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The effect of chlorhexidine mouthwash vs propolis mouthwash on the nitrate-reducing activity of oral bacteria and vascular control in healthy individuals.

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Abstract

Nitrate and nitrite molecules are involved in the synthesis of nitric oxide (NO), a gaseous vasodilatory molecule, via the nitrate-nitrite-NO pathway. Oral bacteria are responsible for the reduction of nitrate to nitrite making them important regulators in NO production and vascular control. This paper aimed to investigate whether a laboratory-made propolis mouthwash maintained the oral nitrate reducing capacity (ONRC) of commensal bacteria compared with a chlorhexidine (CHX) mouthwash and the effects of this on vascular function in healthy individuals. Twenty-eight healthy participants visited the laboratory on two occasions where anthropometric, ONRC, blood pressure and vascular function data were collected. Between laboratory visits a CHX mouthwash or a propolis mouthwash were used for seven days, twice-daily. It was found that propolis mouthwash used for seven days maintained the ONRC of commensal bacteria in healthy individuals (pre; 343.4 ±251.8μM vs post; 331.9 ±225.1μM, $p=0.71$) and that CHX mouthwash significantly abolished this activity, lowering levels by 67.7% in seven days (pre; 399.7 ±356.4μM vs post; 129.1 ±171.3μM, $p<0.001$). No significant changes in blood pressure or vascular function were seen following mouthwash use despite bacterial changes. It is unclear whether these insignificant findings are down to methodological issues or external physiological pathways. It was concluded that propolis mouthwash and CHX mouthwash affect the ONRC of commensal bacteria differently. Propolis mouthwash seems to preserve the ONRC therefore is less detrimental to the oral microbiome than CHX mouthwash suggesting potential uses in dentistry and as a therapeutic for hypertensive individuals.

Keywords: propolis mouthwash, chlorhexidine mouthwash, chlorhexidine, oral bacteria, oral nitrate reducing capacity, vascular control.

Introduction

Nitric oxide (NO) is a small gaseous molecule which has many physiological roles in the body including acting as a potent vasodilator, making it essential for normalising blood pressure and vascular control (Archer, 1993; Stauss & Persson, 2000). NO can be produced by different routes including the nitrate-nitrite-NO pathway. Research into this pathway discovered that the interaction between nitrate and nitrite in the oral cavity can form NO in the blood and tissues (Lundberg, Weitzberg & Gladwin, 2008). Around 25% of dietary nitrate, from sources such as green leafy vegetables, is extracted into the salivary glands and taken to the oral cavity to be reduced to nitrite by commensal anaerobic bacteria. Swallowed saliva will enter the acidic environment of the stomach providing optimal conditions for nitrite reduction to NO (Lundberg et al, 1994). Mammals including humans lack the specific enzymes needed to reduce nitrate in the oral cavity. Therefore, a symbiotic relationship is seen with oral commensal bacteria which contribute to NO production and thus vascular control in humans whilst humans provide nitrate for these bacteria to respire (Lundberg, Weitzberg & Gladwin, 2008).

Antiseptic mouthwash containing chlorhexidine (CHX) is widely used because it decreases the growth of harmful bacteria in the oral cavity. However, it can also hinder the growth and activity of other oral bacterial species in the microbiota including commensal nitrate-reducing bacteria (Petersson et al, 2009). As a result, mouthwash containing CHX is shown to raise blood pressure which is possibly down to eradicating these nitrate-reducing bacteria and therefore disrupting the nitrate-nitrite-NO production pathway (Bryan, Tribble & Angelov, 2017; Kapil et al, 2013). Furthermore, blood pressure reduction has been shown following nitrate supplementation confirming that the pathway has implications in vascular control (Kapil et al, 2010). This may be particularly important as hypertension affects more than 1 in 4 people in the UK and can lead to an array of additional health problems like heart disease and stroke (Public Health England, 2017). Perhaps by increasing nitrate and nitrite bioavailability or allowing nitrate-reducing oral bacteria to colonise, blood pressure can be normalised through NO production, helping manage hypertension without the reliance on medication (Bryan, Tribble & Angelov, 2017).

Propolis is a substance naturally made by bees to protect and repair their hives. It is composed of resins, waxes, fatty acids, aromatic oil, some flavonoids, minerals and vitamins (Khurshid et al, 2017; Więckiewicz et al, 2013). Propolis has shown potential uses in medicine and dentistry due to its anti-bacterial properties. Studies have shown it to be as effective as CHX mouthwash at reducing plaque growth and improving the gingival index whilst showing a slightly better improvement in inflammation than CHX mouthwash (Dodwad & Kukreja, 2011). Propolis has also presented anti-fungal, anti-viral and anti-inflammatory properties (Khurshid et al, 2017). Mouthwashes containing propolis are available to buy, however, it seems unknown in the literature whether propolis mouthwash can preserve the nitrate-reducing activity of oral bacteria and the effects of this on an individual's cardiovascular health, unlike CHX mouthwash which seems detrimental to these measures. Therefore, this paper aims to investigate whether a laboratory-made propolis mouthwash can maintain the oral nitrate reducing capacity (ONRC) of commensal bacteria when compared with CHX mouthwash and the effects of this on vascular function in healthy individuals. It is hypothesised that those using a propolis mouthwash for seven days will have a preserved ONRC and a lowered or

normalised blood pressure whilst those using CHX mouthwash will show a reduction in ONRC and a rise in blood pressure.

Methodology

Study population

Sixteen healthy participants were recruited from the University of Plymouth to complete this study. Participants gave written informed consent prior to the study. Questionnaires were completed by participants to ensure they were not already using mouthwash and that there were no allergies to propolis. Forty-eight hour dietary recalls were completed so similar meal compositions could be had prior to both laboratory visits as it is known that diet can influence oral nitrate and nitrite levels (Ashworth et al, 2019). Ethical approval was granted by the University of Plymouth Faculty of Health and Human Sciences Research Ethics Committee.

Protocol

Participants visited the Nutrition, Exercise and Health laboratories on two separate occasions. During the first visit, participants arrived in the morning under fasted conditions and baseline measures were collected. A ten millilitre (ml) mouth rinse containing nitrate and water was held in the participant's mouth for five minutes and collected back into a falcon tube. This sample was analysed to determine how much nitrate was reduced to nitrite by the participant's oral bacteria which shows the ONRC.

Following this, basic anthropometric measures were collected including height, weight, BMI and body fat percentage using the Tanita body composition analyser (TBF-300 MA, Tokyo, Japan). Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) data were recorded in a supine position. Three measures were taken after an initial 10 minute adaption period to obtain an average value using a sphygmomanometer (ProBP 3400, Welch Allyn, New York, USA). Vascular function was measured using a near-infrared spectroscopy (NIRS) device in combination with a reactive hyperaemia test (NIRO-200NX, Hamamatsu City, Japan). This involved a cuff being attached to a participant's upper left arm and a NIRS probe being attached further down the arm to measure changes in light absorption at different wavelengths so that blood flow and oxygen levels could be monitored throughout the test (Soares & Murias, 2018). The reactive hyperaemia test consisted of baseline levels being measured for two minutes then the cuff expanding for five minutes to restrict blood flow to the tissues at a pressure of 200mmHg (occlusion stage) and then deflating for another five minutes (recovery stage). The NIRS device provided data on the tissue oxygenation index (TOI_1) throughout each test which was used for analysis. This value displays the percentage of blood oxygenated in the tissues.

Once baseline data had been collected, participants received fourteen tubes of ten ml mouthwash to be used twice a day over a seven day period. Instructions for mouthwash use were given to participants ensuring the correct procedure was being followed. Instructions included rinsing the mouth for one minute twice-daily for seven days and not rinsing the mouth with water for five minutes after use. The distribution of CHX and propolis mouthwash was randomised and double blind. Following the seven day interval, participants returned to the lab under the same fasted conditions and data collection was repeated.

Laboratory procedures

Storage of ONRC samples

The ONRC samples collected were centrifuged at 14 000 rpm for 10 minutes at 4°C and were then transferred into eppendorf tubes. All samples were labelled with participant codes to maintain confidentiality, the trial number, what the sample was, and the date collected. Samples were frozen at -80°C until needed for analysis.

Preparation of mouthwashes

The mouthwashes used in this study were either Corsodyl, a commercial CHX based antiseptic mouthwash (Corsodyl, GlaxoSmithKline, Middlesex, England) or a propolis mouthwash which was made in the laboratory. For this, crude propolis was ground into a fine powder and mixed with 95% ethanol. The solution was shaken using a magnetic stirrer for 72 hours at 20°C and filtered using filter paper. The extraction was centrifuged at 3 500 rpm for 10 minutes at 4°C and filtered again. A rotary evaporator was used to extract the ethanol from the solution at 60°C with low pressure. Water was added to achieve a dilution of 2.5% propolis within the solution. The resulting product was separated into 10ml falcon tubes and stored at -20°C until use. For the purpose of this paper, the CHX based mouthwash is named ‘CHX’ and the propolis mouthwash is named ‘PRO’.

Analysis

ONRC

The ONRC nitrite concentrations in saliva were measured using High-Performance Liquid Chromatography (HPLC) by a Nitric Oxide analyser (ENO-30, Eicom, Japan). The nitrite values were displayed as microvolts (mv) and converted to micromoles (μM) for statistical analysis.

Statistical analysis

Statistical analysis was performed using Minitab® 19 Statistical Software (Minitab LLC, USA). Paired t tests were used to compare ONRC, blood pressure and vascular function (TOI_1) differences within treatment groups, before and after mouthwash use. Independent t tests were used to compare values between the two mouthwash groups. The data was logged, or a Mann Whitney U non-parametric test was used where data wasn’t normally distributed. Statistical significance was set at $p<0.05$.

Results

Participant baseline characteristics

The data from the current study (sixteen participants) was combined with another group’s data (twelve participants) to maximise sample size who followed the same protocol as described above. Table 1 displays all participant baseline characteristics. Subjects in CHX and PRO groups had similar characteristics except those in CHX had a significantly higher baseline DBP compared to PRO (66.0 ±7.8mmHg vs 60.8 ±3.9mmHg respectively, $p=0.04$).

Table 1. Participant baseline characteristics.

	CHX	PRO	Total	P value
Participants (n)	14	14	28	-
Age (years)	27 ±4.0	26 ±6.9	26 ±5.5	0.28
Weight (kg)	63.1 ±12.0	58.6 ±7.0	60.8 ±9.9	0.24
Height (m)	1.7 ±0.1	1.7 ±0.0	1.7 ±0.1	0.27
BMI (kg/m^2)	22.1 ±2.8	21.3 ±2.0	21.7 ±2.4	0.41
Body fat percentage (%)	22.8 ±6.1	22.7 ±7.1	22.6 ±6.4	0.95
SBP (mmHg)	103.3 ±10.4	101.8 ±6.9	102.5 ±8.7	0.67
DBP (mmHg)	66.0 ±7.8	60.8 ±3.9	63.4 ±6.6	0.04
MAP (mmHg)	78.3 ±8.3	74.7 ±4.5	76.5 ±6.8	0.17
HR (bpm)	59.5 ±11.2	62.1 ±8.9	60.8 ±10.0	0.51

Data expressed as means ± standard deviations. CHX; antiseptic CHX based mouthwash. PRO; propolis mouthwash. BMI; body mass index. SBP; systolic blood pressure. DBP; diastolic blood pressure. MAP; mean arterial pressure. HR; heart rate. P value obtained from independent t tests and non-parametric Mann-Whitney U tests.

Effects of mouthwash treatments on salivary ONRC

Table 2 demonstrates that following a seven day period of using propolis mouthwash twice-daily, the ONRC of commensal bacteria post treatment is unchanged compared to baseline values (343.4 ±251.8μM vs 331.9 ±225.1μM respectively, p=0.71). In comparison, the ONRC of subjects using CHX mouthwash for the same amount of time was significantly attenuated by 67.7% compared to baseline values (129.1 ±171.3μM vs 399.7 ±356.4μM respectively, p<0.001). Figure 1 displays the significant differences in bacterial ONRC between mouthwash groups following use where CHX decreased bacterial activity and propolis maintained it (p<0.001).

Effects of mouthwash treatments on blood pressure

The use of CHX and propolis mouthwashes for seven days did not significantly change any blood pressure values when compared to baseline values or to one another (p>0.05; see table 2).

Effects of mouthwash treatments on vascular function

Figures 2 and 3 and table 3 show that using either mouthwash for seven days does not significantly influence the tissue oxygenation index (TOI_1) of participants at any point during the reactive hyperaemia tests when compared to their baseline values or when comparing mouthwashes (P>0.05). Figures 2 and 3 illustrate TOI_1 percentages throughout the tests. The baseline stage of the test lasted for 120 seconds after which the TOI_1 percentage started decreasing in the occlusion stage (lasting from 120 to 420 seconds). At 420 seconds the recovery stage started where percentage tissue oxygenation increased rapidly and slowly levelled out to baseline levels until the end of the test. The maximal TOI_1 value throughout the reactive hyperaemia test before CHX exposure was 84.4% and after was 83.9% (p=0.53). Before PRO exposure the maximal TOI_1 was 83.6% and after was 83.8% (p=0.80).

Table 2. The ONRC and blood pressure data collected pre and post mouthwash use.

	CHX				PRO				Differences between CHX & PRO P value (C)
	Pre	Post	Difference	P value (A)	Pre	Post	Difference	P value (B)	
ONRC- Nitrite (µM)	399.7 ±356.4	129.1 ±171.3	-270.6	<0.001	331.9 ±225.1	343.4 ±251.8	+11.5	0.71	<0.001
SBP (mmHg)	103.3 ±10.4	101.9 ±8.5	-1.4	0.48	101.8 ±6.9	99.5 ±8.2	-2.3	0.06	0.65
DBP (mmHg)	66.0 ±7.8	63.5 ±5.9	-2.5	0.22	60.8 ±3.9	60.2 ±4.2	-0.6	0.62	0.40
MAP (mmHg)	78.3 ±8.3	76.3 ±6.2	-2.0	0.31	74.7 ±4.5	73.4 ±5.3	-1.3	0.23	0.61
HR (bpm)	59.5 ±11.2	58.8 ±12.1	-0.7	0.48	62.1 ±8.9	60.3 ±7.9	-1.8	0.28	0.56

Data expressed as means ± standard deviations. CHX; antiseptic CHX based mouthwash. PRO; propolis mouthwash. SBP; systolic blood pressure. DBP; diastolic blood pressure. MAP; mean arterial pressure. HR; heart rate. A; p value obtained from a paired t test on the pre and post values from CHX. B; p value obtained from a paired t test on the pre and post values from PRO. C; p value obtained from an independent t test on the differences between CHX and PRO mouthwashes.

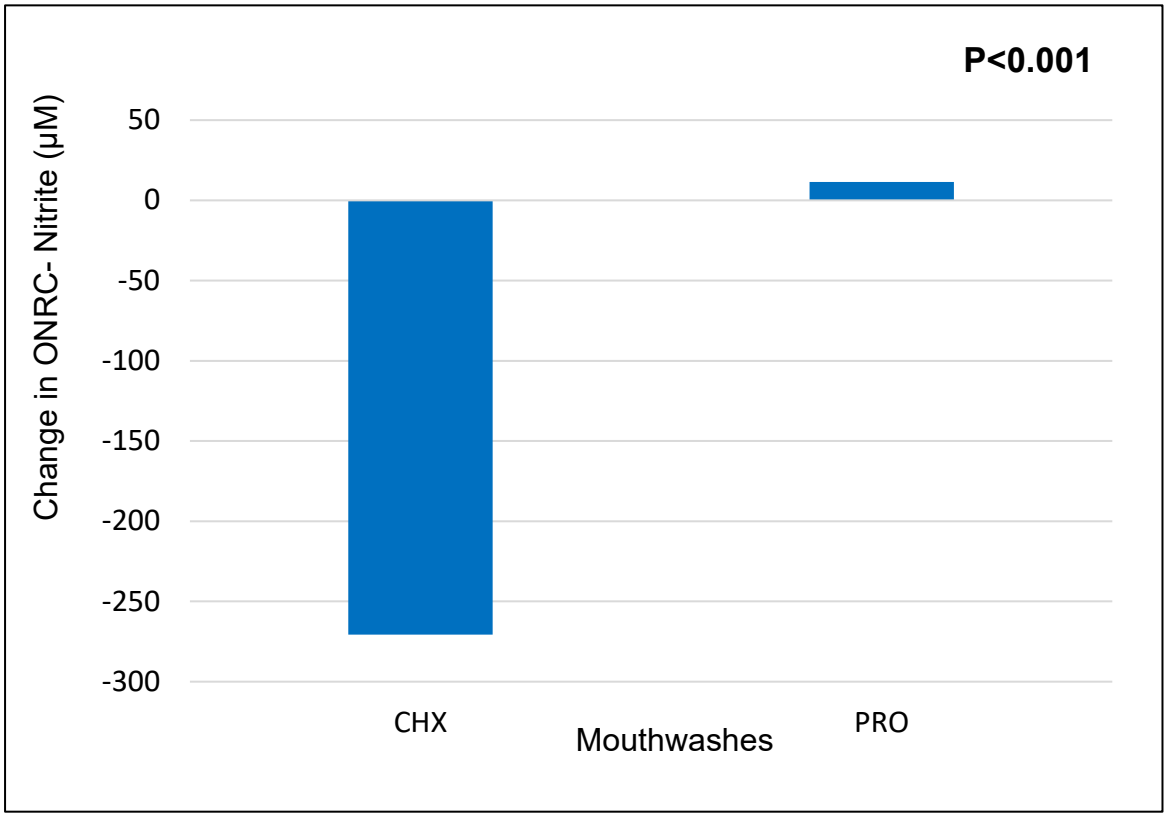

Figure 1. The changes in ONRC before and after mouthwash use.

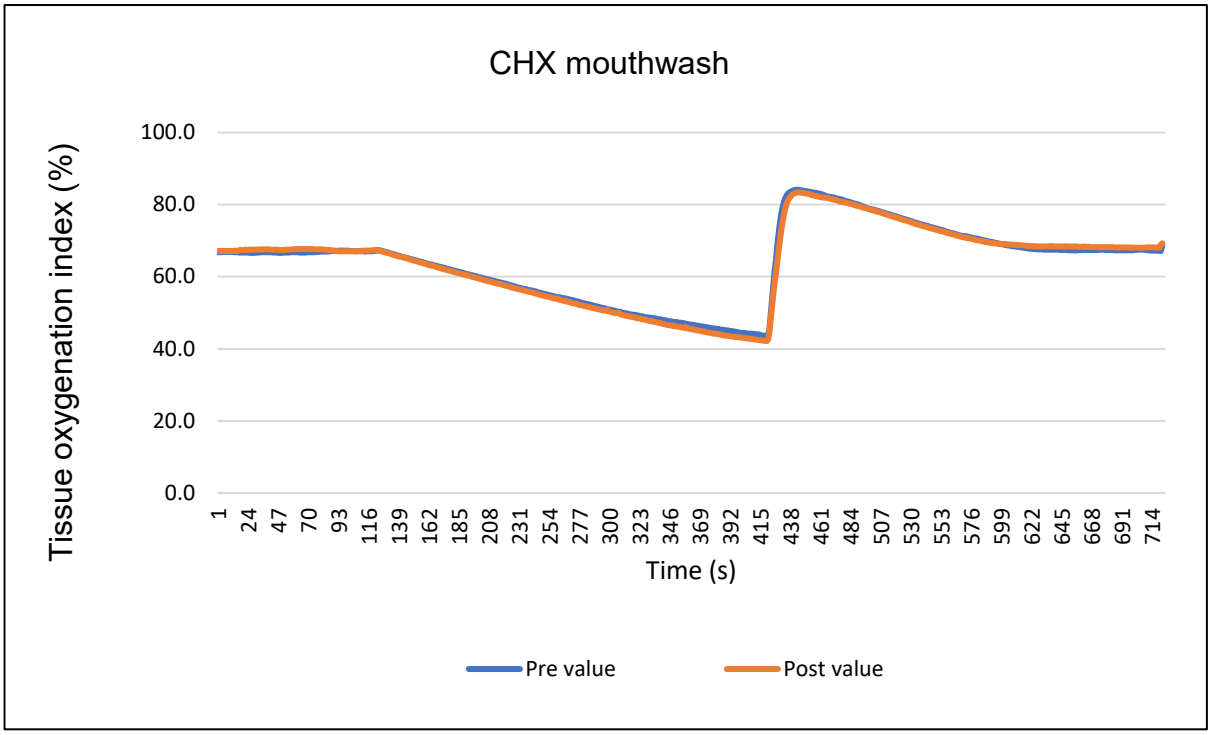

Figure 2. The effects of CHX mouthwash on participant's average percentage tissue oxygenation during a reactive hyperaemia test.

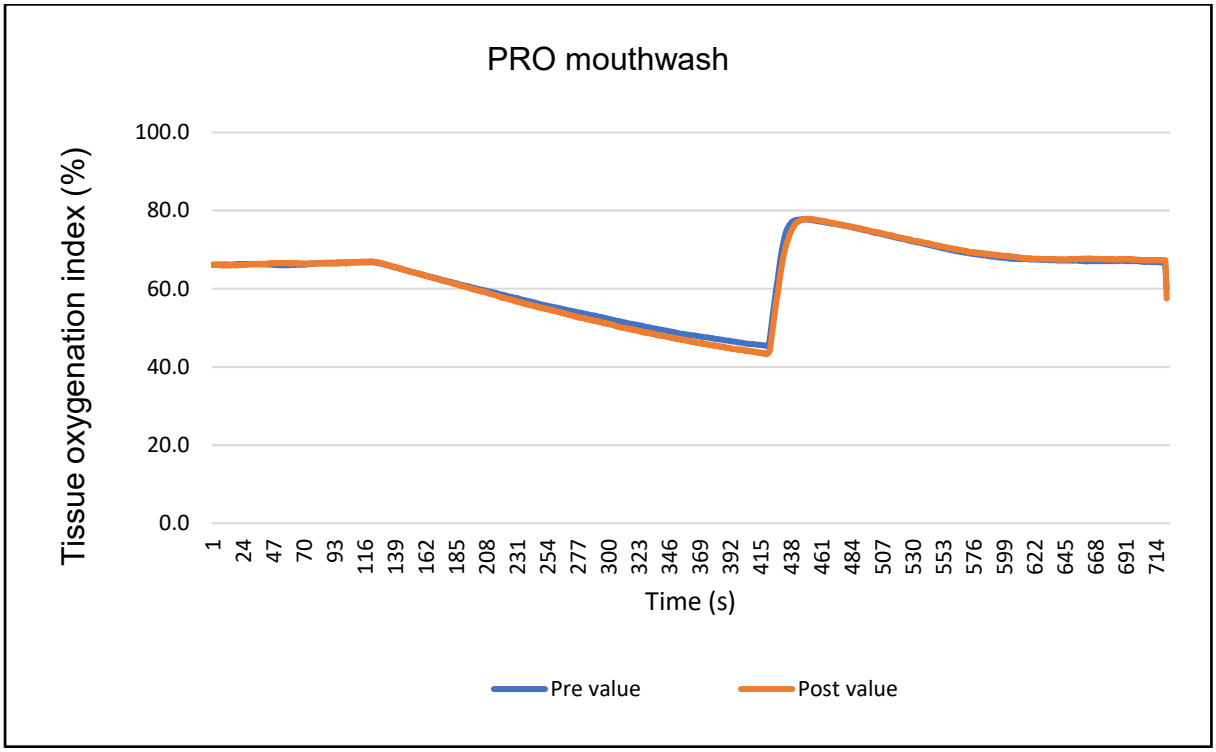

Figure 3. The effects of propolis mouthwash on participant's average percentage tissue oxygenation during a reactive hyperaemia test.

Table 3. Average tissue oxygenation index percentages recorded during the reactive hyperaemia tests for both mouthwashes.

	CHX				PRO				Differences between CHX & PRO P value (C)
	Pre	Post	Differences	P value (A)	Pre	Post	Differences	P value (B)	
Average TOI_1 baseline value (%)	66.9 ±5.9	67.4 ±6.8	+0.5	0.64	71.1 ±3.1	71.2 ±4.5	+0.1	0.94	0.75
Minimum TOI_1 value throughout the test (%)	43.0 ±13.6	41.9 ±13.3	-1.0	0.64	48.4 ±10.0	46.2 ±12.7	-2.2	0.21	0.68
Maximum TOI_1 value throughout the test (%)	84.4 ±2.8	83.9 ±3.4	-0.6	0.53	83.6 ±3.2	83.8 ±2.4	+0.1	0.80	0.93

Data expressed as means ± standard deviations. CHX; antiseptic CHX based mouthwash. PRO; propolis mouthwash. Average TOI_1 baseline value; the first 120 seconds (2 minutes) of the reactive hyperaemia test. A; p value obtained from a paired t test on the pre and post values from CHX. B; p value obtained from a paired t test on the pre and post values from PRO. C; p value obtained from an independent t test on the differences between CHX and PRO mouthwashes.

Discussion

This paper has demonstrated that a laboratory-made propolis mouthwash used for seven days twice-daily can maintain the ONRC of commensal bacteria in healthy individuals. It has also been found that an antiseptic CHX based mouthwash can significantly abolish this bacterial activity, lowering levels by 67.7% in seven days on average. These findings suggest that the hypotheses can be accepted regarding the effects of mouthwashes on bacterial ONRC.

The main findings around ONRC from the current research relate to the nitrate-nitrite-NO pathway and blood pressure. CHX mouthwash has anti-bacterial properties which are unable to differentiate between oral bacteria species. It therefore decreases the ability of commensal bacteria to reduce nitrate to nitrite, suggesting a reduction in NO production (Bryan, Tribble & Angelov, 2017). The literature indicates that a decreased ONRC raises blood pressure mostly likely due to a disruption in NO production as it is a vasodilator (Bryan, Tribble & Angelov, 2017; Kapil et al, 2013). However, contrary to the current hypothesis, no significant increases in blood pressure were seen following CHX use in the current paper. CHX mouthwash may therefore not have affected NO production enough to influence participant's vascular health. Nitrate and nitrite concentrations in the saliva and blood weren't analysed in this paper so it cannot be confirmed that NO levels were directly reduced following a reduction in ONRC caused by CHX mouthwash. This needs confirmation because NO can be formed using alternative routes which may be why no changes in blood pressure were seen following CHX use. Nitric oxide synthase (NOS) enzymes combine L-arginine and oxygen to make NO. Therefore, if oxygen is available, this pathway may be upregulated to produce NO if the nitrate-nitrite-NO pathway is disrupted by using CHX mouthwash (Lundberg, Weitzberg & Gladwin, 2008).

Additionally, vascular function was measured using a reactive hyperaemia test whilst recording tissue oxygenation levels. The maximum TOI_1 percentage reflects the

recovery stage where blood flow increases rapidly in the blood vessels creating a peak in tissue oxygenation. This sudden increase in blood flow causes shear stress in the vessels which stimulates NO release from endothelial cells. NO binds to smooth muscle in the vessel causing a decrease in muscle tension and contraction which dilates the vessel and allows for more blood to flow through to the tissues (Gerovasili et al, 2010; Sandoo et al, 2010). No significant changes to the TOI_1 percentages following CHX mouthwash is unexpected because CHX has been shown to disrupt the nitrate-nitrite-NO pathway therefore indicating less NO would be available to dilate blood vessels. A reduction in vasodilation would produce lower maximum TOI_1 percentages as less blood would be travelling through the vessels to deliver oxygen to the tissues, decreasing vascular function. Healthy participants were used in the current paper who may be able to cope with an attenuated ONRC following CHX mouthwash use. As mentioned previously, it is possible that other pathways could be involved in producing NO meaning a decreased ONRC would not significantly influence NO levels endogenously, therefore leading to little change in vasodilation ability, TOI_1 percentages and vascular function as seen in the current study (Lundberg Weitzberg & Gladwin, 2008). Other population types with existing vascular issues may show different results like those with hypertension. It seems that no previous research has investigated the effects of mouthwashes on vascular function and the reactive hyperaemia test therefore the current findings cannot be compared with those in the literature.

Unlike CHX mouthwash, the laboratory-made propolis mouthwash did not seem to disrupt oral bacteria's ability to reduce nitrate to nitrite as it does not contain the same antiseptic substances. This means NO production in these mouthwash users remained unchanged in theory, even though other pathways for NO production do exist which may have altered NO concentrations as well (Lundberg Weitzberg & Gladwin, 2008). From this paper it seems propolis mouthwash may be less detrimental to oral commensal bacteria than CHX mouthwash. Although no significant differences were found in blood pressure, a 2.3mmHg reduction in SBP following propolis mouthwash use for seven days was seen ($p=0.06$). This reduction may suggest that a propolis mouthwash could potentially help lower individual's SBP. This may be because the ONRC of these participants wasn't attenuated so NO could act as an efficient vasodilator by counteracting rises in blood pressure (Stauss & Persson, 2000). However, no literature seems to be available to confirm these findings around propolis mouthwash and more participants are needed to verify whether this result will be significant.

In the current literature, Petersson et al (2009) found significant reductions in the ONRC of rats when their oral cavities were sprayed with Corsodyl mouthwash containing CHX for seven days. This paper supports the current research findings in relation to the effects of CHX mouthwash on ONRC despite being conducted in rats which may raise generalisation issues when applied to humans. Kapil et al (2013) conducted a similar study in humans and found a 90% reduction in ONRC following CHX mouthwash use for seven days which further supports the current findings. However, they also found a significant rise in participant's blood pressure due to a disruption in nitrite homeostasis and NO production which failed to be seen in the current paper. This may be because they used several measures of blood pressure throughout the trial period including clinical, home and twenty-four-hour ambulatory blood pressure monitoring. In comparison, the current study obtained blood pressure readings before and after the seven day trial, not throughout the trial, at night or at home. Perhaps more readings are needed to provide a more sensitive measurement

in the current paper. Furthermore, Kapil et al (2013) used a cross over study design where participants acted as their own controls by having measures taken during a mouthwash trial and a control trial. This eliminates the possibility of any baseline blood pressure differences causing variation in results unlike in the current study which had significant differences in baseline DBP between participants in different mouthwash groups. Methodological issues in the present study could be leading to insignificant blood pressure results in the CHX group causing discrepancies with the existing literature. It seems that more robust protocols exist within the literature and that the current protocol could be improved based on this.

The preservation of salivary ONRC following the use of propolis mouthwash may have implications in dentistry and vascular health. If the current laboratory-made propolis mouthwash is shown to have anti-bacterial properties against harmful oral pathogens, then it would possess the same properties as popular antiseptic mouthwashes yet preserve the actions of commensal bacteria which have an important role in vascular homeostasis. Therefore, propolis mouthwash would be less detrimental to an individual's oral microbiome and blood pressure control compared to commercial CHX mouthwashes. As mentioned previously, propolis mouthwashes have shown anti-bacterial potential comparable to CHX mouthwashes (Dodwad & Kukreja, 2011; Khurshid et al, 2017). However, in some of these commercial propolis mouthwashes, the solutions contain other substances like menthol which have their own anti-bacterial properties that may not preserve the ONRC of oral bacteria like this mouthwash does (Pattnaik et al, 1997). Therefore, the anti-bacterial properties of the current propolis mouthwash containing only propolis and water need to be verified. Furthermore, the borderline significant reduction in SBP following propolis mouthwash use may imply this mouthwash could be an effective therapeutic for lowering SBP in those with hypertension.

Although the findings from this research present potential implications, some methodological issues should be considered. A limited time frame for conducting this study meant a small sample was gathered, raising issues with generalising findings to a wider population of mouthwash users. Furthermore, a limited number of participants gives less statistical power to measures which may suggest why no significant differences in blood pressure or tissue oxygenation were found following mouthwash use. However, Kapil et al (2013) included nineteen participants and found significant blood pressure changes following CHX mouthwash use which was less than in the current paper suggesting insignificant values were due to other factors as discussed previously. Some participants reported missing mouthwash tubes throughout the trial indicating poor adherence to the study protocol. As these individuals wouldn't have used as much mouthwash as those using all tubes, their changes in ONRC, NO production and therefore blood pressure and tissue oxygenation levels after the study may be different leading to high participant variation. Another point to highlight is that participants used mouthwashes for seven days. It is questionable whether this time frame is long enough to observe sufficient changes in the parameters measured. However, there is evidence to suggest that the microbiome can rapidly adapt to new stimuli. A change in diet can alter the gut microbiome one day after it has reached the distal gut microbiota (David et al, 2014). This suggests that oral commensal bacteria may respond to mouthwash rapidly which explains why a significant reduction in ONRC from CHX mouthwash was observed. Having said this, a rapid change in commensal bacteria action doesn't explain why blood pressure values weren't significantly altered following CHX mouthwash in this study. Measuring blood pressure is highly variable and is

influenced by many factors such as the time of day (Lemmer, 1989) and psychological factors like the mood of an individual (Pollard & Schwartz, 2003). This indicates that blood pressure may be influenced by variables other than ONRC and NO production whilst measures were taken.

This paper highlights that further investigation into blood pressure and vascular function using hypertensive individuals would be valuable. Bondonno et al (2014) found that hypertensive individuals aged 65 years using a CHX based mouthwash for three days had a significantly reduced ONRC, salivary nitrite concentration and increased SBP values. This confirms that a disruption to bacterial NO production following CHX mouthwash is detrimental to blood pressure in those with hypertension but perhaps not for those with healthy values as shown by no significant blood pressure changes in the current findings. For those with hypertension, a reduction in blood pressure would be beneficial to their health and potentially prevent the development of diseases like heart disease. The preserved ONRC effect and almost significant reduction in SBP seen following propolis mouthwash use may lead to an improved NO production and normalised blood pressure in hypertensive individuals that could be clinically significant. In terms of vascular function, hypertension is associated with endothelial dysfunction and disrupted NO production in the vessels (Napoli et al, 2006; Sandoo et al, 2010). No significant changes to tissue oxygenation values were seen in the current findings following mouthwash use however, changes may be more likely in hypertensive individuals because their NO levels are already disrupted. Further disruption to NO production following CHX mouthwash in these individuals may attenuate blood flow to the tissues in the recovery phase of the reactive hyperaemia test due to less vasodilation and therefore less oxygen delivery to the tissues. This could cause significantly lower maximum TOI_1 percentages compared to the healthy participants who showed no differences following CHX use in the current paper.

Future work should include analysing nitrate and nitrite concentrations in saliva and plasma samples which couldn't be included here due to time restrictions. This would clarify whether alterations to the nitrate-nitrite-NO pathway from mouthwashes translate into altered nitrite and NO concentrations endogenously. This is especially important as Burleigh et al (2018) have observed that increases in nitrate-reducing bacteria number and ONRC are associated with increases in salivary nitrite but not plasma nitrite which is the most accurate biomarker to indicate NO bioavailability. As plasma nitrite levels do not seem to be directly increased by bacterial ONRC, NO production may not be either which highlights another possibility as to why blood pressure and tissue oxygenation values weren't significantly influenced by mouthwashes. Lastly, it would be interesting to determine the anti-bacterial properties of the laboratory-made propolis mouthwash as this could have potential uses in dentistry as mentioned previously.

Conclusions

The current paper has addressed its aims by demonstrating that a laboratory-made propolis mouthwash can indeed maintain the ONRC of commensal bacteria when compared with a CHX based mouthwash. The mouthwashes can affect the activity of nitrate-reducing bacteria very differently. Propolis mouthwash's preservation of bacterial ONRC has potential implications as a therapeutic for hypertension and in dentistry as it is shown to be less detrimental to the oral microbiome than commercial CHX based mouthwashes. No significant effects of mouthwashes on

blood pressure or vascular function were found which causes some disagreements with the existing literature. Discrepancies may be down to methodological limitations in the current study or physiological mechanisms which may work to stabilise NO values endogenously despite ONRC changes. Further work is therefore needed to assess why these discrepancies occurred and to verify the potential uses of propolis mouthwash in relation to dentistry and health.

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References

Archer S (1993). Measurement of nitric oxide in biological models. *The FASEB Journal*, 7: 349-360.

Ashworth A, Cutler C, Farnham G, Liddle L, Burleigh M, Rodiles A, Sillitti C, Kiernan M, Moore M, Hickson M, Easton C and Bescos R (2019). Dietary intake of inorganic nitrate in vegetarians and omnivores and its impact on blood pressure, resting metabolic rate and the oral microbiome. *Free Radical Biology and Medicine*, 138: 63-72.

Bondonno C, Liu A, Croft K, Considine M, Puddey I, Woodman R and Hodgson J (2014). Antibacterial Mouthwash Blunts Oral Nitrate Reduction and Increases Blood Pressure in Treated Hypertensive Men and Women. *American Journal of Hypertension*, 28: 572-575.

Bryan N, Tribble G and Angelov N (2017). Oral Microbiome and Nitric Oxide: The Missing Link in the Management of Blood Pressure. *Current Hypertension Reports*, 19.

Burleigh M, Liddle L, Monaghan C, Muggeridge D, Sculthorpe N, Butcher J, Henriquez F, Allen J and Easton C (2018). Salivary nitrite production is elevated in individuals with a higher abundance of oral nitrate-reducing bacteria. *Free Radical Biology and Medicine*, 120: 80-88.

David L, Maurice C, Carmody R, Gootenberg D, Button J, Wolfe B, Ling A, Devlin A, Varma Y, Fischbach M, Biddinger S, Dutton R and Turnbaugh P (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, 505: 559-563.

Dodwad V and Kukreja B (2011). Propolis mouthwash: A new beginning. *Journal of Indian Society of Periodontology,* 15: 121-125.

Gerovasili V, Dimopoulos S, Tzanis G, Anastasiou-Nana M and Nanas S (2010). Utilizing the vascular occlusion technique with NIRS technology. *International Journal of Industrial Ergonomics*, 40: 218-222.

Kapil V, Milsom A, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, Arghandawi S, Pearl V, Benjamin N, Loukogeorgakis S, MacAllister R, Hobbs A, Webb A and Ahluwalia A (2010). Inorganic Nitrate Supplementation Lowers Blood Pressure in Humans. *Hypertension*, 56: 274-281.

Kapil V, Haydar S, Pearl V, Lundberg JO, Weitzberg E and Ahluwalia A (2013). Physiological role for nitrate-reducing oral bacteria in blood pressure control. *Free Radical Biology and Medicine*, 55: 93-100.

Khurshid Z, Naseem M, Zafar MS, Najeeb S and Zohaib S (2017). Propolis: A natural biomaterial for dental and oral healthcare. *Journal of Dental Research Dental Clinics Dental Prospects*, 11: 265–274.

Lemmer B (1989). Temporal aspects of the effects of cardiovascular active drugs in humans. *Cellular and Biochemical Aspects of Chronopharmacology,* 525-541.

Lundberg J, Weitzberg E and Gladwin M (2008). The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. *Nature Reviews Drug Discovery*, 7: 156-167.

Lundberg J, Weitzberg E, Lundberg J and Alving K (1994). Intragastric nitric oxide production in humans: measurements in expelled air. *Gut*, 35: 1543-1546.

Napoli C, De Nigris F, Williams-Ignarro S, Pignalosa O, Sica V and Ignarro LJ (2006). Nitric oxide and atherosclerosis: an update. *Nitric Oxide*, 15: 265–279.

Pattnaik S, Subramanyam VR, Bapaji M and Kole CR (1997). Antibacterial and antifungal activity of aromatic constituents of essential oils. *Microbios*, 89: 39-46.

Petersson J, Carlström M, Schreiber O, Phillipson M, Christoffersson G, Jägare A, Roos S, Jansson E, Persson A, Lundberg J and Holm L (2009). Gastroprotective and blood pressure lowering effects of dietary nitrate are abolished by an antiseptic mouthwash. *Free Radical Biology and Medicine*, 46: 1068-1075.

Pollard TM and Schwartz JE (2003). Are changes in blood pressure and total cholesterol related to changes in mood? An 18-month study of men and women. *Health Psychology*, 22: 47–53.

Public Health England (2017). *Health matters: combating high blood pressure*. [online] Available at: https://www.gov.uk/government/publications/health-matters-combating-high-blood-pressure/health-matters-combating-high-blood-pressure [Accessed 2 Feb. 2020].

Sandoo A, Veldhuijzen van Zanten J, Metsios G, Carroll D and Kitas G (2010). The Endothelium and Its Role in Regulating Vascular Tone. *The Open Cardiovascular Medicine Journal*, 4: 302-312.

Soares R and Murias J (2018). Near-infrared spectroscopy assessment of microvasculature detects difference in lower limb vascular responsiveness in obese compared to lean individuals. *Microvascular Research*, 118: 31-35.

Stauss HM and Persson PB (2000). Role of Nitric Oxide in Buffering Short-Term Blood Pressure Fluctuations. *Physiology,* 15: 229-233.

Więckiewicz W, Miernik M, Więckiewicz M and Morawiec T (2013). Does Propolis Help to Maintain Oral Health? *Evidence-Based Complementary and Alternative Medicine,* 2013: 1-8.