



Linnæus University

Faculty of Health and Life Sciences

Degree project work

Effect of Antibacterial Mouthwash on Basal Metabolic Rate in Humans

A Randomized, Double-blinded, Cross-over Study

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Subject: Chemistry
Level: First Cycle
Nr: 2013:L6

Effect of Antibacterial Mouthwash on Basal Metabolic Rate in Humans

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Examination Project Work, *Chemistry* 15 ECTS

Bachelor of Science

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The Examination Project Work is included in the study program Nutrition and Food Science 180 ECTS

Abstract

The use of mouthwash is a common complement to oral care. However, the physiological implication of this use, besides of effects on oral hygiene, is poorly known. The research of the gut micro flora and its implications on the host is a very active area of research today. Many important connections between the gut micro flora and obesity and diabetes have been found. These billions of bacteria are part of the immune system, they produce essential vitamins and they make inaccessible polysaccharides more digestible to the host, just to mention a few of their symbiotic roles for the host.

A less explored area is the micro flora in the oral cavity. On the back of the tongue, anaerobic bacteria can reduce dietary nitrate to nitrite which then further can be reduced to nitric oxide, NO. NO is important in several important biological functions, e.g. as a signal substance, vasoregulation, mucus production and antibacterial effects. Vegetables as beetroot and spinach are dietary sources with a high nitrate content. Also drinking water and processed meats can be of relevance. Nitrite is added to processed meat for the prevention of botulism but also adds taste and color.

Experiments on humans indicate that mitochondrial efficiency increases after nitrate load, manifested as a decreased oxygen demand during physical exercise. This can also be relevant under conditions where the mitochondrial function is impaired, such as in diabetes and cardiovascular diseases.

First a pilot study was made to evaluate the nitrate reducing effect from the antibacterial mouthwash. The mouthwash proved very effective. The concentrations of nitrate and nitrite in saliva was analyzed by HPLC and saliva from the antibacterial treatment showed greatly reduced concentrations of nitrite and high concentrations of nitrate. Saliva from placebo mouthwash showed high concentrations of nitrite and low concentrations of nitrate as expected.

To study the importance of oral bacteria on metabolism, we performed a randomized, cross-over double-blinded study with 19 healthy males between 22-43 years. During two separate three-day periods they used an antibacterial and placebo mouthwash, respectively. On the fourth day their basal metabolic rate (BMR) was measured with an indirect calorimetric system. Moreover, samples from saliva, urine and blood were collected but these results are not included in this thesis. An earlier, unpublished study has demonstrated that nitrate administration reduces the basal metabolic rate. Accordingly, our aim was to study potential effects on the basal metabolic rate following reduction of the number of oral bacteria by aid of antibacterial mouthwash. Our hypothesis was that the reduced availability of nitrite would decrease the availability of NO in the body and manifest as an increased basal metabolic rate.

The results from indirect calorimetry measurements showed no significant difference between placebo and antibacterial mouthwash, but there may be confounding factors. Further study is needed to assess the potential effects on host metabolism by these bacteria.

SAMMANFATTNING

Att använda munsköljmedel som ett komplement till munhygien är vanligt idag. Även om den antibakteriella effekten är väldokumenterad så är kunskapen om eventuella övriga effekter av munsköljmedel på kroppen ofullständigt kända. Forskningen kring mikrofloran i mag-tarmkanalen pågår för fullt med stort intresse och kopplingar till bland annat diabetes och fetma har hittats. Mag-tarmfloran har stor betydelse för värden, exempelvis utgör den en stor del av vårt immunförsvar, den underlättar metabolismen av annars odigererbara polysackarider och syntetiserar vitaminer. I munhålan finns anaeroba bakterier som gör om nitrat till nitrit. Nitrit i sin tur kan omvandlas till kväveoxid, NO, som har flera viktiga fysiologiska funktioner. Bland annat fungerar NO som signalsubstans, den reglerar blodflöde, stimulerar till mucusproduktion och har dessutom antibakteriella egenskaper. Vi får i oss nitrat via kosten där rödbetor och gröna bladgrönsaker såsom spenat och ruccolasallad är exempel på grönsaker med särskilt högt nitratinnehåll. Det finns indikationer på att mitokondrierna blir effektivare vid nitratintag och detta har visat sig genom minskad syreåtgång vid fysiskt arbete. Detta kan också vara av intresse vid sjukdomstillstånd där mitokondrierna fungerar dåligt som exempelvis vid hjärt-kärlsjukdomar och diabetes.

I denna studie har vi låtit 19 friska män mellan 22-43 år använda bakteriedödande munsköljmedel under en tredagarsperiod för att sedan mäta deras basalmetabolism. Då en tidigare, ännu ej publicerad, studie visat att nitratintag minskar basalmetabolismen ville vi undersöka vad som hände om bakterierna slogs ut, dvs. den bakteriella nitratreducerande effekten skulle utebli. Vår hypotes var att den minskade tillgången på nitrit skulle påverka tillgängligheten av NO i kroppen och detta skulle manifestera sig som en ökad vilometabolism uppmätt genom indirekt kalorimetri.

Eftersom den efterföljande studien var beroende av att vi hade ett välfungerande bakteriedödande munsköljmedel gjordes en pilotstudie där sänkningen av den nitratreducerande förmågan testades. Munsköljmedlet visades vara effektivt då vi genom HPLC kunde se att nivåerna av nitrit i saliv kraftigt hade minskat jämfört med placebo som hade höga nivåer av nitrit. På samma sätt såg vi att nitratnivåerna i saliv steg efter munskölj, med stor sannolikhet på grund av den minskade nitratreducerande förmågan. Ingen skillnad på basalmetabolismen kunde detekteras hos deltagarna efter att de använt placebo- respektive antibakteriellt munsköljmedel.

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1. INTRODUCTION

Antibacterial mouthwash is used on a daily basis by many millions of people world-wide, as a complement to oral hygiene, e.g. to prevent bad breath. Reportedly, 30-45% of adults in USA and the UK use antibacterial mouthwash on a regular basis (1, 2). However, although mouthwash is effective with regard to oral hygiene, very little is known about the long term health effects of such use. The microflora has an intricate relationship with the host and antiseptic treatment effectively reduces the bacterial activity in the mouth. Recent advances in microbiology have shown important metabolic effects of the gut microflora. In the study “Richness of human gut microbiome correlates with metabolic markers” the researchers found that individuals with a low bacterial richness was associated with a higher content of adipocytes, insulin resistance, dyslipidaemia and a more pronounced inflammatory phenotype compared to individuals with a high bacterial richness (3). If oral bacteria has the same impact on overall health this finding would have broad implications on the use of antibacterial mouthwash.

Today obesity is a quickly expanding problem around the world. Although our genes and environmental factors play a role, the major underlying factor to the development of obesity is an excess of energy intake as related to expenditure. Scientists are now searching for other factors for therapeutic and preventive uses against obesity (4). One such preventive strategy is to explore ways of modulating energy expenditure.

In our gut trillions of anaerobic bacteria and archaea live and perform metabolic actions which make inaccessible polysaccharides accessible and produce essential vitamins (5). The amount of energy from the diet can increase by actions of the microbiota. Moreover, an altered gut microbiota is associated with obesity and type-2 diabetes (6). Bäckhed and coworkers found environmental factors associated with the regulation of fat storage in the gut microbiota when comparing germ-free and conventionally raised mice (7). When germ free mice received gut microbiota from obese mice their gain in body fat was significantly higher as compared to that of germ free mice which had received gut microbiota from lean mice (8).

The wide-spread use of antibacterial mouthwash (9) in combination with the fact that oral commensal bacteria are of central importance in the conversion of dietary nitrate (NO_3^-) and

nitrite (NO_2^-), which is further metabolized to nitric oxide (NO) by different pathways (2) makes this an interesting field to explore. NO participates in several biological functions including vasoregulation, vascular homeostasis, cell signaling events, modulation of mitochondrial respiration and host defense (2, 10). The exploration of gut microbes is in progress and the body of knowledge is constantly increasing. However, little is known in this context about oral bacteria and their eventual impact on the host physiology and metabolism remains to be explored.

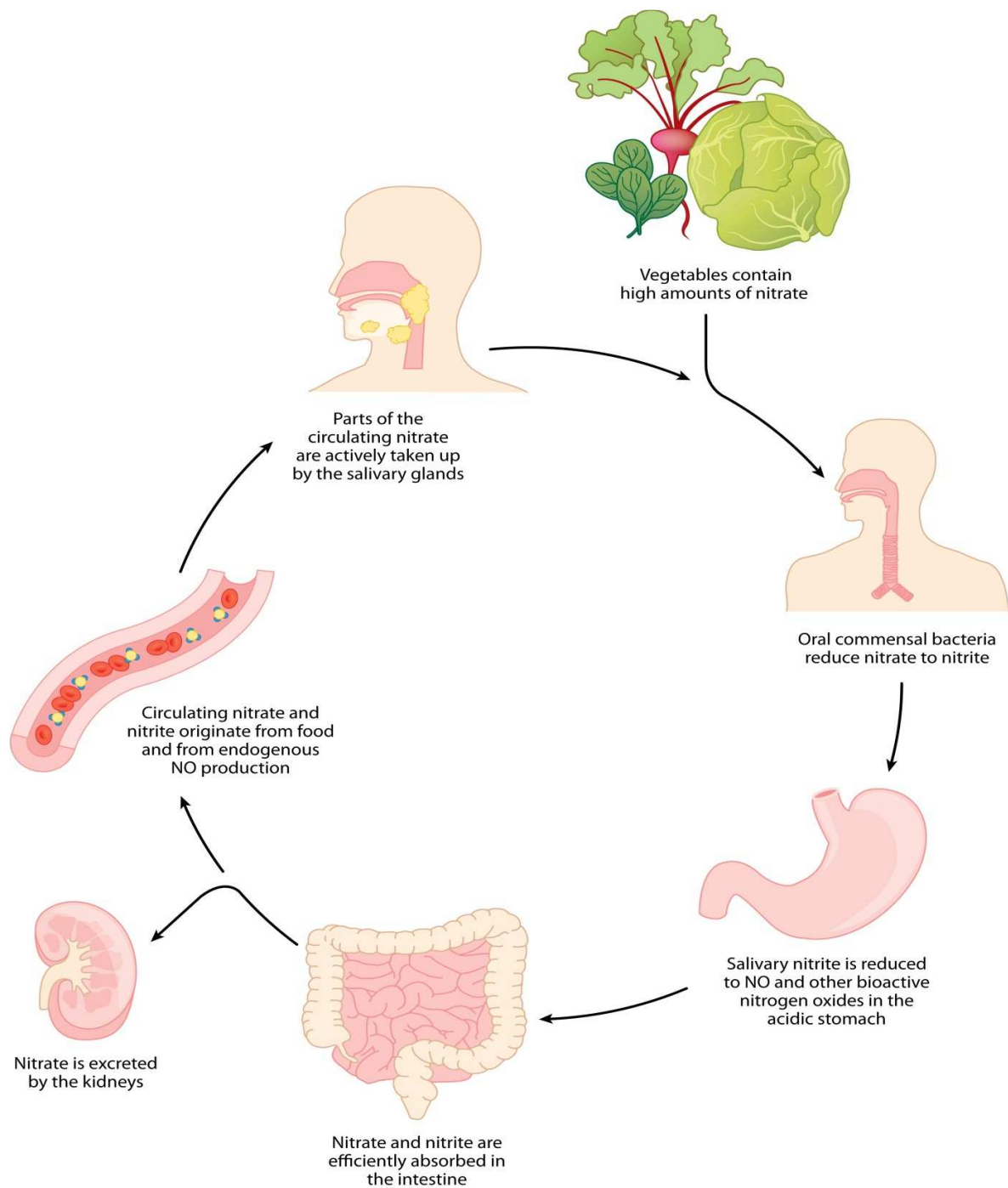
1.1. Dietary sources of nitrate and nitrite

Green leafy vegetables and beetroots are examples of dietary sources with very high content of inorganic nitrate (11). Vegetables account for 60-80% of the dietary nitrate (12). Drinking water (15-20%) and food stuff such as processed meats (10-15%) to which nitrite is added for prevention of botulism and improvement of taste and color, are examples of other sources of dietary nitrate and nitrite (13). Nitrate and nitrite are also found in cigarette smoke and in car exhausts (14).

Our intake of nitrate and nitrite is strictly controlled due to the claimed risk for cancer and methemoglobinemia in infants. The fear of carcinogenic activity might be exaggerated as a causal link between a high nitrate intake and cancer is lacking (15). At the same time a high intake of fruit and vegetables is recommended. These recommendations do not take into account that the contents of nitrate can vary much depending on which vegetables that are ingested (12).

1.2. Nitrate, nitrite and NO

Dietary nitrate is efficiently taken up by the blood where up to 25 % is concentrated in the salivary glands (see figure 1.), and a nitrate concentration at least tenfold higher than in plasma has been found (14). From the salivary glands the nitrate is secreted into the oral cavity where nitrate is reduced to nitrite by anaerobic bacteria. These bacteria belong to the symbiotic microflora and in particular, these are found in the crypts back of the tongue. (16). They are facultative anaerobic bacteria and use nitrate as a terminal electron acceptor during respiration. The levels of nitrite in saliva is more than a thousand times higher than in plasma (17).



A Weitzberg E, Lundberg JO. 2013.
R Annu. Rev. Nutr. 33:129–59

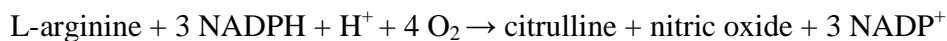
Figure 1. Dietary nitrate is absorbed in the small intestine and reach the bloodstream. The circulating nitrate is partly excreted in the kidneys, but 25 % is taken up by the salivary glands and excreted with the saliva. Oral bacterial converse parts of the nitrate to nitrite. The saliva enters the acidic stomach and the nitrite is further

metabolized to NO. Parts of the nitrite pass the stomach intact and will reach the circulation and tissues, where it will be further reduced to NO (18). (Courtesy of Jon Lundberg)

When the nitrite reaches the acidic stomach some of it will be further reduced to nitric oxide (19). NO stimulates mucus production and has antibacterial features. Nitrite can also react with amines and form potentially harmful nitrosamines (20).

Nitrite is taken up by the blood and will via the circulation reach organs and tissues where it can be reduced to NO. There are several pathways for the reduction of nitrite, both non-enzymatic and enzymatic. Deoxyhemoglobin in blood and deoxygenated myoglobin in tissues, xanthine oxidoreductase, Cytochrome *c* oxidase in mitochondria, eNOS and simple reduction by protonation are some of the many putative pathways for nitrite reduction to NO (18).

Apart from the non-enzymatic reduction of nitrate to nitrite and NO, a well-known enzymatic pathway of NO production exists: Nitric oxide synthases (NOS) is a family of enzymes that synthesizes nitric oxide in the body. These enzymes catalyze the oxidation, (see below) of L-arginine to L-citrulline and NO with oxygen as co-substrate. The nitric oxide synthases exist in three isoforms; neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) (17).



There is an important difference between the classical NOS dependent reaction and the $\text{NO}_3^- - \text{NO}_2^- - \text{NO}$ – pathway. The classical reaction is inhibited by hypoxia but the $\text{NO}_3^- - \text{NO}_2^- - \text{NO}$ -pathway on the other hand is favored during hypoxia. Further, the conversion from NO_2^- to NO is favored at low pH and may function as a complement to the classical pathway, which is oxygen dependent (21, 22).

1.3. Established effects of dietary nitrate administration

Various biological effects of nitrate administration have been identified; these include reduced oxygen cost during exercise (17), more efficient mitochondria (as described below) (23), reduced blood platelet formation (24) and brings improved revascularization in chronic ischemia (25).

Ingestion of dietary nitrate reduces blood pressure acutely but it needs to be studied in a long-term fashion (18, 24, 25).

It is now clear that supplementation with dietary nitrate can alter the physiological response to exercise and improve physical performance (23, 26, 27).

In a recently unpublished study from our laboratory supplementation with dietary nitrate a significant reduction of the basal metabolic rate (BMR) with 4.4% was observed, and increased nitrate and nitrite concentrations in both plasma and saliva (28). Earlier reports from our laboratory indicate improved human skeletal muscle mitochondrial efficiency after nitrate supplementation. This effect was explained by reduced proton leakage over the inner mitochondrial membrane due to down regulation of two mitochondrial proteins; adenine nucleotide translocase (ANT) and uncoupling protein 3 (UCP-3) (17). The P/O ratio, which is the amount of oxygen consumed per ATP produced, is the classical way to measure the mitochondrial oxidative phosphorylation efficacy (29), the P/O ratio was improved after nitrate administration.

1.4. Basal metabolic rate

Basal metabolic rate (BMR) is the energy needed for the body to maintain basic processes. For most individuals in western societies BMR is the largest components of the total body energy demand (30). There is a high variation in BMR between individuals and the reasons are unknown (31). However, several studies have shown that BMR is well correlated to the fat free mass (FFM) (32-34). If the fat mass (FM) is large it can contribute to the BMR. However, the contribution is yet a small part (35). BMR may also be influenced by age, sex and hormones such as triiodothyronine (T_3) and thyroxine (T_4).

In a study with 150 subjects the BMR was shown to vary significantly between the subjects but little for each individual (31). The study concluded that BMR is largely affected by FFM whereas the contributions from FM and age are minor parts. However, a large part could not be explained.

Over- and underproduction of thyroid hormones leads to changes in BMR (36, 37) but if changes within the euthyroid range have a significant impact on BMR is uncertain. Some studies have demonstrated a connection between T_3 and BMR (38, 39), while no connection has been observed in others (40). Nitrate, among other anions, can work as a competitive inhibitor for thyroid uptake of iodide by binding to the sodium-iodide symporter on the thyroid follicles. Hence, nitrate can possibly reduce the levels of T_3 and T_4 (41).

1.5. Two opposing theories how metabolic rate affects life span and ROS production

”Rate of living” and ”Uncoupling to survive” are two different, completely contrary predictions linking energy metabolism and aging. Both theories highlight the role of reactive oxygen species (ROS). The “rate of living” theory (42) associates high metabolism with a shorter lifespan. The theory is based on the observation that a higher oxygen metabolism results from an increased flux of electrons in the mitochondrial electron transport system (ETS). Of the total oxygen consumption a set amount (0.1-4%) is believed to be used for the formation of ROS. A lower energy metabolism would give a lower ROS formation, meaning less molecular damage and therefore a slower aging rate.

The ”Uncoupling to survive” (43) hypothesis suggests a positive association between lifespan and oxygen metabolism. In the ETS, ROS is primarily produced at complex I and complex III (43). The production of ROS at complex III increases if the membrane potential in the mitochondria increases. Thus, a lower membrane potential would effectively decrease ROS production. A lower membrane potential can be achieved by releasing some of the protons through the inner mitochondrial membrane, i.e. uncoupling of the mitochondria. There are several proton leak pathways, two of them are adenine nucleotide translocase (ANT) (44) and the uncoupling proteins (UCP) (45).

2. AIM

First a pilot study was performed to examine the amount of nitrates and nitrite after mouthwash with placebo and antiseptic chlorhexidine mouthwash to determine the efficacy of the

antibacterial effect. The end-points of this pilot study were to confirm that the nitrate reducing capacity in the oral cavity were lost using this protocol.

In a randomized, double-blinded, cross-over study we aimed to investigate the effects of antibacterial mouthwash during a three-day period on the basal metabolic rate in 19 healthy non-tobacco using male volunteers.

It is known that the mitochondrial efficiency is one factor that determines BMR (46). With this new data as a background, we intended to investigate if normal levels of nitrate and nitrite were important in the control of BMR. Therefore, we aimed to investigate if the nitrate reducing capacity of oral bacteria, without concurrent nitrate supplementation, is an important determinant of BMR in health male subjects. Our hypothesis was that the antibacterial mouthwash effectively reduced saliva and plasma levels of nitrite and thus increased BMR.

3. MATERIALS AND METHODS

This work contains a pilot study where the aim was to evaluate the antibacterial effect of chlorhexidine based mouthwash with respect to the levels of nitrate and nitrite. The analysis method was HPLC.

The basal metabolic rate was measured using indirect calorimetry.

3.1. Study population

Pilot study

Four healthy volunteers (three males, one female, range 30-49 years) participated in this study.

A baseline saliva sample was collected, immediately followed by antibacterial mouthwash or placebo for one minute. Saliva was again sampled after 60, 120 and 180 minutes. This procedure was followed with placebo mouthwash and chlorhexidine mouthwash. During these three hours the participants were fasting.

The mouthwash and metabolic rate study

19 healthy, non-tobacco using, male volunteers were recruited with mean age 29 (range 22-43) and mean weight 82.4 kg (range 71.2-107.35). Variations in the metabolic rate because of the menstrual cycle are significant (47), therefore women were excluded. Recruitment was achieved via personal networking. The blinding was performed so the primary researcher and participants were unaware of its content.

3.2. The mouthwash

The antibacterial mouthwash ingredient is based on the ingredients in Corsodyl® with chlorhexidine as active substance (48). The ingredients used in the antibacterial mouthwash are chlorhexidine (0.2 %), ethanol 95 %, menthol and distilled water. The placebo mouthwash had the same mixture except for chlorhexidine. The amount of menthol was adjusted so it effectively masked the taste of chlorhexidine.

The taste and antibacterial effect were tested in the pilot study in a cross-over design.

3.3. Mouthwash and BMR

During two separate three-day periods the subjects were instructed to rinse their mouth with the 10 ml mouthwash for two minutes, three times a day after a meal. During these three days they were instructed to follow a nitrate-restricted diet, i.e. they were told to avoid green leafy vegetables, beetroot and cured meats. On day three they were also instructed to avoid protein rich food and to be physically inactive. They were also told to start fasting from 8.00 pm. The next morning (Day 4) they were instructed to wash their mouths with mouthwash one last time immediately when they woke up. Fasting and physically inactive they were told to come to the lab for measuring their basal metabolic rate by indirect calorimetry (see table 1). Each visit lasted two hours.

Table 1. Schematic view of the mouthwash protocol, exactly the same procedure was followed for both test trials (placebo and chlorhexidine mouthwash). The test trials were separated with a minimum of a three day washout period.

Day	Mouthwash	Diet	Physiological activity
1	Three times	Nitrate restricted	No restrictions
2	Three times	Nitrate restricted	No restrictions
3	Three times	Nitrate and protein restricted diet. Fasting from 8.00 pm	No exercise, as physical inactive as possible
4		Fasting until test is finished (no coffee/tea)	Arrive to the lab in a physical inactive manner

3.4. Experimental protocol

Upon arrival to our laboratory our study participants were first instructed to empty the bladder (not collected), and then body weight was measured on a digital scale to the nearest 50 g, where after they drank 5 ml of water per kg bodyweight. The test leader was talking to the participants in a calm manner informing again what was going to happen during the trial. The BMR was measured at 22-23 °C with an indirect calorimetric system connected to a ventilated hood in a quiet room just including the test leader and one participant at a time. The participant was instructed to lie down on the bed without a pillow, lying completely still and quiet, but staying awake during the 30 minutes of measurement.

Before each measurement the ambient conditions were measured and the gas analyzers and the inspiratory flow meter were calibrated.

A blood sample was taken about one hour after the subject had emptied the bladder. Saliva was collected and stored on ice while the samples were transferred to and stored in -80 °C freezer. The tubes in which blood were collected for nitrate and nitrite analysis were checked for nitrate

contamination. Immediately after the blood collection, the blood from these tubes was transferred to a falcon tube with 0.72 μ L nitrite-free EDTA and then immediately centrifuged for 10 minutes in 4 °C to avoid auto-oxidation of nitrite. Immediately after centrifugation the plasma was collected and transferred on ice to the – 80 C freezer.

The first test was followed by an at least three day wash-out period before the second test trial, i.e. if the first trial was on a Monday, the second trial was earliest the following Monday. The first and second test days were performed at the same time of the day.

3.5. Equipment

The basal metabolic rate was measured with an indirect calorimetric system. A ventilated hood is placed over the face of the person and the expired air is led into an instrument which measures ventilation and volume of carbon dioxide and oxygen. The method is based on the difference in inspired and expired air and accounts for the amount of oxygen which is taken up and the amount of carbon dioxide produced in the body.

3.6. Molecular analysis

In the pilot study nitrate and nitrite concentrations in saliva were analyzed with HPLC. Nitrate and nitrite concentrations in plasma and saliva from the BMR study have not yet been analyzed because of time constraints.

3.7. Statistical analysis

Graph Pad Prism 5.0 was used for the statistical analyses. P values <0.05 were considered significant. Students paired t-test was used to analyze group differences for single measurements. A one-way ANOVA was used for repeated measures and time effects.

3.8. Ethical considerations

For this work; results from blood, urine and saliva are not included but the participants in this work did leave such samples and therefore this aspect will be considered.

The mouthwash with the antibacterial agent chlorhexidine can cause mild pain in the mouth and a loss of taste but will go back to normal after the use of mouthwash stops (48).

A blood sample was taken and can be associated with discomfort due to the needle penetrating the skin. It is possible to feel claustrophobic and uneasy during the BMR-measurement because of the ventilated hood.

Each participant received oral and written information about the experimental approach. They gave their written agreement for participating and signed agreements letting us store their blood samples, urine and saliva. The participants could, whenever they wanted to, terminate their participation without any further questions. All participants who finished the experiment received compensation. The ethical approval number from Local Ethics Committee in Stockholm is 2013/520-31/2.

4. RESULTS

4.1. Pilot study

Antibacterial mouthwash efficiently reduced nitrite levels in saliva

The results from the pilot study clearly show that the antibacterial effect is efficient and that the placebo mouthwash had no effect. Due to the antibacterial effect nitrate levels in saliva were significantly increased 1, 2 and 3 hours after the antibacterial mouthwash (see figure 2a) and the amount of nitrite in saliva was decreased (see figure 2b) The placebo mouthwash had in contrast to the chlorhexidine mouthwash, a low amount of nitrate (see figure 2a) and a high amount of nitrite but no significant time effect (see figure 2b).

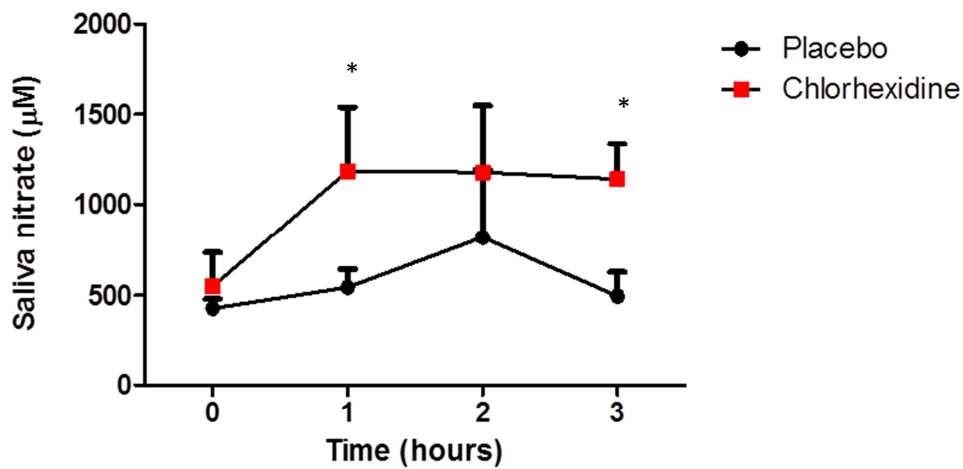


Figure 2a. Nitrate concentrations in saliva after treatment with placebo mouthwash and chlorhexidine mouthwash. The amount of nitrate in saliva was higher after treatment with chlorhexidine mouthwash and lower after treatment with placebo mouthwash. (*=significantly different compared to placebo)

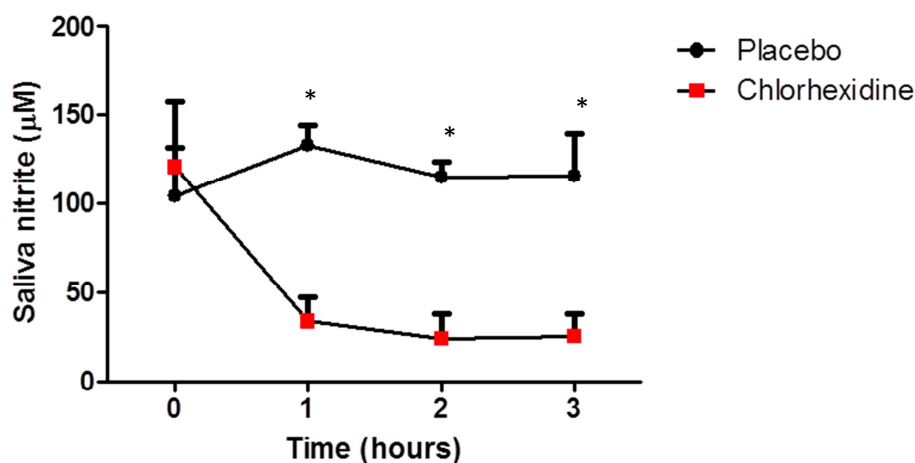


Figure 2b. Nitrite in saliva after treatment with placebo mouthwash and chlorhexidine mouthwash. The amount nitrite in saliva was lower after treatment with chlorhexidine mouthwash and higher after treatment with placebo mouthwash. (*=significantly different as compared to placebo)

4.2. BMR and mouthwash

Effect on gas exchange and BMR by mouthwash

There was no significant ($P=0.93$) difference in mean volume of consumed oxygen (VO_2) after use of placebo mouthwash ($221 \pm 23 \text{ ml min}^{-1}$) and chlorhexidine mouthwash ($222 \pm 18 \text{ ml min}^{-1}$, see figure 3). There was no significant difference ($P=0.18$) in mean volume of exhaled carbon dioxide (VCO_2) after use of placebo mouthwash ($202 \pm 17 \text{ ml min}^{-1}$) and chlorhexidine mouthwash ($198 \pm 17 \text{ ml min}^{-1}$, see figure 4). There was no significant difference ($P=0.20$) in respiratory exchange ratio (RER) after use of placebo mouthwash (0.92 ± 0.06) and chlorhexidine mouthwash (0.89 ± 0.06 , see figure 5).

There was no significant ($P=0.15$) difference in mean body weight (kg) after placebo mouthwash ($82.25 \pm 7.90 \text{ kg}$) compared to after chlorhexidine mouthwash ($82.62 \pm 8.07 \text{ kg}$, see figure 6). The changes in urine volume showed no significant ($P=0.92$) difference after treatment with placebo mouthwash ($402 \pm 205 \text{ ml}$) and chlorhexidine mouthwash ($408 \pm 225 \text{ ml}$, see figure 7).

No significant ($P=0.34$) difference in resting heart rate (bpm) was seen between study participants receiving placebo mouthwash ($55 \pm 6 \text{ bpm}$) and those receiving chlorhexidine mouthwash ($54 \pm 7 \text{ bpm}$, see figure 8).

There was no significant ($P=0.87$) difference in energy expenditure ($\text{kcal } 24\text{h}^{-1}$) between participants receiving placebo mouthwash ($1579 \pm 153 \text{ kcal } 24\text{h}^{-1}$) and those receiving chlorhexidine mouthwash ($1574 \pm 121 \text{ kcal } 24\text{h}^{-1}$, see figure 9) groups. However, we found a significant difference ($P=0.0009$) in energy expenditure between the first visit ($1621 \pm 122 \text{ kcal } 24\text{h}^{-1}$) and the second visit ($1532 \pm 139 \text{ kcal } 24\text{h}^{-1}$, see figure 10).

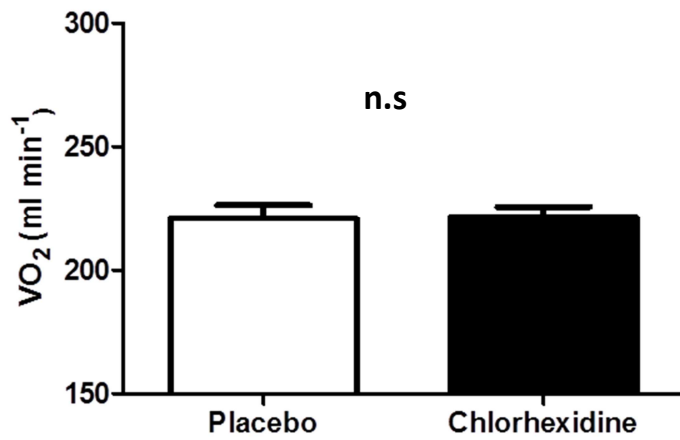


Figure 3. VO₂ (ml min⁻¹) after placebo mouthwash and chlorhexidine mouthwash. No difference could be observed between the participants receiving placebo mouthwash and those receiving chlorhexidine mouthwash.

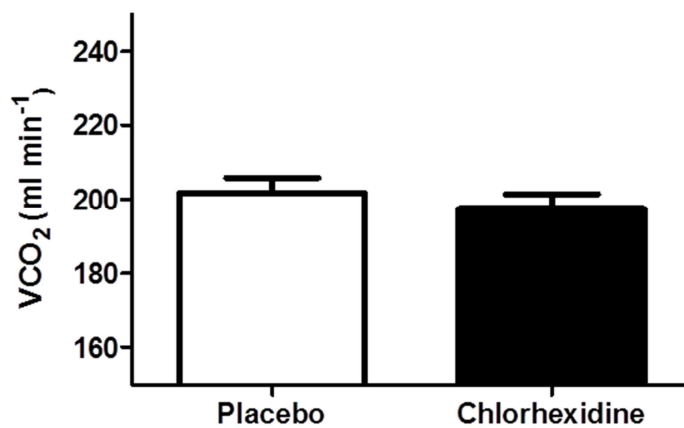


Figure 4. VCO₂ (ml min⁻¹) after treatment with placebo mouthwash and chlorhexidine mouthwash, respectively. No significant difference was observed in VCO₂ (ml min⁻¹) between the participants receiving placebo mouthwash and those receiving chlorhexidine mouthwash.

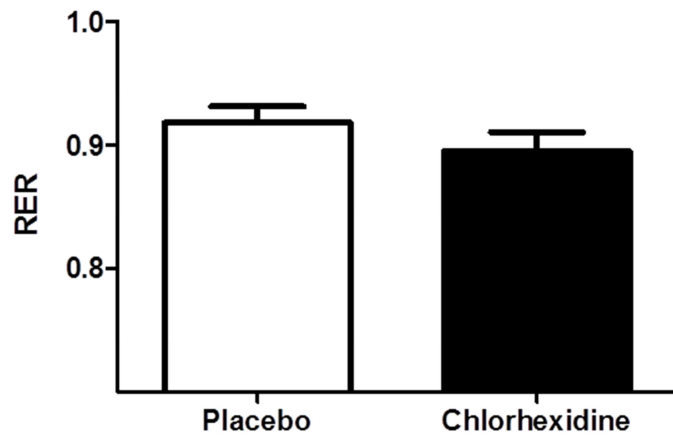


Figure 5. RER (respiratory exchange ratio) after use of placebo mouthwash and chlorhexidine mouthwash. A small difference, albeit not significant, in the RER was seen between participants receiving placebo mouthwash and those receiving chlorhexidine mouthwash.

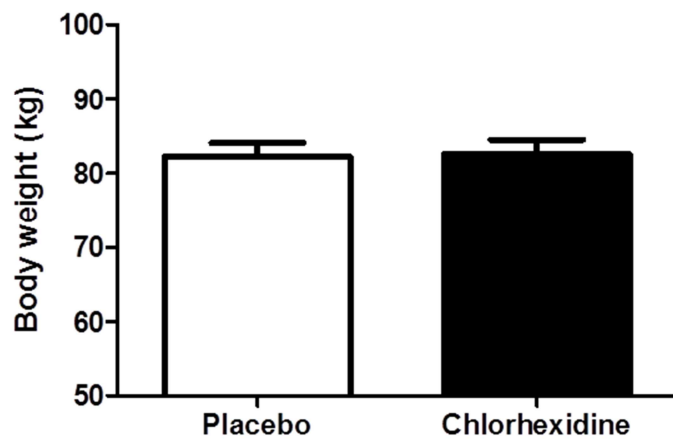


Figure 6. Body weight (kg) after placebo mouthwash and chlorhexidine mouthwash. There was no significant difference in the body weight (kg) after treatment with the placebo mouthwash and after the chlorhexidine mouthwash, respectively.

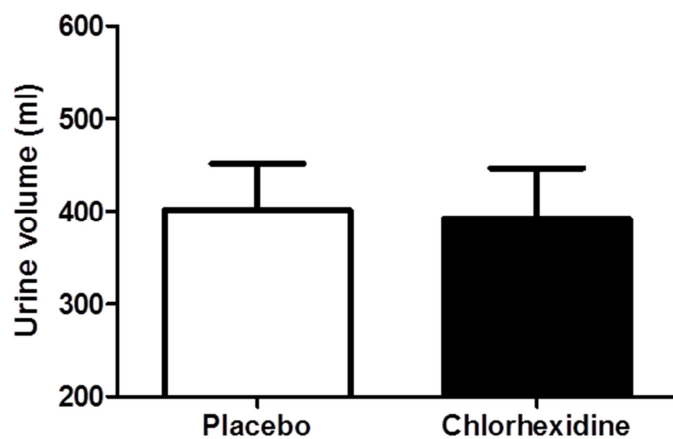


Figure 7. Urine volume after placebo compared to chlorhexidine mouthwash. No significant difference in urine volume (ml) was seen between study participants receiving placebo mouthwash and those receiving chlorhexidine mouthwash.

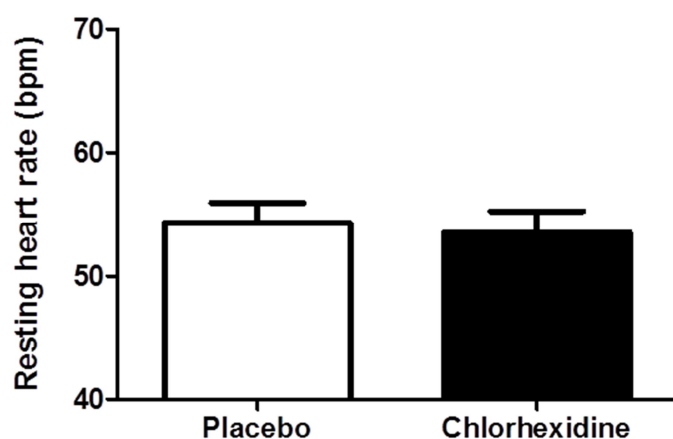


Figure 8. Resting heart rate (bpm) after use of placebo mouthwash and chlorhexidine mouthwash, respectively. No significant difference in resting heart rate could be observed between (beats per minute, bpm) the placebo mouthwash and the chlorhexidine mouthwash groups.

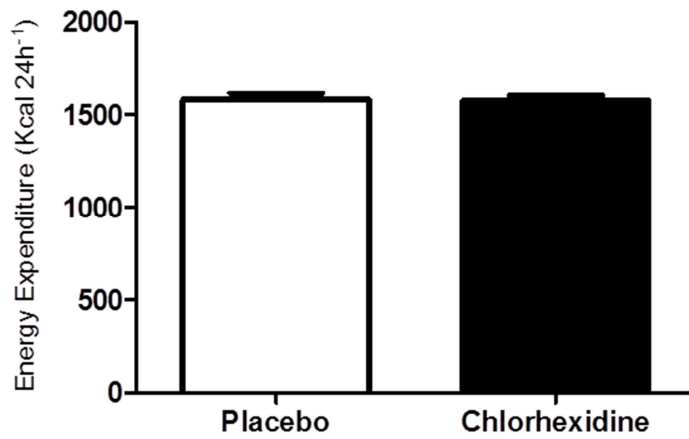


Figure 9. Energy expenditure (kcal 24h⁻¹) after placebo mouthwash and chlorhexidine mouthwash. There was no difference in energy expenditure between the two groups.

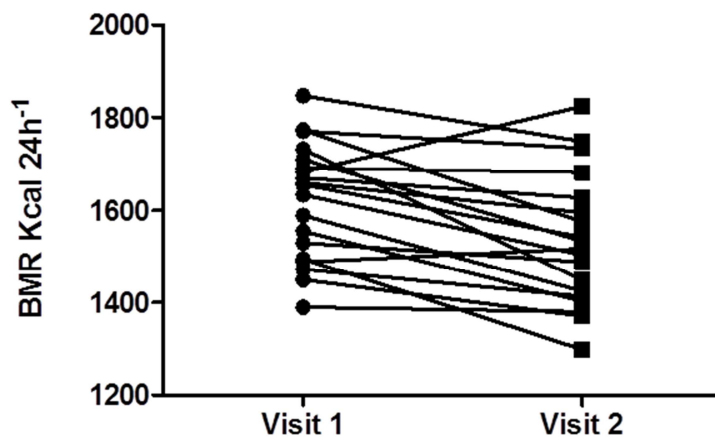


Figure 10. Energy expenditure (kcal 24h⁻¹) between the first visit and the second visit. The BMR was higher at visit 1 compared to visit 2 with exception for two subjects, the difference was significant (P=0.0009).

5. DISCUSSION

Our hypothesis was that the antibacterial mouthwash would increase the basal metabolic rate in healthy, non-tobacco using men in the age between 22-43 years. The pilot study showed that the nitrate conversion by the oral bacteria declined when the chlorhexidine based mouthwash was used compared to placebo. However, BMR seems to not have been affected. Thus, the antibacterial effect was effective according to the nitrate reduction, but it did not affect the basal metabolic rate as hypotized.

Until blood and saliva samples have been analyzed it is not possible to know if the compliance was good. If the study participants failed to use the antibacterial mouthwash the bacteria could still reduce nitrate to nitrite. It is also possible that the low nitrite levels remaining even after antibacterial mouthwash (see figure 2b) are sufficient to keep basal metabolic rate at a normal level. Another possibility is that the basal levels of nitrate and nitrite are below the limit where the physiological effects occur. In that case, the effect seen after nitrate administration (17) is close to pharmacological levels and not something that is achieved through a normal diet. It is also possible that the antibacterial mouthwash indeed was effective but that there was no real effect or the effect was below the detection limit of our method.

A major setback in this study was the finding that the BMR was higher at the first test occasion compared to the second occasion (see figure 10). This was seen in 17 of totally 19 participants. There are many possible explanations for this. The major reason is probably the new situation. None of the participants had done the calorimetric measure before. The canopy hood can be perceived as claustrophobic. In the ventilated hood the CO₂ content is higher (0.5 -1 %) than in the atmosphere (0.038 %) and this can affect persons who are sensitive to the higher CO₂ content. This can induce hyperventilation which in turn can lead to higher BMR through the increased respiratory work. An increased sympathetic activation may also be triggered according to the new situation. In addition, the blood sample was drawn after the BMR measurements which may have caused anticipation stress in some of the subjects. Almost all participants (15 of 19) had met the test leader before the trial day to reduce social stress during the main tests.

There was no significant difference ($P < 0.05$) between study participants receiving placebo mouthwash and those receiving chlorhexidine mouthwash with respect to VO₂, VCO₂, RER,

urine volume, body weight (kg), resting heart rate (bpm), or energy expenditure ($\text{kcal } 24 \text{ h}^{-1}$).

The significantly higher BMR in the first trial compared to the second trial warrants more familiarization visits in future studies to ensure that the participants do not feel stress during the main measurement. Most likely, a longer run-in period is needed, where participants can try the indirect calorimeter before the main tests to reduce psychological stress. If the participants can familiarize themselves with the situation the results will better match the actual energy expenditure.

In conclusion, no difference in BMR was seen between study participants receiving antibacterial mouthwash and those receiving placebo. However, due to the practical issues with a higher BMR at the first visit the result of this study has to be interpreted with caution.

6. ACKNOWLEDGEMENTS

Thanks to all of you!

Supervisor Filip Larsen

Supervisor Håkan Andersson

Tomas Schiffer

Professor Eddie Weitzberg and Professor Jon Lundberg

Carina Nihlén, Annika Ohlsson and Meta Stensdotter

Cecilia Jädert

Karin Eriksson

Ming Li and Annette Ebberyd

Micke Elm and Eva Näsström

All 19 participants who did this work possible for me to do.

Fredrik Eriksson who listened to me and advised me to contact Filip Larsen.

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