or as a side effect to certain medications (AHA, 2017). Conditions commonly associated with high blood pressure include chronic kidney disease (CKD), diabetes, obstructive sleep apnea, hormonal imbalances (i.e. thyroid disorders, Cushing's syndrome, hyperaldosteronism), and stress (AHA, 2017). Other risk factors include family history,

stiffening and narrowing of the arteries as a result of prolonged plaque build-up, race/ethnicity, overweight or obese weight status, alcohol and/or tobacco use, physical inactivity, and dietary sodium consumption. (AHA, 2017).

Pathophysiology of Hypertension. The pathogenesis of essential hypertension is highly complex and involves the interaction and cooperation of multiple organ systems and biochemical pathways. Although there are many interrelated factors that may contribute to hypertension, those most commonly observed include dietary sodium intake, obesity, insulin resistance, the renin-angiotensin system, and the sympathetic nervous system (Beevers, 2001). Genetic predisposition may also be a contributing factor to the pathogenesis of hypertension (Beevers, 2001).

Blood pressure is a product of cardiac output and total peripheral resistance to flow. Normal blood pressure maintenance depends upon controlled cardiac output and controlled peripheral resistance. Cardiac output refers to the amount of blood pumped by the heart through the circulatory system each minute and peripheral vascular resistance refers to the flow of blood in peripheral arterial blood vessels (Beevers, 2001). Peripheral vascular resistance is mediated by vasoconstrictors and vasodilators. Vasoconstrictors are compounds that facilitate the contraction and subsequent narrowing of the muscular wall of the blood vessels, and vasodilators are compounds that facilitate the dilation and subsequent widening of the muscular wall of blood vessels. Common vasoconstrictors include endothelin [ET], angiotensin II [Ang II], and catecholamines (Foëx & Sear, 2004). Common vasodilators include nitric oxide [NO], prostaglandins, and kinins (Foëx & Sear, 2004).

Essential hypertension is usually caused by an imbalance in cardiac output and controlled peripheral resistance, most often as a result of normal cardiac output and increased peripheral vascular resistance (Beevers, 2001). However, it can result from both increased cardiac output and peripheral vascular resistance (Foëx & Sear, 2004).

Dietary Sodium Intake. Development of hypertension is largely determined by the quality of dietary intake. Therefore, nutritional intervention is warranted as a valid preventive strategy against the spread of hypertension. The effects of nutrients and foods on hypertension risk are well established, specifically with regards to dietary sodium intake. Sodium is a key nutrient that plays specific physiological roles, such as muscle contraction, fluid balance, and proper nerve conduction. However, sodium consumed in excess can result in poor cardiovascular outcomes. Excessive dietary sodium intake is acknowledged as a major risk factor for numerous cardiovascular pathologies including chronic kidney disease, cerebrovascular accidents, and most notably hypertension. This section will briefly review evidence that links excess sodium consumption with hypertension and cardiovascular disease.

In 2018, Dolmatova et al. explored the relationship between dietary sodium intake and hypertension among U.S. adults using NHANES data from 1999-2012. During this time, sodium intake was measured using 24-hour dietary recalls and was compared to adults greater than 20 years old who self-reported a diagnosis of hypertension. Over the 13-year study period, it was observed that sodium consumption increased 14.2% among adults with hypertension and was most notable among Hispanic and African-American study participants (Dolmatova, Moazzami, & Bansilal, 2018). It was concluded that sodium intake increased significantly among those with hypertension and aggressive

approaches to reduce our national sodium consumption is needed. (Dolmatova et al., 2018).

Increased dietary sodium intake has been significantly associated with increased central systolic blood pressure and aortic stiffness in young and middle-aged adults, as shown in a 2017 study by Muth et al. This controlled feeding cross-over study included 85 normotensive adults that received a 7-day low sodium diet intervention and 7-day high sodium diet intervention to assess effects of sodium intake on blood pressure and arterial stiffness. Findings indicated a significant increase in central systolic blood pressure in both the young and middle-aged groups after the high sodium diet intervention and increased forward and reflected wave amplitudes in the middle-aged group (Muth, Brian, Chirinos, Lennon, Farquhar, & Edwards, 2017). It was concluded that systolic blood pressure can be significantly impacted by increased levels of sodium consumption, specifically in adults 41-60 years of age (Muth et al., 2017).

Reduced dietary sodium intake, particularly in conjunction with a diet high in fruits, vegetables, and low-fat dairy products, has been linked to a decrease in blood pressure. The Dietary Approaches to Stop Hypertension (DASH) diet was designed to treat and prevent the onset of hypertension through the consumption of nutrient-rich foods. The New England Journal of Medicine published a large study in 2001 that observed the effects of the standard American diet versus the DASH diet in 412 participants. Findings indicated a significantly lower systolic blood pressure in those following the DASH diet, with a mean systolic blood pressure approximately 7.1 mmHg lower in participants without hypertension and 11.5 mmHg lower in participants with hypertension (Sacks et al., 2001). Researchers concluded that reduction of sodium intake

and DASH diet adherence lowers blood pressure substantially, with greater effects when adhered to in combination (Sacks et al., 2001).

Obesity. Epidemiological data indicates a strong relationship between obesity and hypertension. In combination, they are associated with high morbidity and high mortality rates. Prevalence of high blood pressure among obese people is greater than 60%, and accounts for 78% of incident hypertension in men and 64% in women (Demarco, Aroor, & Sowers, 2014). Hypertension prevalence also increases with BMI and is displayed in both men and women (Demarco et al., 2014). Progression from a state of normal blood pressure to one of hypertension is likely due to a combination of lifestyle, environmental, dietary, and genetic factors. However, the link between increased blood pressure with obesity is largely associated with excess weight gain. It is estimated that for every 5% increment in weight gain, the risk of developing high blood pressure increases between 20-30% (Demarco et al., 2014). An excess of visceral adipose tissue commonly seen in obese patients can lead to insulin and leptin resistance, which has also been associated with an increased risk for hypertension development and will be discussed further below.

Insulin Resistance. Metabolic abnormalities often co-exist with hypertension development. One such abnormality is insulin resistance, which can be defined as the inability of insulin to properly regulate glucose uptake and utilization by our cells. Insulin resistance typically precedes hypertension and decreases in arterial wall elasticity have been observed in patients who are insulin resistant (Wang, Han, & Hu, 2017).

The state of glucose metabolism is reflected from fasting insulin concentrations, and hyperinsulinemia is often used as a biomarker of insulin resistance. In a meta-analysis involving 10,230 patients with hypertension identified from a total pool of

55,059 patients, Wang et al. observed the relationship between insulin resistance and risk of hypertension. Findings from this meta-analysis suggest that fasting insulin concentrations and insulin resistance is associated with an increased risk of high blood pressure in the general population (Wang et al., 2017). It was concluded that early intervention of insulin resistance may serve as a tool in identifying patients at high risk for hypertension development (Wang et al., 2017).

Renin-Angiotensin System. The renin-angiotensin system (RAS) serves as a feedback mechanism that regulates sodium balance and extracellular fluid volume. Renin is a hormone responsible for converting angiotensinogen to angiotensin I (Ang I). Ang I is rapidly converted to Ang II, a vasoconstrictor mentioned previously that promotes blood vessel constriction and a subsequent increase in blood pressure (Yim & Yoo, 2008). Ang I is converted to Ang II by angiotensin converting enzyme (ACE). Ang II also stimulates the release of aldosterone, a hormone secreted from the adrenal glands that promote sodium and water retention (Yim & Yoo, 2008). Ang II and aldosterone work in combination to raise blood pressure. Inappropriate activation of RAS serves as an essential mechanism for development of hypertension, and introducing an anti-RAS regimen is often used as a therapeutic technique to lower blood pressure (Yim & Yoo, 2008). This class of medications is known as the ACE inhibitors, with examples including enalapril, lisinopril, and ramipril (Yang & Xu, 2017).

Sympathetic Nervous System. The sympathetic nervous system (SNS) also plays an important role in maintaining normal blood pressure. When the SNS is activated, it can cause arteriolar constriction and arteriolar dilation that leads to short-term alterations in blood pressure levels (Beevers, 2001). This usually occurs in response to physical

activity or increased stress levels. It remains unclear if the release of epinephrine and norepinephrine during this response plays a role in essential hypertension development, however medications that hinder the SNS response have been shown to provide therapeutic effects in lowering blood pressure (Beevers, 2001).

Lifestyle Modifications and Pharmacotherapies. Hypertension is currently managed through lifestyle changes and medications. Lifestyle changes that are often recommended include following the DASH diet, decreasing sodium consumption, maintaining a healthy weight, maintaining a regular physical activity regimen, and limiting or eliminating alcohol and tobacco use (AHA, 2017).

Medications approved to treat hypertension fall into the following categories: thiazide diuretics, ACE inhibitors, Angiotensin II receptor blockers (ARBs), and calcium channel blockers. Thiazide diuretics act on the kidneys to stimulate sodium and water elimination by inhibiting reabsorption of sodium and chloride ions, which subsequently reduces blood volume (Musini, Gueyffier, Puil, Salzwedel, & Wright, 2017). ACE inhibitors, as mentioned previously, block the conversion of Ang I to Ang II, thereby relaxing blood vessels through decreased cardiac output and blood vessel resistance (Musini et al., 2017). ARBs block the activation of Ang II after it has already been formed, which reduces production and secretion of aldosterone and causes vasodilation (Musini et al., 2017). Calcium channel blockers relax muscle cells of the blood vessels by reducing the contraction force of the heart and reducing aldosterone production, which also causes vasodilation (Musini et al., 2017).

Hypertension in Developing Countries. Hypertension continues to be a significant burden on populations around the world. Data from various national surveys

now supports the finding that there has been a considerable increase in cardiovascular disease globally, with hypertension being the most common (Tibazarwa & Damasceno, 2014). It is likely that the increasing prevalence of hypertension is due to increased urbanization, leading to a shift to poor dietary habits and increased social stress levels (Ibrahim, 2018). In addition to urbanization, developing countries have a number of other risk factors associated with hypertension including high rates of illiteracy, poverty, poor dietary choices, poor access to adequate healthcare, and high costs of pharmaceuticals (Ibrahim, 2018). Additionally, priority is often given to more acute illnesses, prevention of dangerous infectious diseases, and maternal and fetal health rather than to the prevention of chronic disease development (Ibrahim, 2018). While hypertension prevalence was almost unfounded among developing countries in 1940, it is now estimated that most of the global burden of hypertension occurs in developing countries that account for over 80% of the world's population (Ibrahim, 2018). It is essential in the coming decades that governments, along with the appropriate outreach organizations, work to provide preventive programs aimed at educating the public about modifiable risk factors for hypertension. According to a study published in the *Journal of Hypertension* in 2009 that aimed to review the quantitative differences in hypertension prevalence and treatment of hypertension in developed versus developing countries, scientists found that the prevalence, treatment, and control of hypertension in developing countries are now comparable to that of developed countries (Pereira, Lunet, Azevedo, & Barros, 2009). Unfortunately, it is expected that prevalence of hypertension in developed countries will decrease with a simultaneous increase in hypertension prevalence in developing countries in the coming years (Pereira et al., 2009).

NITRIC OXIDE

History and Discovery. Nitric oxide (NO) was first discovered in 1772 by Joseph Priestly as a simple and colorless gas consisting of one oxygen atom and one nitrogen atom (Yetik-Anacak & Catravas, 2006). In the 1980's, scientists began to investigate the mechanism of blood vessel dilation as a method of blood pressure control. While Robert Furchgott was studying the effects of acetylcholine on vasodilation, he discovered that blood vessel relaxation only occurred in the presence of endothelium-derived relaxing factor (EDRF) when the endothelium was intact (Furchgott & Zawadzki, 1980). EDRF, which promotes smooth muscle cell relaxation, was later proved to be NO (Yetik-Anacak & Catravas, 2006). This discovery revolutionized cardiovascular biology and provided new potential strategies for blood pressure management.

Function. NO is a signaling molecule that is involved in many physiological processes. It plays a pivotal role in the regulation of blood flow through the blood vessels and regulation of the cardiovascular system. Although NO also plays essential roles in maintaining healthy immune and nervous systems, this section will focus on NO's involvement in the vasculature.

There are three layers that make up the components of the vascular wall of blood vessels: the internal layer of endothelial cells, the medial layer composed of vascular smooth muscle cells, and the tunica externa, also called the adventitia (Zhao, Vanhoutte, & Leung, 2015). The intimal layer of endothelial cells lines the interior surface of all blood vessels and is the primary barrier between blood flowing through the vessel's lumen and other tissues (Zhao et al., 2015). The endothelium releases endothelium-derived NO, which plays a critical role in regulating blood vessel diameter (Zhao et al.,

2015). The medial layer of smooth muscle cells functions to regulate the constriction and dilation of the vasculature through mechanical or pharmacological stimulates (Zhao et al., 2015). The external adventitia functions to maintain cellular adherence to the surrounding tissues and provide a means of signal trafficking between the blood vessel and the surrounding organ (Zhao et al., 2015).

Within the blood vessel wall, NO is produced primarily through the endothelial nitric oxide synthase (eNOS) pathway, which will be discussed in detail in the next section. NO's primary role of vascular homeostasis and vasodilation is facilitated by its continuous low-level release from the endothelium (Bondonno, Croft, & Hodgson, 2016). This release of NO diffuses into the underlying smooth muscle cells, activating guanylate cyclase (sGC) that catalyzes the conversion of guanosine triphosphate into cyclic guanosine monophosphate (cGMP) (Bondonno et al., 2016). cGMP activates protein kinase G (PKG) and phosphorylates various downstream cellular targets that lower calcium concentrations, thereby promoting vascular relaxation and vasodilation (Bondonno et al., 2016). Decreased production and bioavailability of NO can result in different cardiovascular pathologies, most notably hypertension and atherosclerosis (Bondonno et al., 2016). In addition to vasodilation and maintenance of vascular tone, NO also inhibits proliferation and migration of smooth muscle cells, enhances endothelial cell proliferation, suppresses platelet aggregation, and prevents adhesion of leukocytes and monocytes to the endothelium (Lei, Vodovotz, Tzeng, & Billiar, 2013).

Production Pathways. There are two primary pathways that exist to produce NO that impact vasculature regulation. These pathways include the classical endothelial nitric oxide synthase (eNOS) pathway in which NO is produced from L-arginine and the more

recently discovered nitrate-nitrite-nitric oxide pathway as a method of enterosalivary nitrate metabolism.

eNOS is expressed in the endothelial cells in order to maintain blood vessel dilation, control blood pressure, and exert vasoprotective effects on the vascular wall. This isoform of NOS is primarily responsible for the production of NO that regulates vascular function (Zhao et al., 2015). L-arginine is the most well-known substrate for NOS and requires several co-factors in order to catalyze NO production, these including tetrahydrobiopterin (BH4), flavin mononucleotide, flavin adenine dinucleotide, calmodulin, and heme (Zhao et al., 2015). The NOS-catalyzed reaction from L-arginine to NO is found below:

$$2\ \text{L-arginine} + 3\ \text{NADPH} + 3H^{+} + 4O_2 \rightarrow 2\ \text{citrulline} + 2NO + 4H_2O + 3NADP^{+}$$

eNOS can be initiated in both calcium-dependent and calcium-independent mechanisms, which are activated by a variety of different stimuli such as shear stress, acetylcholine, bradykinin, and histamine (Zhao et al., 2015). Acetylcholine, bradykinin, and histamine are all agonists that increase calcium concentration within the cell, which binds to calmodulin to promote electron flux from the reductase domain of the enzyme to the oxygenase domain to produce NO (Zhao et al., 2015). eNOS can also be phosphorylated without an increase in calcium concentration, which facilitates the same electron flux from the reductase to oxygenase domain (Zhao et al., 2015).

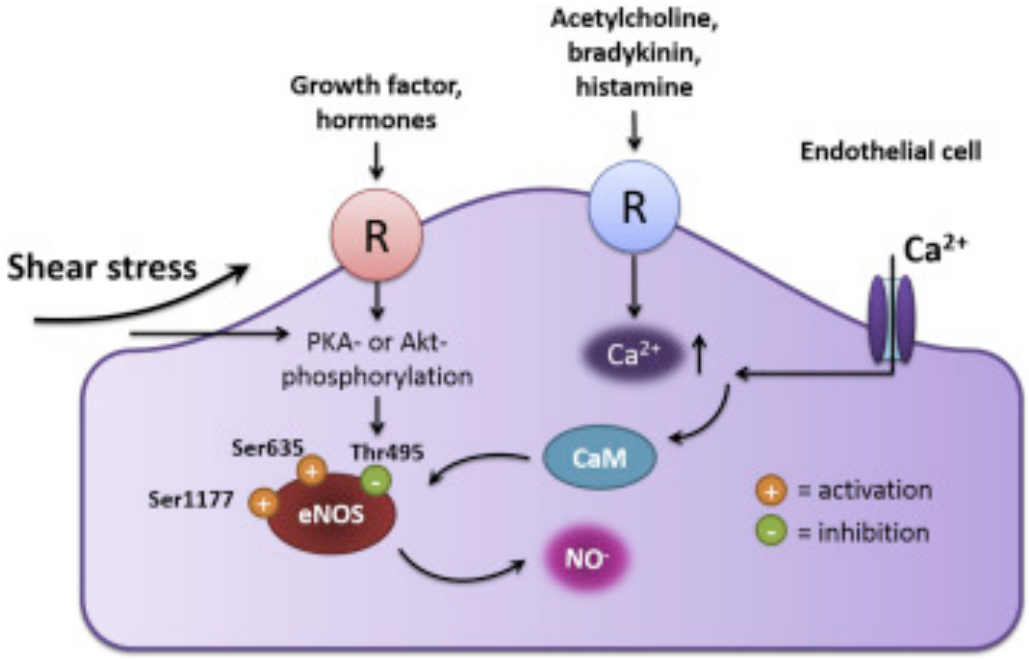

Figure 1. Endothelial Nitric Oxide Synthase Activation (Zhao et al., 2015)

This pathway can decline with age as the enzyme becomes less functional over time. A decrease in functionality can also occur due to physical inactivity, tobacco use, and poor diet (Zhao et al., 2015).

Polymorphisms in the eNOS gene do exist, and can influence regulation of blood pressure, vasodilation, and vascular tone. A recent meta-analysis consisting of over 63,000 subjects explored the correlation between the G894T and T-786C eNOS single nucleotide polymorphisms (SNP) that have previously been associated with an increased risk for hypertension (Xie, Shi, Xun, & Rao, 2017). Findings from the study suggest that the G894T and T-786C SNPs had a significant correlation with an increased risk for hypertension, especially for essential hypertension and gestational hypertension (Xie et al., 2017).

The other pathway is the enterosalivary nitrate-nitrite-nitric oxide pathway, which is more recently discovered and is dependent on oral nitrate-reducing bacteria. It was previously believed that nitrate and nitrite were inert end-products of NO derived from the eNOS pathway, however inorganic dietary nitrate and nitrite consumption is now considered to be another significant origin of exogenous NO that is bioavailable and can participate in various vasoactive signaling processes (Koch, Gladwin, Freeman, Lundberg, Weitzberg, & Morris, 2017). Nitrate from dietary sources requires an initial reduction to nitrate by a nitrate reductase enzyme that mammals do not produce on their own (Bryan et al., 2017). In the oral cavity, commensal facultative anaerobic bacteria serially reduce dietary nitrate to nitrite and NO, which provides the human host with bioactive form of NO that can be used downstream for vasodilation and blood vessel relaxation (Bryan et al., 2017). This bacterial nitrate reduction pathway will be discussed in further detail below.

Dietary Nitrate Sources. Over recent years, dietary nitrate consumption has become increasingly popular due to its effects on blood vessel dilation and exercise performance through the nitrate-nitrite-nitric oxide pathway (Lidder & Webb, 2013). Current known benefits of dietary nitrate consumption include a reduction in blood pressure, platelet aggregation inhibition, preservation of adequate endothelial function, and enhanced exercise performance in already healthy individuals (Jones, 2014). Newer studies are also beginning to reflect reduction in arterial stiffness, inflammation, and intimal thickness, as well as protection against ischemia-reperfusion injury as further benefits of dietary nitrate consumption (Lidder & Webb, 2013). Dietary nitrates are found to be particularly high in the Mediterranean and Japanese traditional cuisine, which

include high amounts of green leafy vegetables (Lidder & Webb, 2013). The consumption of lettuce, spinach, and other green leafy vegetables has been associated with a reduction in myocardial infarction incidence, coronary heart disease, and stroke (Lidder & Webb, 2013).

It is currently estimated that 85% of dietary nitrate consumed comes from vegetables and the remaining 15% comes from our drinking water (Lidder & Webb, 2013). Vegetables with the highest nitrate content include spinach, lettuce, radish, beetroot, and Chinese cabbage (Lidder & Webb, 2013). The nitrate content of vegetables can be largely influenced by environmental/agricultural factors such as humidity, temperature, sunlight exposure, water content during growth, nitrogen fertilization and fixation, and genetic factors such as nitrate reductase activity (Lidder & Webb, 2013). This section will explore the substantial evidence in the literature that indicates dietary nitrate consumption exhibits protective cardiovascular effects.

McDonagh et al. observed the effects of dietary inorganic nitrate supplementation on nitrate metabolism and subsequent blood pressure response in ten healthy normotensive adults. All subjects consumed equal doses of nitrate from concentrated beetroot juice, non-concentrated beetroot juice, a solid beetroot flapjack, a soluble beetroot crystal drink, and a control drink. Blood pressure, salivary nitrate/nitrite levels, and urinary nitrate/nitrite levels were measured before and after ingestion at various time increments. Results indicated that the concentrated beetroot juice was most effective in reducing blood pressure, however all conditions achieved elevated plasma nitrite concentrations (McDonagh, Wylie, Webster, Vanhatalo, & Jones, 2018).

In a 2012 double-blind, randomized, crossover study by Kelly et al., effects of short-term dietary nitrate supplementation in older adults on blood pressure, O2 uptake kinetics, muscle function, and cognitive function was studied. The study included 12 healthy, older adults that consumed either a nitrate-rich concentrated beetroot juice or a nitrate-depleted beetroot juice placebo for a total of 3 days. Blood pressure and plasma nitrite levels were measured before and after the intervention. Plasma nitrite levels were significantly increased after nitrate supplementation, and systolic and diastolic blood pressure were both significantly reduced (Kelly et al., 2012). There was also a speeding effect of nitrate supplementation on VO2 response time (Kelly et al., 2012). Findings from this study suggest that dietary nitrate can reduce blood pressure and improve oxygen uptake kinetics in healthy older adults.

Liu et al. explored the relationship between dietary nitrate consumption and arterial stiffness and blood pressure in 26 healthy adults aged 38-69 in a randomized controlled crossover trial. Two energy-matched meals were given to the subjects in random order that contained high levels of nitrates from spinach or low nitrates as a control meal. There was a significant increase in salivary nitrate and nitrite concentrations after the spinach meal (Liu et al., 2013). Additionally, significant results for higher artery elasticity, lower systolic blood pressure, and lower pulse pressure were observed after the spinach meal (Liu et al., 2013).

In 2016, Jonvik et al. assessed the impact of different nitrate-rich vegetables on blood pressure and plasma nitrate/nitrite concentrations in healthy adults. Using a semi-randomized crossover design, this study included 18 participants that consumed different beverages that contained equal concentrations of nitrates: beetroot juice, an arugula salad

beverage, and a spinach juice. Increases in plasma nitrate and nitrite concentrations and reductions in blood pressure were observed after consumption of all beverages, indicating that ingestion of various nitrate-rich vegetables can all be effective in improving cardiovascular health (Jonvik, Nyakayiru, Pinackers, Senden, JC van Loon, Verdijk, 2016).

Kapil et al. observed the blood pressure lowering effects of dietary nitrate in hypertensive patients in a randomized, double-blind, placebo-controlled study in 2015 as a follow-up to a study that demonstrated reductions in blood pressure after single dose dietary inorganic nitrate administrations in normotensive healthy volunteers. This study included 68 hypertensive adults that received daily nitrate supplementation in the form of beetroot juice or a placebo drink given as a nitrate-free beetroot juice. Clinic, ambulatory, and home blood pressure recordings were taken to assess changes in the nitrate group compared to placebo. Findings indicated an average blood pressure reduction of 7.7/2.4 mmHg in the clinic, 7.7/5.2 mmHg ambulatory, and 8.1/3.8 mmHg at home (Kapil, Khambata, Robertson, Caulfield, Ahluwalia, 2015). Additionally, arterial stiffness was reduced by an average of 0.59 m/s and endothelial function was improved by 20% in the nitrate group (Kapil et al., 2015). This provided some of the first evidence of positive cardiovascular effects after dietary nitrate supplementation in a hypertensive population.

This trend was observed again in a 7-day, double-blind, randomized, placebo-controlled, crossover trial by Kerly et al. in 2018. In this study, all subjects had treated, uncontrolled hypertension. Participants also received either a nitrate-rich beetroot juice or a nitrate-depleted beetroot juice for a total of 7 days, with ambulatory blood pressure taken before and after each condition. The nitrate group demonstrated significantly

increased plasma nitrite concentrations and significantly decreased 24-hour systolic and diastolic blood pressure compared to the placebo group (Kerley, Dolan, James, & Cormican, 2018). This data supported the existing evidence that dietary nitrate supplementation can induce an anti-hypertensive effect in patients with uncontrolled hypertension.

In another randomized, placebo-controlled, crossover study, Raubenheimer et al. investigated the effects of a nitrate-rich beetroot juice versus a nitrate-depleted beetroot juice on blood pressure, hemostasis, and vascular inflammation markers in 12 healthy older adults between 51-71 years. As in previous studies, significant increases in plasma nitrate/nitrites and significant decreases in systolic and diastolic arterial blood pressure were observed in the nitrate group (Raubenheimer et al., 2017). In this study, blood coagulation and vascular inflammation markers were also observed after the intervention and found reduced blood monocyte-platelet aggregation, reduced CD11b-expressing granulocytes, and slightly increased numbers of CD14++ and CD16+ monocytes after the nitrate-rich beetroot juice consumption (Raubenheimer et al., 2017). These findings suggest that dietary nitrate supplementation may also have an effect on platelets and various immune cells that result in decreased vascular inflammation (Raubenheimer et al., 2017).

It is important to note the distinction between plant-based and animal-based nitrates. Recent evidence has suggested that dietary nitrate consumption from processed meats has been linked to an increased risk for heart disease and various cancers of the digestive tract while nitrate-rich vegetables have been linked to improved cardiovascular

functioning (Bedale, Sindelar, Milkowski, 2016). These opposing views have sparked controversy regarding the perceived health benefits of increased nitrate consumption.

Nitrates and nitrites are commonly added to cured and processed meats such as bacon, salami, sausages, and other sandwich meats as a preservative. This is done in an effort to halt bacterial growth and maintain the freshness of food (Gassara, Kouassi, Brar, & Belkacemi, 2015). In contrast, leafy green vegetables are naturally high in nitrates. Dietary nitrate sources are reduced to nitrites by commensal bacteria that exist as part of the oral microbiome. These nitrites travel to the stomach, where they can then be converted into bioavailable NO or combine with secondary and tertiary amines to produce N-nitroso compounds (Gassara et al., 2015). These N-nitroso derivatives have further been linked to a wide range of cancers, thus indicating a potential harmful effect of nitrate consumption (Gassara et al., 2015). It is believed that dietary nitrate from plant sources contributes significantly to NO formation, whereas dietary nitrate from animal sources can combine with proteins and heme present in meat, leading to higher production rates of N-nitroso derivatives (Bedale et al., 2016).

ORAL MICROBIOME

Complexity of Microbial Composition. The oral microbiome is extremely complex and is comprised of various colonies of bacterial species that reside on our teeth, tongue, cheeks, tonsils, and other physical structures of the mouth. The human microbiome has received significant attention in recent times, however the focus has remained largely on gastrointestinal microbiota. Evidence continues to emerge regarding the composition of the oral microbiome and its role in human health.

Microbe discovery began in the 1700s, however oral bacteria first became apparent when Antonie van Leeuwenhoek examined dental plaque through a microscope and identified the first microorganisms of the mouth (Gao, Xu, Huang, Jiang, Gu, & Chen, 2018). With technological advances in microscopy, knowledge on the oral microbiome has significantly expanded and oral microorganisms have been found to colonize a variety of habitats within the oral cavity. The average adult oral cavity contains between 50 to 100 billion bacteria, contributing to an approximate 700 predominant oral bacterial species (Krishnan, Chen, & Paster, 2017). This estimation comes largely from culture-independent molecular studies, and the majority of oral bacterial taxa have not yet been grown in vivo (Krishnan et al., 2017).

The oral microbiome is especially unique because the bacteria are easily accessible and understanding their interactions with microbiomes in other locations of the body can expand upon existing data that supports it role in health improvement. The oral microbiome has been known to influence various factors associated with human health and can be influenced by time, age, diet, and extreme environmental exposure. Several studies discussed below elaborate on these factors.

In 2014, Costello et al. examined the variation in bacterial communities in different bodily habitats across space and time. The researchers surveyed bacteria from 27 different sites in 9 healthy adults across four different time periods. It was determined that bacteria are personalized, and that compositional variation is systematic across different habitats within the body (Costello, Lauber, Hamady, Fierer, Gordon, & Knight, 2009). These patterns suggest there are a variety of implications for medical practice, in

both therapies and preventive strategies, related to the functions of habitat-specific microbial species. (Costello et al., 2009).

Bacterial colonies have also been shown to be quite diverse among different age groups. In a comparative study of oral bacterial composition from healthy females selected at ages 8, 28, and 56, it was determined that colonization of specific bacterial species differed in different age groups (Anukan & Agbakoba, 2017). This study revealed that varying bacterial organisms may be associated with health and disease across the lifespan (Anukan & Agbakoba, 2017).

Lassalle et al. explored the relationship between commensal bacterial balance and pathogen load linked to diet in a population of hunter-gatherers and traditional farmers. Saliva samples were obtained from three different hunter-gatherer and farmer populations from different communities. Findings revealed that transitions in diet from different communities are likely a contributing factor to the emergence of oral pathogens that play a role in certain disease states (Lassalle et al., 2017).

In 1976, Brown et al. observed the effects of extreme environmental influence on intraoral microbial populations. Oral health factors were assessed from Skylab mission crew members before and after their flights. Pre-flight and post-flight samples of microbial populations indicated that specific anaerobic microflora (*Streptococci*, *Neisseria*, *Lactobacilli*, and *Enteric bacilli*) all demonstrated significant elevations, suggesting that extreme environment exposure can influence microbial populations within the oral cavity (Brown et al., 2976).

Oral microbial dysbiosis has also been linked to a number of whole-body systematic diseases. Because the oral cavity serves as the initial point of entry to the rest

of the gastrointestinal and respiratory tract, it has been associated with a number of gastrointestinal diseases including inflammatory bowel disease, liver cirrhosis, and various gastrointestinal cancers (Gao et al., 2018). There is also emerging evidence that links oral microbiomes to the health of the nervous system, with connections to Alzheimer's disease risk and other forms of dementia (Gao et al., 2018). Endocrine system diseases such as diabetes, polycystic ovary syndrome (PCOS), complications due to infertility, obesity, chronic low-grade inflammation, and insulin resistance have also been shown to be correlated to significant shifts in oral bacterial composition (Gao et al., 2018). Additionally, the oral microbiome is strongly linked to a variety of immunity functions and has been implicated in disorders such as rheumatoid arthritis and human immunodeficiency virus (HIV) (Gao et al., 2018). Although there is evidence to support the role of the oral microbiome in a variety of physiological systems, the primary interest of the oral microbiome in the following sections will be its implications to the cardiovascular system.

Bacterial Nitrate Reduction. One specific function of oral bacteria is to facilitate the conversion of dietary nitrate to nitrite in the mouth, a component of the nitrate-nitrite-nitric oxide pathway that has recently gained substantial recognition in the literature for its importance in cardiovascular health maintenance. Though the classical nitric oxide synthase/L-arginine pathway has been traditionally viewed as the primary method of NO production for the purpose of regulating blood flow and blood vessel dilation, studies are now showing that dietary nitrate consumption can be converted into nitrite by oral bacteria that is later reduced to a bioavailable form of NO (Bryan et al., 2017). This bacterial reduction through the nitrate-nitrite-nitric oxide pathway further facilitates

vasodilation and subsequent reductions in both systolic and diastolic blood pressure (Bryan et al., 2017).

In order for nitrate consumed through the diet to become bioavailable, it must first be reduced to nitrite and later reduced to NO. Humans lack the specific reductase enzymes capable of catalyzing this reduction (Bryan et al., 2017). Instead, this process is performed by facultative anaerobic bacteria present in the saliva (Bryan et al., 2017). Although almost 75% of nitrate ends up being excreted in the urine, there is still 25% that remains concentrated in saliva that can be reduced by the commensal bacteria colonizing the oral cavity (Lundberg, Weitzberg, & Gladwin, 2008). Salivary nitrate is reduced to nitrite through a two-electron reduction by nitrate reductase enzymes that occurs during anaerobic respiration of facultative anaerobic bacteria in the oral cavity (Bryan et al., 2017). This process is detailed in the figure below:

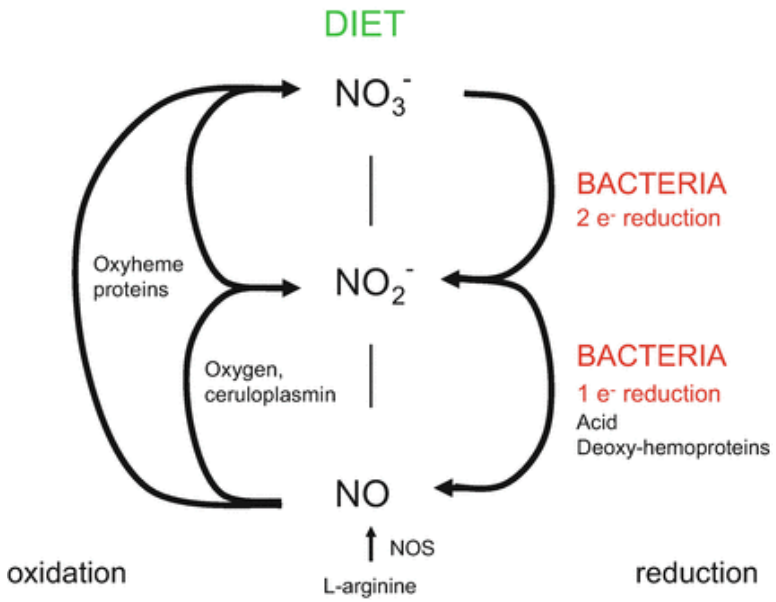

Figure 2. Bacterial Reduction of Nitrate (Bryan et al., 2017)

Analysis of bacterial communities from samples of tongue scrapings have identified 14 bacterial species with the highest rates of nitrate reduction activities: *Granulicatella adiacens*, *Haemophilus parainfluenzae*, *Actinomyces odontolyticus*, *Actinomyces viscosus*, *Actinomyces oris*, *Neisseria flavescens*, *Neisseria mucosa*, *Neisseria sicca*, *Neisseria subflava*, *Prevotella melaninogenica*, *Prevotella salivae*, *Veillonella dispar*, *Veillonella parvula*, and *Veillonella atypica* (Bryan et al., 2017). Of these species, *Veillonella* species from the tongue scraping samples were found to have the highest nitrate reducing effects (Bryan et al., 2017).

Eradication of Nitrate-Reducing Bacteria through Mouthwash Use. The cardiovascular protective effects of nitrate consumption and the method by which dietary nitrate is converted to a bioactive form of NO has been discussed in detail. NO homeostasis achieved through increased dietary nitrate consumption has been linked to reductions in blood pressure, vascular immunity, and vascular regeneration in healthy and hypertensive adults. However, the absence of commensal oral bacteria that facilitate nitrate reduction to NO may eliminate these beneficial effects. The eradication of these bacteria through mouthwash use may hinder the nitrate-nitrite-nitric oxide pathway and result in increased blood pressure. Therefore, reconsidering mouthwash use may be a potential therapeutic target in the efforts toward reducing the exceedingly high rates of hypertension. This section will present findings from the limited research available regarding mouthwash use and vascular health.

In 2009, Petersson et al. observed that gastroprotective and blood pressure lowering effects of dietary nitrate consumption were abolished when treating animal models with an antiseptic mouthwash. In this study, rats were given nitrate-supplemented

drinking water and were sprayed twice daily with a commercial antiseptic mouthwash. Rats sprayed with the antiseptic mouthwash displayed a drastic reduction in viable nitrate-reducing oral bacteria and had significantly less circulating NO levels (Petersson et al., 2009). Additionally, decreases in blood pressure that were seen previously with nitrate supplementation were absent in the mouthwash-treated rats (Petersson et al., 2009). These results suggested that antiseptic mouthwash use may interrupt the effects of dietary nitrate consumption and thus cause an increase in blood pressure (Petersson et al., 2009).

A similar study by Woessner et al. was performed in humans in 2015 in which resting blood pressure, plasma nitrate concentrations, and salivary nitrate concentrations were observed in normotensive adults using increasing strengths of mouthwash after a dietary nitrate load. Commercially available mouthwashes (Listerine, Cepacol, and Chlorhexidine) were compared to a control (water). This study included 12 normotensive healthy males that consumed nitrate-rich beet juice, and then gargled with the assigned mouthwash 15 minutes later. Concentration measures were then observed every hour for 4 hours. Plasma and salivary nitrate levels increased above baseline after consumption of the beetroot juice, however mouthwash treatment appeared to inhibit the effect of the nitrate-nitrite-nitric oxide pathway and revealed subsequent responses in resting blood pressure (Woessner, Smoliga, Tarzia, Stabler, Bruggen, & Allen, 2016). The stronger mouthwashes (Chlorhexidine and Cepacol) showed significant inhibition of plasma and saliva nitrate concentration rise and led to higher systolic blood pressure readings (Woessner et al., 2016). This study demonstrated the significant role of the nitrate-nitrite-

nitric oxide enterosalivary pathway and the capability of antiseptic mouthwash to hinder its blood pressure lowering effects.

Bondonno et al. observed the effects of antibacterial mouthwash use on oral nitrate reduction and blood pressure in a population of hypertensive adults. In this study, 15 hypertensive men and women with a mean age of 65 years participated in a 3-day crossover trial in which they were instructed to use an antibacterial mouthwash followed by measures of salivary nitrate, salivary nitrite, plasma nitrite, and blood pressure. Compared to the control group, significant decreases in oral nitrate to nitrite reduction and salivary nitrite levels were observed (Bondonno et al., 2015). Additionally, significant increases in systolic blood pressure were seen at an average of 2.3 mmHg but changes in diastolic blood pressure were not observed (Bondonno et al., 2015). Findings suggest that interrupting the nitrate-nitrite-nitric oxide pathway through mouthwash use was associated with a small increase in blood pressure in a population of hypertensive adults (Bondonno et al., 2015).

In 2017, Mitsui and Harasawa observed the effects of other antibacterial treatments in addition to mouthwash on salivary nitrate concentrations and the nitrate-reducing capacity of oral bacteria. This study involved 12 participants that mouth-washed with an essential oil blend, povidone-iodine, 0.0025% chlorhexidine, or water as a control. After mouth washing, participants consumed 110 mg of nitrates by eating 100 g of lettuce. Saliva collections were taken at 1, 5, and 10-hour intervals to assess bacterial species present and salivary nitrate concentrations. Results indicated that the essential oil, povidone-iodine, and water treatments had little effect on nitrate concentration, however

Veillonella dispar populations were significantly inhibited following the chlorhexidine wash (Mitsui & Harasawa, 2017).

Sundqvist et al. examined the effects of antiseptic mouthwash use on oral bacterial nitrate conversion to nitrite, resting metabolic rate, plasma nitrite, and blood pressure in a 2016 randomized, double-blind, crossover study. In this study, the sample was comprised of 17 female participants who were healthy and normotensive, with a mean age of 23 years. Participants received two separate 3-day interventions in which they consumed a low-nitrate diet and rinsed daily with either a chlorhexidine mouthwash or a placebo mouthwash. 24-hour ambulatory blood pressure was measured along with blood, urine, and saliva samples after each intervention. Results indicated that the chlorhexidine treatment reduced oral nitrate to nitrite conversion but exhibited no significant effects on resting metabolic rate or blood pressure (Sundqvist, Lundberg, & Weitzberg, 2016). It was concluded that mouthwash use, although effective in decreasing nitrate to nitrite bacterial conversion, was not associated with resting metabolic rate or blood pressure changes in healthy normotensive females (Sundqvist et al., 2016).

CONCLUSION

The U.S. is currently experiencing high rates of hypertension that are expected to increase in the coming years. Hypertension is a health concern that has been associated with significant increases in the risk for development of cardiovascular disease, heart attack, and stroke. Addressing the current hypertensive crisis is essential. Simple and effective strategies for the average individual to prevent and reduce high blood pressure need to be further studied.

Mouthwash use has been associated in some cases, primarily those who are already hypertensive, with increases in blood pressure and decreases in healthy cardiovascular biomarkers such as plasma nitrate and NO. There may be cause for discouraging the continued use of antiseptic mouthwash in susceptible individuals. However, more research needs to be conducted to observe the effects of commercially available mouthwash products on cardiovascular health. Additionally, the potential to target the enterosalivary nitrate-nitrite-nitric oxide pathway could provide useful therapeutic strategies as we continue the search for sustainable methods of reducing rates of hypertension.

CHAPTER 3

METHODS

Participants

Participants included adults, age >30 years, with mildly elevated blood pressure. Inclusion criteria for blood pressure was relaxed due to recruitment difficulties in order to ensure adequate study participation. Subjects were recruited from the Arizona State University (ASU) community for this seven-week mouthwash and sodium intervention. Participants were required to be adults who were generally healthy and free of any chronic disease (heart disease, renal disease, liver disease, stroke, or diabetes), non-smoking, and taking no systemic medication other than a contraceptive pill. Participants were excluded if they had any recent antibiotic use or a history and/or recent treatment of any oral health conditions such as gingivitis, periodontitis, or halitosis.

Recruitment

Subjects were recruited via ListServs provided by ASU, verbal announcements throughout the nutrition department, electronic messages, and posted paper flyers. During the recruitment phase, subjects who expressed interest in the study were emailed a brief survey regarding their demographics, blood pressure, their current oral hygiene routine, and their use or non-use of mouthwash products. The completion of this survey determined whether the individual would be eligible to participate in the intervention. Subjects that met inclusion and exclusion criteria were scheduled for their first screening visit.

Study Design

This study was a randomized, controlled, crossover trial with a total of 10 participants. The participants were randomized via simple randomization to receive treatments A, B, and C in a random order, with each treatment lasting one week followed by a one-week washout period. Treatment A was the control and consisted of no prescribed mouthwash use or sodium intervention. Treatment B consisted of mouthwash use alone with no sodium intervention. Treatment C consisted of mouthwash use paired with a high sodium diet of at least 6 grams of sodium per day. The mouthwash used during Treatments B and C was Cepacol Mouthwash, an over-the-counter antibacterial mouthwash containing the active ingredient cetylpyridinium chloride. This active ingredient has been shown to target the bacterial membrane walls of oral bacterial most similarly to Chlorhexidine, a prescription-strength mouthwash (Woessner et al., 2016). Participants were instructed to brush their teeth twice per day in the morning and in the evening, followed by rinsing with the mouthwash for at least one minute. Participants were instructed to refrain from using any sonic or high-speed electric toothbrushes during the duration of the trial. During the high sodium treatment, participants were instructed to consume two Best Maid pickles per day, each containing 2760 mg of sodium totaling approximately 6000 mg of added sodium, in addition to the prescribed mouthwash use.

In addition to the three treatments, participants were instructed to reduce their sodium intake the day before they came in for testing of blood pressure and salivary nitrate levels. They were instructed to remain sedentary on the morning of testing and refrain from consuming caffeine prior to testing. Participants were allowed to consume a light breakfast consisting of plain or buttered toast, or a small bowl of cereal with milk.

Outcome measures included blood pressure and salivary nitrate levels. These measures were analyzed to determine if there was an effect on the dependent variables.

Study Protocol

The screening visit with participants occurred prior to the start of the trial. All individuals participating in the study signed the consent form and anthropometric measurements for blood pressure, body weight, height, body mass index (BMI), and waist circumference were obtained. Prior to measuring blood pressure, individuals were advised to relax for 10 minutes and fully empty their bladder. Blood pressure measurements were taken three times using a calibrated blood pressure cuff. The blood pressure readings were then averaged and recorded. This method was used during all blood pressure measurements throughout the trial. Height was measured using a freestanding height rod. Waist circumference was measured around the umbilicus using a research-grade tape measure. Additionally, participants completed a validated food frequency questionnaire assessing sodium intake to discern their current average sodium consumption (Charlton, Steyn, Levitt, Jonathan, Zulu, & Nel, 2008).

After this screening visit, participants underwent a two-week washout period where they were instructed to remove mouthwash from their current oral hygiene routine. During this two-week washout period, participants came in two more times to record additional blood pressure readings that were then averaged and used to obtain an accurate baseline blood pressure measurement.

After the two-week washout period, participants began following their assigned treatment and followed up at the end of each week to measure their blood pressure and salivary nitrate levels. In addition, food frequency questionnaires were completed to

assess for sodium intake at each visit. Participants were incentivized by receiving a $50 Amazon gift card at the end of the study for their participation.

Laboratory Analysis

Salivary nitrate concentrations were measured using a commercially available Nitric Oxide Assay Kit Catolog No. EMSNO. Participants provided saliva samples at the beginning and end of each treatment week. Saliva was analyzed for nitrite concentration in a two-step process: converting nitrate to nitrite using nitrate reductase followed by the addition of a Griess Reagent to convert nitrite into a purple azo compound whose absorbance can be used to obtain a photometric measurement of nitrite concentration.

Statistical Analysis

Data was interpreted by the Statistical Package for Social Sciences (SPSS Version 25 for Mac, 2017) and reported as mean ± standard deviation. Because the sample size is less than 50, the Shapiro Wilks test was used to assess the data for normality. If normality assumption was not met ($p > 0.05$), data was transformed using log or square root transformation. For comparison of means, a repeated measure analysis of variance (ANOVA) test was used to assess relationships. If the data were unable to be log or square root transformed, the non-parametric Wilcoxon sign-rank test was conducted. The results were analyzed to determine if statistical significance (defined as a $p < 0.05$) was present.

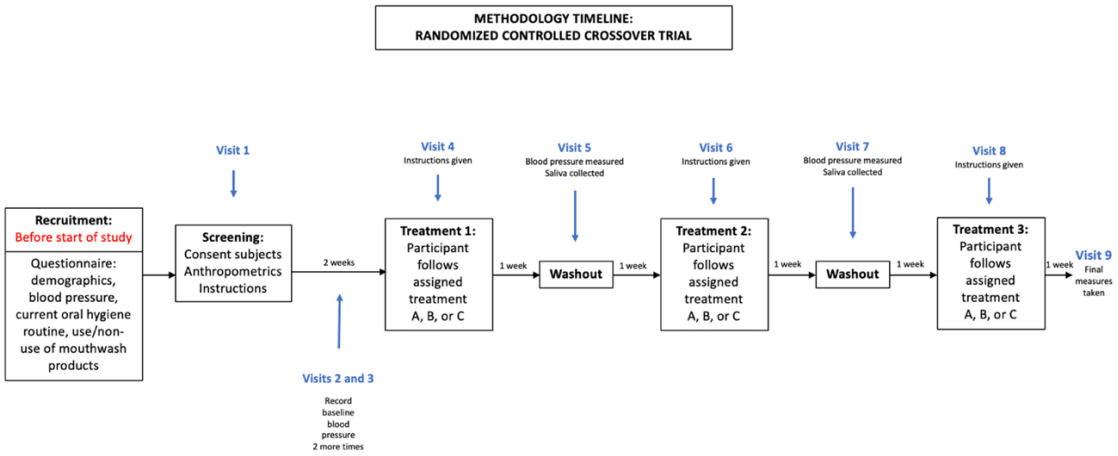

Figure 3. Methodology Timeline

CHAPTER 4

RESULTS

A total of 40 people responded to the online recruitment questionnaire and indicated interest in study participation. Of the 40 interested subjects, 15 were excluded due to age restrictions and 2 were excluded due to recent history of an oral condition. Inclusion criteria for blood pressure was relaxed due to recruitment difficulties in order to ensure adequate study participation. Of the remaining eligible respondents, a total of 10 subjects were scheduled to meet for the initial screening visit and enrolled in the trial. There were no participants who withdrew throughout the duration of the study. Thus, a total of 10 participants completed the 7-week intervention. Baseline characteristics were measured at the initial screening visit and included age, weight, height, waist circumference, body mass index (BMI), blood pressure, and dietary sodium intake. At baseline, blood pressure did not correlate with age, weight, height, waist circumference, or BMI so it was not necessary to control for these variables. Baseline characteristics are summarized in Table 1 and are provided as mean±SD. Pre, post, and change data for each outcome measure are presented as mean±SD for the control, mouthwash, and mouthwash + pickles intervention weeks. Change data for each outcome were normally distributed and were compared between groups using repeated measures ANOVA.

Table 1. Subject Characteristics and Demographics (n=10)

	Mean±SD	Minimum	Maximum
Gender M/F	1/9[1]		
Age (years)	47.3±12.5	37	64
Weight (kg)	66.7±13.2	50.2	93.7
Height (cm)	162.0±7.7	153.0	175.0
Waist Circumference (cm)	83.2±9.4	70.5	95.3
Body Mass Index (kg/m^2)	25.4±4.4	20.9	32.5
Average Baseline Systolic Blood Pressure (mmHg)	114.9±12.5	95.7	138.0
Average Baseline Diastolic Blood Pressure (mmHg)	75.2±9.1	62.3	92.0
Average Baseline Daily Sodium Intake (mg)	2638.4±1426.6	855	5024

1. Data represented as a number.

Blood Pressure

It was hypothesized that blood pressure would increase from baseline after mouthwash use and that blood pressure would increase to a greater degree after mouthwash use combined with high sodium intake than mouthwash use alone. When comparing the effects of the mouthwash and mouthwash + pickles conditions, there were no statistically significant differences in systolic or diastolic blood pressure ($p=0.468$ and $p=0.859$, respectively), as outlined in Table 2. Given these findings, the hypothesis was not supported.

Table 2. Pre, Post, and Change Values in Systolic and Diastolic Blood Pressure During Control, Mouthwash, and Mouthwash + Pickles Phases of the Study[1] (n=10)

	Pre	Post	Change	Change
		Mean±SD		p-value
Systolic Blood Pressure (mmHg) Control	113.3±11.9	118.1±13.9	4.8±11.5	0.468
Systolic Blood Pressure (mmHg) Mouthwash	117.0±12.3	116.2±16.2	-0.8±6.9	
Systolic Blood Pressure (mmHg) Mouthwash + Pickles	116.3±10.8	117.0±12.0	0.7±7.6	
Diastolic Blood Pressure (mmHg) Control	74.6±8.8	75.9±10.5	1.3±8.8	0.859
Diastolic Blood Pressure (mmHg) Mouthwash	75.1±11.3	74.9±13.2	-0.2±5.7	
Diastolic Blood Pressure (mmHg) Mouthwash + Pickles	75.2±8.2	76.7±8.6	1.5±9.4	

2. Data are the mean±SD; p-value represents repeated measures ANOVA for change data between treatments.

Salivary Nitrite Concentration

It was hypothesized that salivary nitrite levels would decrease from baseline after mouthwash use. When comparing change data between conditions, there were no statistically significant differences in salivary nitrite concentrations during the study (p=0.493), as outlined in Table 3. Given these findings, the hypothesis was not supported.

Table 3. Pre, Post, and Change Values in Salivary Nitrate and Nitrite Levels During Control, Mouthwash, and Mouthwash + Pickles Phases of the Study[1] (n=10)

	Pre	Post	Change	Change
	Mean±SD			p-value
Salivary Nitrite Concentration (μmol/L) Control	665.63±564.28	553.96±304.84	-111.67±419.20	0.493
Salivary Nitrite Concentration (μmol/L) Mouthwash	558.97±260.85	593.18±260.69	34.21±254.08	
Salivary Nitrite Concentration (μmol/L) Mouthwash + Pickles	505.76±299.91	575.92±253.63	70.16±291.82	

Sodium

Sodium intake was assessed throughout the study via the Sodium Food Frequency Questionnaire. Dietary sodium intake was estimated at baseline and before and after each intervention. There appeared to be a significant increase in sodium intake during the mouthwash + pickle intervention (p=0.000), however sodium intake did not appear to change significantly throughout the study when the pickles were not accounted for.

Table 4. Pre, Post, and Change Values in Daily Sodium Intake During Control, Mouthwash, and Mouthwash + Pickles Phases of the Study[1] (n=10)

	Pre	Post	Change	Change
		Mean±SD		p-value
Daily Sodium Intake (mg) Control	1750.9±987.6	1453.6±929.0	-297.3±272.6	0.000
Daily Sodium Intake (mg) Mouthwash	1366±781.4	1364.2±821.9	-2.4±427.0	
Daily Sodium Intake (mg) Mouthwash + Pickles	1768.0±1094.8	7669.7±793.5	5901.7±622.3	

1. Data are the mean±SD; p-value represents repeated measures ANOVA for change data between treatments.

CHAPTER 5

DISCUSSION

This 7-week randomized, controlled, crossover trial sought to examine the effects of antiseptic mouthwash use and sodium intake on blood pressure and salivary nitrate levels in a population of prehypertensive adults. Repeated measures ANOVA was used to compare change data for each outcome measure. Due the nature of the crossover design protocol, all subjects received both of the intervention conditions and the control condition of the trial. All subjects also adhered to a 1-week washout period between each treatment.

Blood Pressure

This study concluded that there was no significant change in systolic or diastolic blood pressure from the start to the end of each intervention ($p=0.468$ and $p=0.859$, respectively). This finding suggests that antiseptic mouthwash use may not induce hypertensive effects in this population. These findings align with previous research in normotensive adult populations. In a similar study with a crossover design, results in a population of normotensive female participants indicated that while mouthwash use reduced oral nitrate to nitrite conversion, there were no significant changes in systolic or diastolic blood pressure (Sundqvist et al., 2016). The present study did use an older population than Sundqvist et al. (mean age of 47.3±12.5) and study interventions were increased from 3 days to 7 days. In contrast, other studies performed in hypertensive populations did have results that indicated mild systolic blood pressure elevations following the use of antiseptic mouthwash (Woessner et al., 2016). Additionally, frequency of tongue cleaning has been shown to impact the composition of the human

tongue microbiome and enterosalivary circulation of nitrate. According to a 2019 study, tongue cleaning frequency did serve as a predictor of increases in systolic blood pressure induced by chlorhexidine mouthwash use (Tribble et al., 2019), however frequency of tongue cleaning was not taken into account throughout the duration of the present study. To date, there is also evidence in the literature to support the enhancement of flow-mediated vasodilation through upregulation of the eNOS pathway following vinegar (acetic acid) intake. In a 2010 study, researchers examined the effect of acetate on eNOS in human umbilical vein endothelial cells and found that phosphorylated eNOS was significantly increased following acetate exposure and maximum forearm blood flow in response to shear stress was also increased following vinegar administration compared to placebo (Sakakibara et al, 2010). These results suggest that in the present study, acetic acid-induced eNOS phosphorylation following vinegar consumption from the pickles may have contributed to an upregulation of flow-mediated vasodilation and therefore counteracted any systolic blood pressure increasing effects following mouthwash use. Further, it is important to note that in the present study inclusion criteria were relaxed to allow for adequate participant recruitment. Consequently, blood pressure of study participants more closely reflected normotensive values (mean blood pressure of 114.9/75.2 mmHg) than studies that demonstrated blood pressure changes in a hypertensive population.

Salivary Nitrite Concentration

Contrary to what was hypothesized, there was no significant change in salivary nitrite concentration from the start to the end of each intervention ($p=0.493$). These findings were not in alignment with previous research in which researchers observed

decreases in salivary nitrite concentration following treatment with mouthwash. In a 2016 study, researchers observed a stepwise reduction in plasma and salivary nitrite following the introduction of increasing strengths of mouthwash after a dietary nitrate load from beet juice (Woessner et al., 2016). It is important to note that this study was acute and observed salivary nitrite concentrations each hour over a total duration of four hours. However, in a 3-day randomized controlled crossover trial following hypertensive men and women, treatment of antibacterial mouthwash use did result in significant decreases in oral nitrate to nitrite reduction, indicating a trend towards reduced salivary nitrite concentrations and subsequent increases in blood pressure (Bondonno et al., 2015). Frequency of tongue cleaning may have also been a factor in enterosalivary circulation of nitrate and nitrite due to changes in microbial composition within the mouth. Oral microbial communities have the capacity to supplement host nitric oxide production through the enterosalivary nitrate-nitrite-nitric oxide pathway. Increased tongue cleaning may result in reduced production of bacterial nitrite reductases and thus contribute to changes in oral nitrite concentration (Tribble et al., 2019), however as stated previously this was not accounted for in the current study.

Strengths

There were several notable strengths of the present randomized controlled crossover study. Strengths of this study include the crossover design in which participants act as their own control and allowed the researcher to assess within-subject changes following each of the three treatment conditions. Participants were asked to comply with a washout phase between each treatment condition, allowing adequate time to return to baseline blood pressure and salivary nitrate levels in order to enhance accurate

comparison between treatment conditions. Participants were provided with calendars to track adherence to the study protocol, which indicated 100% participant compliance for twice daily mouthwash use and consumption of the high-sodium pickles. Additional strengths include exclusion of subjects currently taking antihypertensive medications and those who have recently taken or are currently taking antibiotics. Research in the literature has confirmed that antihypertensive medication use will interfere with blood pressure readings and antibiotic use may impact composition of oral microbiome and influence bacterial nitrate conversion in the mouth (Bryan et al., 2017).

Limitations

Consistent with all research, inherent limitations did exist in this study. During this research trial include the small sample size of only 10 subjects, which may have lacked sufficient power to produce statistically significant results. Sodium intake was monitored using a self-reported food frequency questionnaire and therefore, is subject to biases such as participant recording error. Additionally, frequency of tongue cleaning was not monitored throughout the duration of the study and has been shown to influence systolic blood pressure readings and composition of the oral microbiome. Future research seeking to assess the effects of mouthwash use on blood pressure may benefit by recruiting a population of prehypertensive and hypertensive individuals with a blood pressure ranging from 120/80 mmHg to 140/90 mmHg, obtaining a 24-hour urine collection to more accurately assess sodium status, collecting data on participant weight each week to more closely monitor fluid status, and recruiting a larger sample of participants to ensure the study is adequately powered in order to produce statistically significant results.

Conclusion

The results of this randomized controlled crossover trial suggest that twice daily use of an over-the-counter strength antiseptic mouthwash rinse does not significantly increase systolic or diastolic blood pressure or induce significant changes in salivary bacterial nitrate to nitrite reduction. Significant increases in sodium consumption were observed following pickle consumption (~6000 mg of added sodium per day), however this did not seem to induce significant changes in blood pressure or salivary nitrite concentrations.

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APPENDIX A

IRB APPOVAL

ASU Knowledge Enterprise Development

APPROVAL: EXPEDITED REVIEW

Carol Johnston
Nutrition
602/496-2539
CAROL.JOHNSTON@asu.edu

Dear Carol Johnston:

On 12/5/2018 the ASU IRB reviewed the following protocol:

Type of Review:	Initial Study
Title:	The effects of antiseptic mouthwash use on systemic blood pressure regulation and salivary nitrate concentrations in adults consuming a high sodium diet: a randomized controlled crossover trial
Investigator:	Carol Johnston
IRB ID:	STUDY00009333
Category of review:	(3) Noninvasive biological specimens, (4) Noninvasive procedures, (7)(b) Social science methods, (7)(a) Behavioral research
Funding:	Name: Graduate College (GRAD)
Grant Title:	
Grant ID:	
Documents Reviewed:	• protocol, Category: IRB Protocol; • online survey tool (screener), Category: Screening forms; • Dietary sodium measure (FFQ), Category: Measures (Survey questions/Interview questions /interview guides/focus group questions); • health history questionnaire, Category: Screening forms; • verbal script/flyer, Category: Recruitment Materials; • consent, Category: Consent Form;

The IRB approved the protocol from 12/5/2018 to 12/4/2019 inclusive. Three weeks before 12/4/2019 you are to submit a completed Continuing Review application and required attachments to request continuing approval or closure.

If continuing review approval is not granted before the expiration date of 12/4/2019 approval of this protocol expires on that date. When consent is appropriate, you must use final, watermarked versions available under the "Documents" tab in ERA-IRB.

In conducting this protocol you are required to follow the requirements listed in the INVESTIGATOR MANUAL (HRP-103).

Sincerely,

IRB Administrator

cc:
Karrol Shaw

APPENDIX B

RECRUITMENT QUESTIONNAIRE

Mouthwash, Dietary Sodium, and Blood Pressure

Thank you for your interest in this research study conducted by ASU Nutrition Professor Carol Johnston and Kassie Shaw, a nutrition Masters student. This research study will examine the influence of mouthwash use on blood pressure. We are inviting your participation in the screening process, which will consist of answering questions regarding health history, demographics, and scheduling availability. You have the right to not answer any question, and to stop participation at any time.

We are recruiting healthy adults 30 years of age or older. Your participation in this survey is voluntary. If you choose not to participate or to withdraw from the survey at any time, there will be no penalty. Your responses to this survey will be confidential. If you meet the criteria for this study, you will be contacted to schedule an in-person appointment at the downtown campus of Arizona State University.

If you have any questions concerning the research study, please contact Dr. Carol Johnston: carol.johnston@asu.edu. If you have any questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk, you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Office of Research Integrity and Assurance, at (480) 965-6788.

By completing this survey, you are agreeing to be contacted by investigators (via email) to schedule an appointment, should you qualify.

1. Please provide your email address

2. Please select your gender

- ○ Male
- ○ Female

3. What is your age?

Age (years)

4. Please enter your height and weight

Height (inches) (note 5 feet = 60 inches)

Weight (pounds)

5. Has a doctor ever told you that you have high blood pressure?

- ◯ Yes
- ◯ No

6. Do you know your blood pressure?

- ◯ Yes
- ◯ No

If yes, what was the last reading you remember?

7. Do you have any active disease state that is currently requiring medication?

- ☐ Yes
- ☐ No

8. Have you ever been diagnosed with an oral condition such as gingivitis, periodontitis, or halitosis?

- ☐ Yes
- ☐ No

9. If female, are you pregnant, lactating, or do you anticipate becoming pregnant?

- ◯ Yes
- ◯ No

10. If you smoke, please select how many cigarettes you smoke per day

- ◯ 0
- ◯ 1-5
- ◯ 6-10
- ◯ >10

11. How many times per week do you participate in vigorous, highly intense exercise?

12. Are you following a special diet (such as vegetarian or gluten-free)?

- ◯ Yes
- ◯ No

If yes please specify:

13. Do you take any dietary supplements daily? (e.g., vitamin, mineral, and/or herb)

- ◯ Yes
- ◯ No

If yes (please specify)

14. Do you currently use mouthwash daily?

- ◯ Yes
- ◯ No
- ◯ If yes, list the type of mouthwash

15. Are you willing to follow a specific mouthwash schedule during the 9-week study (e.g., some weeks you would use mouthwash 2x daily but other weeks you would not be allowed to use mouthwash)?

- ◯ Yes
- ◯ No

16. Are you able to brush your teeth 2x daily for 2 minutes each during the 9-week trial?

- ◯ Yes
- ◯ No

17. Are you able to eat pickles daily for one week during the trial? (Note, the pickles will be provided.)

- ◯ Yes
- ◯ No

18. Are you willing to provide saliva samples during the study? (e.g., spit into a test tube)

- ◯ Yes
- ◯ No

19. Are you able to eat pickles daily for one week during the trial? (Note, the pickles will be provided.)

- ◯ Yes
- ◯ No

20. Are you willing to meet with investigators on the ASU Downtown Campus (near VanBuren and 5th Streets) on 9 occasions (10-20 minutes per visit)?

- ◯ Yes
- ◯ No

APPENDIX C

INFORMED CONSENT

THE EFFECTS OF ANTISEPTIC MOUTHWASH USE ON SYSTEMIC BLOOD PRESSURE REGULATION AND SALIVARY NITRATE CONCENTRATIONS

INTRODUCTON

The purposes of this form are (1) to provide you with information that may affect your decision as to whether or not to participate in this research study, and (2) to record your consent if you choose to be involved in this study.

RESEARCHERS

Dr. Carol Johnston, a Nutrition professor at Arizona State University Downtown Campus, and Kassie Shaw, a nutrition graduate student, have requested your participation in a research study.

STUDY PURPOSE

The purpose of the research is to examine the effects of mouthwash use on blood pressure and salivary nitrate.

DESCRIPTION OF RESEARCH STUDY

You have indicated to us that you are at least 30 years of age, a non-smoker and generally healthy. You have not been diagnosed with a chronic disease or disease of the oral cavity, you do not take prescription medications, you have not taken antibiotics in the recent month, and (if female) you have not recently been pregnant or lactating. Participants are asked to maintain their usual diet and physical activity throughout the study but to moderate sodium intake the day prior to study visits. On the morning of the study visit, participants are to restrict caffeine consumption prior to testing and to stay rested (no exercise). Participants are to refrain from all mouthwash use during the study unless provided by the study investigator. Participants will need to manually brush their teeth twice daily and are not to use electric/sonic toothbrushes. Study interventions include (1) the use of mouthwash twice daily for one week and (2) the use of mouthwash and the consumption of pickles (3 per day) for one week.

At the initial screening visit, you will complete a food questionnaire and a brief health history questionnaire to demonstrate the absence of medical conditions that may impact the study. Your weight, height, girth, and blood pressure will be measured. This first meeting will take about 20 minutes. Over the next 2 weeks you will be asked to return to the test site for a blood pressure measurement (10 minutes). The interventions occur during weeks 3, 5, 7 and you will come to the test site before and after these treatments (6 visits total). At these 6 visits, blood pressure will be taken, a small saliva sample will be collected, and further instructions will be provided. The procedures on all test days are identical and will take 15 minutes. You may eat a small breakfast the morning of these 6 visits (e.g., a slice of buttered toast or a small bowl of cereal and milk), but you need to refrain from eating at least 1 hour ahead of your visit. On test days, you will travel to ASU (the Nutrition labs at the ABC1 Building on the ASU Downtown campus) in the morning.

About 22 subjects will participate in this study and will receive a $50 gift card at the end of the trial.

RISKS

No physical risks are anticipated. Your blood pressure will likely fluctuate during the study.

BENEFITS

There is no direct benefit for participating in this trial. If you would like your personal data, you can sign a standard release form to receive your results.

NEW INFORMATION

If the researchers find new information during the study that would reasonably change your decision about participating, then they will provide this information to you.

CONFIDENTIALITY

All information obtained in this study is strictly confidential unless disclosure is required by law. The results of this research study may be used in reports, presentations, and publications, but your name or identity will not be revealed. In order to maintain confidentiality of your records, Dr. Johnston will use

subject codes on all data collected, maintain a master list separate and secure from all data collected, and limit access to all confidential information to the study investigators.

WITHDRAWAL PRIVILEGE
You may withdraw from the study at any time for any reason without penalty or prejudice toward you. Your decision will not incur negative treatment to you by the researchers.

COSTS AND PAYMENTS
The all test foods will be given to you during the study free of charge. You will receive a $50 gift card at the end of the trial.

COMPENSATION FOR ILLNESS AND INJURY
If you agree to participate in the study, then your consent does not waive any of your legal rights. However, in the event of harm, injury, or illness arising from this study, neither Arizona State University nor the researchers are able to give you any money, insurance coverage, free medical care, or any compensation for such injury. Major injury is not likely but if necessary, a call to 911 will be placed.

VOLUNTARY CONSENT
Any questions you have concerning the research study or your participation in the study, before or after your consent, will be answered by Dr. Carol Johnston; 500 N. 3rd Street Phoenix, AZ 85004; 602-827-2265.

If you have questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk, you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Research Compliance Office, at 480-965 6788.

This form explains the nature, demands, benefits and any risk of the project. By signing this form you agree knowingly to assume any risks involved. Remember, your participation is voluntary. You may choose not to participate or to withdraw your consent and discontinue participation at any time without penalty or loss of benefit. In signing this consent form, you are not waiving any legal claims, rights, or remedies. A copy of this consent form will be given to you.

Your signature below indicates that you consent to participate in the above study.

Subject's Signature	Printed Name	Date

Contact phone number	Email

INVESTIGATOR'S STATEMENT
"I certify that I have explained to the above individual the nature and purpose, the potential benefits, and possible risks associated with participation in this research study, have answered any questions that have been raised, and have witnessed the above signature. These elements of Informed Consent conform to the Assurance given by Arizona State University to the Office for Human Research Protections to protect the rights of human subjects. I have provided the subject/participant a copy of this signed consent document."

Signature of Investigator_____ Date_____

APPENDIX D

HEALTH HISTORY QUESTIONNAIRE

MEDICAL HISTORY QUESTIONNAIRE

MEDICAL HISTORY QUESTIONNAIRE ID#_____

Height _____ft. _____ in. Weight: _____lbs. Waist: _____ ins. To be completed by investigator

Age: _____

Gender: □ Male □ Female

Current smoker: □Yes □ No

1. Have you been diagnosed with any type of oral disease or condition? Y N

2. Have you been diagnosed with 'dry mouth'? Y N

3. Have you been diagnosed with other chronic diseases (such as heart disease, neurological disease, autoimmune disease, or cancer)? Y N

4. Have you been diagnosed with high blood pressure? Y N

5. What is your blood pressure? _____

6. Have you taken antibiotics in the recent several months? Y N
   If yes, what was the date of the last dosage: _____

7. Do you take any medications regularly? Y N
   If yes, please list what kind and how frequently:

Medication	Dosage	Frequency

8. Do you currently take supplements (vitamins, minerals, herbs, etc.)? Y N
   If yes, what supplements and how often?

   _____
   _____
   _____
   _____

9. Do you have medical conditions that you see a physician for on a regular basis? Y N
   Please explain:

   _____
   _____
   _____
   _____

OVER→

10. Do you have any food allergies? Y N
If yes, please describe _____
_____

11. Do you follow a special diet? (weight gain/loss, vegetarian, low-fat, etc.) Y N
If yes, please describe _____
_____
_____

12. Are you OK with eating 3 pickles per day for one week? Y N

13. Will you have any problems restricting mouthwash use, sonic or electric toothbrushes? Y N

14. Do you have any difficulty chewing or swallowing? Y N

15. Will you have a problem spitting into a test tube to collect saliva? Y N

16. Typically, how many alcoholic beverages to you consume? (circle most appropriate answer)

0 1-2 weekly 3-4 weekly 5-6 weekly 1 daily 2 daily >2 daily

17. Please circle ***the number of times*** you did the following kinds of exercises for ***more than 15 minutes*** last week.

Mild exercise (minimal effort):
Easy walking, golf, gardening, bowling, yoga, fishing, horseshoes, archery, etc.

Times per week: 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14+

Moderate exercise (not exhausting):
Fast walking, easy bicycling, tennis, easy swimming, badminton, dancing, volleyball, baseball, etc.

Times per week: 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14+

Strenuous exercise (heart beats rapidly):
Running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling, etc.

Times per week: 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14+

APPENDIX E

SODIUM FOOD FREQUENCY QUESTIONNAIRE

Appendix: Salt intake questionnaire

NUTRITIONAL AND LIFESTYLE HABITS The following questions are about your dietary and life-style habits. All your answers will be strictly confidential	***Office use***
Study number:	| | | **3**

During the **PAST 7 days (1 week)** did you eat any of the following? IF YES, ASK HOW OFTEN
(*if no, circle never*) **[**DO NOT PROMPT THE ANSWER OPTIONS BELOW**]**

Food item	***NEVER***	1-3 times per **week**	4-6 times per **week**	1 time a **day**	2 times a **day**	3+ times a **day**		
White bread / white bread rolls	0	1	2	3	4	5		4
Brown /wholewheat bread / Rolls	0	1	2	3	4	5		
Breakfast Cereal (processed)	0	1	2	3	4	5		
Breakfast Cereal (minimally processed - weetbix, muesli, etc.)	0	1	2	3	4	5		
Crackers (ProVita etc)	0	1	2	3	4	5		
Cookies, biscuits,rusks	0	1	2	3	4	5		
Cake / scone / muffin / puddings / pancake / fruit pie / koeksister	0	1	2	3	4	5		
Roti / samoosa / springroll / doughnut	0	1	2	3	4	5		
Pizza	0	1	2	3	4	5		
Pasta/noodle dishes with cheese sauces (macaroni cheese, lasagne, noodle salad etc.)	0	1	2	3	4	5		
Popcorn	0	1	2	3	4	5		
Crisps (Simba and Niknaks etc.)	0	1	2	3	4	5		
Beef sausage (*boerewors*)	0	1	2	3	4	5		
Polony /salami / bacon / salami / pork sausages (processed meat, cooked, smoked and canned)	0	1	2	3	4	5		
Meat or chicken pies/sausage rolls	0	1	2	3	4	5		
Chicken - battered (KFC etc). and chicken burger only	0	1	2	3	4	5		
Meat and meat dishes (steaks, minced meat, cottage pie, mince, meatballs, stew, bobotie, etc.)	0	1	2	3	4	5		20
Gravy, made with stock or gravy powder	0	1	2	3	4	5		
Biltong/dry *wors* /fish biltong	0	1	2	3	4	5		
Milk (all types, also dairy fruit juice, malted milk, milk shakes)	0	1	2	3	4	5		
Maas (fermented milk)	0	1	2	3	4	5		
Cheese	0	1	2	3	4	5		
Yoghurt	0	1	2	3	4	5		
Eggs	0	1	2	3	4	5		
Tinned fish (pilchards/tuna, etc.)	0	1	2	3	4	5		
Other fish and seafood	0	1	2	3	4	5		
Potato chips/french fries and potato salad	0	1	2	3	4	5		
Canned vegetables, incl. Baked beans, tomato paste, sweetcorn, etc.	0	1	2	3	4	5		
Soup (all types)	0	1	2	3	4	5		

Food item	***NEVER***	1-3 times per **week**	4-6 times per **week**	1 time a **day**	2 times a **day**	3+ times a **day**		
Salad dressing/mayonnaise	0	1	2	3	4	5		
Ice cream (all types)	0	1	2	3	4	5		
Margarines, all types, also butter	0	1	2	3	4	5		
Chutney / atchar / chakalaka / Worcester sauce	0	1	2	3	4	5		
Savoury sauces (mushroom, monkey gland, white, cheese)	0	1	2	3	4	5		
Tomato sauce	0	1	2	3	4	5		
Salt	0	1	2	3	4	5		
Aromat / Fondor /mustard	0	1	2	3	4	5		
Peanuts	0	1	2	3	4	5		
Peanut butter	0	1	2	3	4	5		
Marmite/Bovril	0	1	2	3	4	5		
Chocolate sweets and sauce	0	1	2	3	4	5		
Beer and cider	0	1	2	3	4	5		

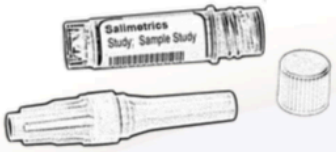
APPENDIX F

SALIVA COLLECTION INSTRUCTIONS

How to Collect Saliva: Collection Methods and Devices

Collection Methods and Devices: Adults & Children 6 + Years of Age

Passive Drool



Passive drool is highly recommended because it is both cost effective and approved for use with almost all analytes. If research participants are not willing or able to drool saliva into a vial, the SalivaBio Oral Swab may be used as an alternative collection method, but only for certain analytes (see instructions).

To avoid problems with analyte retention or the introduction of contaminants, use only high quality polypropylene vials for collection, such as our 2 ml cryovials (Salimetrics Item No. 5002.01). The vials used must seal tightly, must be able to withstand temperatures down to -80ºC, and must be externally threaded to allow for use of the Saliva Collection Aid (SCA – Salimetrics Item No. 5016.02) to effectively guide drool directly into the cryovial.

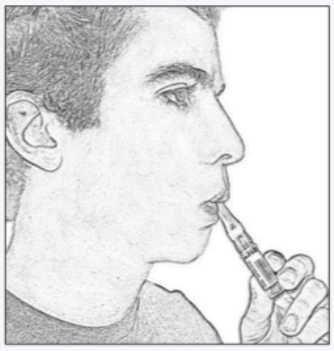
*If you are collecting saliva for biomarker analysis and think that the sample may be used at some point for genetic analysis, please see **Collection for DNA Analysis** before proceeding.*

Materials List:

1. Cryovials
2. Saliva Collection Aid
3. Bar-Coded Labels
4. Cryostorage Box (2")

*Secretory IgA & DHEA-S concentrations in saliva are affected by saliva flow rate, and α-amylase may also be affected. (6,9,10) We recommend recording mL/min during collection. See **Effects of Flow Rate and Mouth Location**, or contact us for details.*

Instructions for Collecting Saliva:



1. Remove cap from cryovial
2. Remove SCA from packaging and place securely into cryovial.
3. Instruct participants to allow saliva to pool in the mouth. Some find it helpful to imagine eating their favorite food.
4. With head tilted forward, participants should drool through the SCA to collect saliva in the cryovial. (We advise using a vial with twice the capacity of the desired sample volume.)
5. Repeat until sufficient sample is collected. **Reserve air space in the vial to accommodate the expansion of saliva during freezing**. Collection of samples to be analyzed for multiple analytes may require additional cryovials.
6. Replace cap onto cryovial.

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