Effect of Antibacterial Mouthwash on Muscle Function in Healthy Young Men and Women

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1.0 Background

Approximately $1,400,000,000 worth of antiseptic/antibacterial mouthwash is sold in the US each year.[1] Thus, although the exact figure is uncertain a large number of people must use such products. Antiseptic/antibacterial mouthwash use can improve oral health, by diminishing plaque and reducing gingivitis. However, such products may also have detrimental effects. In particular, prior research has demonstrated that antibacterial mouthwash can destroy the nitrate (NO_3^-) -reducing bacteria normally found in the mouth.[2] These facultative anaerobes reduce salivary NO_3^- to nitrite (NO_2^-), which after being swallowed and absorbed into the circulation can be further reduced by, e.g., deoxyhemoglobin, to form nitric oxide (NO).[3] NO is a key signaling molecule involved in a variety of physiological responses, including, but not limited to, the regulation of blood flow/pressure. As a result, interruption of the bacteria-dependent enterosalivary pathway of NO production via twice-daily use of chlorhexidine-containing mouthwash (i.e., Corsodyl©) for 7 d has been shown to decrease plasma NO_2^- levels by 25% and increased systolic blood pressure by 2-3 mmHg.[4] The latter change is potentially clinically significant, as it would raise the chance of dying of ischemic heart disease and stroke by 7 and 10%, respectively.[5] Similar changes in plasma NO_2^- and systolic blood pressure have been found following use of cetylpyridinium-containing mouthwash (i.e., Cepacol©).[1] It is therefore clear that chronic use of antibacterial mouthwash may have unintended negative consequences. On the other hand, use of Listerine©, an antiseptic (vs. antibacterial) mouthwash, has minimal effect on blood pressure.[1]

In addition to influencing blood flow/pressure, NO is involved in the regulation of numerous other physiological functions, e.g., neural transmission, immune function, mitochondrial respiration, etc. This includes the regulation of muscle contractility, i.e., the force and speed of muscle contraction. Specifically, NO may (or may not) slightly suppress isometric force production, by interfering with excitation-contraction coupling at the level of Ca^{2+} release and/or by directly inhibiting actomyosin via S-nitrosylation of cysteine residues.[6] Indirectly, however, NO causes an overall "slow-to-fast" shift characterized, in part, by increases in the rate of force development, maximal shortening velocity, and maximal power of both single muscle fibers and isolated muscles of animals.[6] These stimulatory effects of NO are thought to be the result of activation of soluble guanylyl cyclase and thus enhanced production of cyclic GMP.[6,7]

Previously, we have demonstrated that the acute ingestion of NO_3^--rich beet juice can *increase* plasma NO_3^-/NO_2^- and breath NO and *enhance* muscle contractile function in healthy young and middle-aged individuals,[8] athletes,[9] and patients with heart failure.[10] It is not known, however, whether a *reduction* in NO bioavailability resulting from use of antibacterial mouthwash can *inhibit* muscle function. Such knowledge is obviously relevant not only in the context of athletic performance but also to clinical populations (e.g., the elderly) in whom baseline muscle strength, speed, and power are impaired, thus limiting activities of daily living and increasing the risk of falls.

2.0 Rationale and Specific Aims

Specific Aim #1: To determine the effects of antibacterial mouthwash on maximal knee extensor speed and power in healthy young men and women.

We hypothesize that use of an antibacterial mouthwash will significantly reduce maximal knee extensor speed and power (primary endpoints). This will be accompanied by significant reductions in salivary NO_3^-/NO_2^- and breath NO levels (secondary endpoints).

We will test these hypotheses using isokinetic dynamometry and salivary and breath sampling in **n = 24** healthy young men and women (n = 8/group).

3.0 Inclusion/Exclusion Criteria

Inclusion:

- Men and Women age 18-30 years old
- No current use of mouthwash

Exclusion:

- Men and Women <18 or 30> y of age
- Unable to provide informed consent
- Current users of mouthwash
- Current antibiotic use
- Current smokers
- Stage II hypertension (resting blood pressure >140/>90)

An answer of yes to any of the seven questions on the first page of the Physical Activity Readiness Questionnaire (PAR-Q) indicating that the subject is not physically ready for exercise without a medical exam. These exclusions include the following:

- If participant's doctor has ever said that he/she has a heart condition and that he/she should only do physical activity recommended by a doctor
- Pain in chest when doing physical activity
- In past month, chest pain when not doing physical activity
- If participant has ever lost balance because of dizziness or has ever lost consciousness
- Bone or joint problem that could be made worse by change in physical activity
- Currently on prescribed drugs for blood pressure or heart condition.
- If the participant knows of any other reason why he/she should not do physical activity.

Session exclusion criteria:

- These criteria do not apply to the first study visit, only for visits 2 and 3. Subjects will be instructed to not smoke, perform exercise on the day of the visit, and to not attend the visit in a fasting or hungry state. They will also be asked to not consume caffeine or alcohol or use chewing gum in the 24 hours prior to the visit. They will be asked to avoid consuming foods (e.g., spinach, beets, collard greens) high in NO_3^- during the evening prior to visits. Subjects will also be instructed to not use any other mouthwash products other than those provided during the study. Subjects will be excluded from a session if their resting blood pressure is greater than 140/90.

4.0 Enrollment/Randomization

Study participants will be given a consent form ahead of the screening visit. The study participant will be given time to review the consent form, and a study staff member will contact the potential subject to answer any questions they may have. If interested, then the potential subject will be scheduled for a meeting where the consent form will be reviewed again. The potential subject will be given as much time

as they wish to consider participation before signing the consent form and if they wish to move forward with participating, then the consent form will be signed by both the study participant and the person who reviewed the consent and obtained the participant's signature. A copy of the signed consent form will be provided to the subject. Both the participants and the investigators will be blinded to the order of treatment, which will be randomized in four blocks of four using randomization.com.

5.0 Study Procedures

Study Design

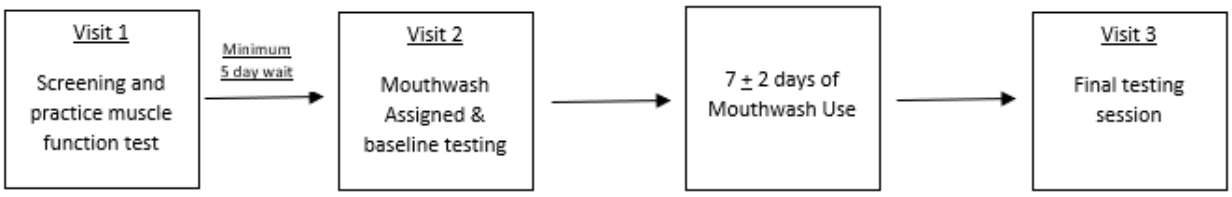

Figure 1. Overall study design.

Study Procedures

Study Visit 1 (Screening and muscle function testing practice): The consent statement will be reviewed with the subject including the procedures of the study and the risks of participating. Subjects will also be instructed on the requirements while in the study including not exercising on the day of study visits prior to the visit and to not attend the visit in a fasting or hungry state. They will also be asked to not consume caffeine or alcohol or use chewing gum in the 24 hours prior to the visit. They will be asked to avoid consuming foods (e.g., spinach, beets, collard greens) high in NO_3^- during this 24 hour period and to not use any other mouthwash products other than those provided during the study. Subjects who decide to participate will sign the consent and complete a screening to determine if they are eligible. If they are determined to be eligible they will then be asked to practice the muscle function test that will be used during the study that assesses the contractile function, i.e., maximal knee extensor speed and power, of the knee extensors of the subject's dominant leg. This is done using a Biodex 4 Isokinetic Dynamometer (Biodex Medical Systems, Shirley, NY).

Study Visit 2 (Baseline Testing): participants will be asked to return and undergo baseline testing. This will include 1) collection of a saliva sample for a measurement of NO_3^- and NO_2^-, and, 2) assessment of the muscle function of the knee extensors (see Study Methods). The subjects will then be randomly assigned (via randomization.com) in four blocks of four subjects each to receive either an antibacterial mouthwash (Cepacol©, Reckitt Benckiser, Parsippany, NJ) or, as a control solution, an alcohol-free "natural" mouthwash (Tom's of Maine©, Kennebunk, ME). Participants will be asked to rinse their mouth as directed on the product's packaging for 30 s twice per day for 7 days and to record the usage of the mouthwash on a provided form. Subjects will then return for Study Visit 3. Note that although the two products differ significantly in terms of packaging, color, taste, etc., the subjects will be told that the study is simply to compare the two products, with no expectation as to the outcome.

Study Visit 3 (Final Testing), participants will be asked to return to undergo the same procedures as described above for Study Day 2.

Study Methods

Measurement of Salivary Nitrate & Nitrite: Saliva samples will be obtained using a salivary sample collection kit (Salivette, Sarstedt, Newton, NC). The NO_3^- and NO_2^- of each sample will be determined using high performance liquid chromatography (ENO-30, Eicom USA, San Diego, CA).

Measurement of skeletal muscle contractile functions: A Biodex 4 System isokinetic dynamometer (Biodex Medical Systems, Shirley, NY) will be used to measure the subject's muscle contractile properties as previously described. Briefly, participants will be asked to perform three maximal isometric knee extensions with their dominant leg at a joint angle of 70 degrees. Each isometric contraction will last 5 s, with 15 s of rest in between. After 2 min of additional rest, the subject will perform three maximal isokinetic efforts at angular velocities of 1.57, 3.14, 4.71, and 6.28 rad/s, with 2 min of rest between each set. To eliminate artifacts, the dynamometer data will be "windowed" to isolate the isokinetic phase and smoothed using using a nine point weighted moving average filter, after which the highest torque generated at each velocity will be multiplied by the velocity to determine the peak power at that velocity. The resulting power-velocity data will then be fit with a 2nd order polynomial function (i.e., $y=ax^2+bx+c$) which will be solved to determine the subject's maximal knee extensor velocity (Vmax; $=-b/a$) and power (Pmax; $=(4ac-b^2)/4a$).

Risks

Likely: Muscle soreness from using the Biodex Isokinetic Dynamometer. Subjects may have muscle soreness in their quadriceps because the quadriceps is responsible for extension of the knee. They might also experience soreness in their knee joint. Fatigue may also be experienced from exercising the muscles.

Very rarely, an exercise test, such as the muscular function test, may be associated with serious complications including, but not limited to:

- Fainting and disorders of the heart beat (too fast or too slow) which may require hospitalization;
- heart attack, stroke, or death.

The risk of arrhythmia and heart attack are rare and the risk of death during exercise testing is extremely rare. The estimated risk of a fatal event during maximal exercise testing ranges from 0.3 to 0.8 per 10,000 tests. Approximately 1.4 nonfatal events occur per 10,000 tests.[11] The risk should be lower in this study as these subjects since they are young, apparently healthy adults have been screened for signs and symptoms of cardiovascular disease.

We will make every effort to minimize these rare risks by observing and monitoring during testing. However, no guarantees can be made.

Mouthwash may cause surface staining of the teeth, an increase in systolic blood pressure, and the taste may be unpleasant to subjects.

Another risk of this study is the possible loss of confidentiality, which is minimal. Even though the risk is small, a link exists between your protected health information and your sample. In addition to the risks listed above, there may be some unknown or infrequent and unforeseeable risks associated with participation in this study. You will be informed in a timely manner of any new information, findings or

changes to the way the research will be performed that might influence your willingness to continue your participation in this study.

There also may be other side effects that we cannot predict.

Mitigation of Risks

To minimize the risk of muscle soreness, subjects will warm up by pedaling a stationary cycle for 5 min against minimal resistance.

To minimize the risk of exercise testing, subjects will be screened using an accepted exercise risk screening tool (Physical Activity Readiness Questionnaire - PAR-Q) to ensure subjects are at minimal risk. High risk individuals (those reporting cardiovascular, pulmonary, or metabolic disease) will be excluded from this study.

If any of the following occurs, the subject will be excluded from participating in any test involving physical activity on that day:

- Resting blood pressure systolic > 140 mm Hg or diastolic > 90 mm Hg
- Resting heart rate > 120 beats/min or < 45 beats/min

Blood pressure and heart rate will be taken before and after the isokinetic test.

During exercise testing subjects will be instructed on proper technique including avoiding the valsalva maneuver (holding their breath during exertion) which has been shown to increase blood pressure. Subjects will be instructed that they may stop the tests at any time with no adverse consequences. Subjects will also be instructed to talk with the experimenter about any discomforts that occur during the physical tests. If for any reason the participant reports an injury, chest pain, excessive shortness of breath, abnormal heart rhythm or dizziness, the test will be terminated and they will be referred to their doctor, or the PI will call the doctor or other health care provider.

Additionally, all exercises will occur in an air conditioned facility because environmental extremes can be poorly tolerated during exercise. Also, every precaution will be taken to provide fluids, rest and other measures to insure each participant is comfortable, safe, and secure in the testing/exercise environment. In the unlikely event that an emergency may occur, a CPR certified person will be available for all testing sessions.

To simplify scheduling, the isokinetic muscle assessments will be completed in either *The National Institute of Fitness and Sport* (location of the human performance laboratory), or on an identical system in IU-Health University Hospital, 550 University Boulevard, Indianapolis IN 46202, on the 5th floor adjacent to the Clinical Research Center. *The National Institute of Fitness and Sport* has set up provisions to respond to a medical emergency which include: 1) Evaluate the condition of the individual (consciousness, breathing, and pulse), 2) Call 911 or instruct another staff or lab member to call 911 and state "We have an emergency situation at our facility. The individual appears to have (give

condition). Please send a paramedic team to the National Institute for Fitness and Sport located at 250 University Blvd. We are South of the Natatorium on the IUPUI campus. Someone will meet you at the main entrance to the building." For a life threatening emergency, we are instructed to dial "799" for a NIFS building page. Say "There is a medical emergency in the (given location)." Then hang-up. and 3) initiate CPR procedures if needed. A fully equipped automated external defibrillator is also available in the building. The NIFS emergency procedures will be located in the lab and all research staff will be trained on the emergency procedures. Within the hospital, emergency equipment and trained personnel are available to deal with any emergency.

To minimize the loss of confidentiality, all records will be kept in a locked file cabinet inside an office which has locking doors. All study procedures will be completed behind closed doors inside the Human Performance Laboratory located in the National Institute of Fitness and Sport at 250 University Boulevard, Indianapolis IN 46202, or in IU-Health University Hospital, 550 University Boulevard, Indianapolis IN 46202, on the 5th floor adjacent to the Clinical Research Center.

6.0 Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others

The following standard definitions will be used for this study:

Adverse event (AE): Any untoward or unfavorable medical occurrence in a human study participant, including any abnormal sign (e.g. abnormal physical exam or laboratory finding), symptom, or disease, temporally associated with the participants' involvement in the research, whether or not considered related to participation in the research.

Serious Adverse Event (SAE): Any AE that results is place the participant at immediate risk of death from the event as it occurred, or results in inpatient hospitalization, prolongation of existing hospitalization, persistent or significant disability/incapacity, congenital abnormalities or birth defects, or death.

Unanticipated problem: As Defined by DHHS 45 CFR part 46, any incident, experience, or outcome that meets all of the following criteria: 1) is unexpected, in terms of nature, severity, or frequency, given (a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the study population; 2) is related or possibly related to participation in the research (in this guidance document, possibly related means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and 3) suggests that the research places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

AEs will be graded according to the following scale:

- Mild: An experience that is transient, and requires no special treatment or intervention. The experience does not generally interfere with usual daily activities. This includes transient laboratory test alterations.
- Moderate: An experience that is alleviated with simple therapeutic treatments. The experience impacts usual daily activities. Includes laboratory test alterations indicating injury, but without long-term risk.

- Severe: An experience that requires therapeutic intervention. The experience interrupts usual daily activities. If hospitalization (or prolongation of hospitalization) is required for treatment it becomes an SAE.

Attribution of AEs and SAEs will be categorized as:

- Not related: The AE is clearly not related to the study procedures (i.e., another cause of the event is most plausible and/or a clinically plausible temporal sequence is inconsistent with the onset of the event).
- Possibly related: An event that follows a reasonable temporal sequence from the initiation of study procedures, but that could readily have been produced by a number of other factors.
- Related: The AE is clearly related to the study procedures.

Reporting of AEs, SAEs, and unanticipated problems will follow the guidelines of the IU HRPP Policy on Reportable Events. Specifically, the following events will be reported promptly (i.e., within five business days) to the IRB:

- *AEs (including SAEs)* that are assessed by the PI or co-investigators as (1) unexpected, (2) related or possibly related to participation, AND (3) suggests that the research places subject(s) or others at greater risk of harm than was previously known;
- *Major protocol deviations* that may, in the opinion of the PI, (1) impact subject safety, (2) affect the integrity of the data, OR (3) affect study participant' willingness to participate in the study;
- *Noncompliance*, which includes any action or activity associated with the conduct or oversight of the research that fails to comply with federal or state regulations, institutional policies governing human study participant research, or the requirements or determinations of the IRB.

Unanticipated problems that do not meet the criteria for prompt reporting will be reported at time of protocol renewal to ensure the IRB has a full understanding of the conduct of the research.

7.0 Study Withdrawal/Discontinuation

A participant may withdraw from the study at any time verbally or by providing this request in writing as described in the informed consent document. As outlined in the consent document, if the participant/patient wishes to withdraw consent, the PI will:

- no longer use and take reasonable steps to destroy all saliva samples and information
- not take back any research / analyses already completed

8.0 Statistical Considerations

Non-PHI primary data will be stored electronically on both the measurement instruments and as Excel and/or Prism data files on the PI's university-supplied, DUO-protected laptop computer. The laptop is backed up automatically by UITS, whereas the instrument data files will be backed up manually on a monthly basis. Other data sources include the subjects' sex, age, and body mass will be collected as paper records and merged with the primary data as needed. Data quality will be assured by random auditing of single entry results and extraction/examination of data used for subsequent statistical analysis every 6 months.

Statistical analyses will be performed using Prism© version 8.0.1 (GraphPad Software, La Jolla, CA). Normality of data distribution will first be tested using the D'Agostino-Pearson omnibus test. Analysis of individual endpoints will then be conducted using a mixed model analysis of variance (ANOVA) approach, with treatment and trial number as fixed effects and subject as a random effect to account for repeated measurements. Post-hoc testing will be performed using the Šidák-Holms multiple comparison approach. A P value of <0.05 will be considered significant.

As this is a pilot experiment, we lack the preliminary data necessary to perform a formal sample size/power analysis. In previous research,[8] however, we have observed than an *increase* in NO bioavailability resulting from ingestion of NO_3^--rich beet juice *increased* (P<0.05) Vmax and Pmax by 11.4±16.5 and 6.3±8.7%, respectively. Assuming similar intersubject variability when NO bioavailability is *reduced* by use of an antibacterial mouthwash, our chosen sample size will allow us to detect any effect size ≥1.3 with an alpha of 0.05 and 1-beta of 0.80.

9.0 Privacy/Confidentiality Issues

All study activities will be performed within areas respective of the participants' right to privacy.

Although there can be no absolute guarantee of confidentiality, every practical precaution will be taken.

Each study participant will be assigned a unique ID. Samples and information will be de-identified using this unique ID.

10.0 Follow-up and Record Retention

Study recruitment will be ongoing. The duration of the entire study is expected to be about 8 mo.

Any remaining saliva samples will be de-identified and discarded. The de-identified data will be retained on computer files indefinitely. Hard copy study documents will be kept in a locked, file cabinet in a locked room. De-identified electronic study information will be stored on a specified password-protected network that is backed up daily. Only the study team and the relevant personnel will have access. Files will be kept on site until 2 y after study completion, and then sent to a secure archiving facility. Files will be kept as long as required by law.

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