

Original

The effects of essential oil, povidone-iodine, and chlorhexidine mouthwash on salivary nitrate/nitrite and nitrate-reducing bacteria

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Abstract: Dietary nitrate is reduced to nitrite and nitric oxide by microbial flora, and this activity is beneficial to vascular health. It has been reported that this bacterial process is inhibited by chlorhexidine mouthwash, although the effects of other products are largely unknown. This study examined the effects of several treatments on salivary nitrate/nitrite and nitrate-reducing bacteria. Twelve university staff and students performed mouth-washing with water (control), essential oil, 0.35% povidone-iodine, or 0.0025% chlorhexidine and then ate 100 g lettuce (110 mg nitrate content), followed by collection of saliva and tongue bacteria at the baseline, and 1, 5, and 10 h thereafter. The individual treatments were separated by an interval of one week. Salivary nitrate/nitrite was measured by the calorimetric method, and a representative nitrate-reducing bacterial species, *Veillonella dispar*, was detected and semi-quantified using a polymerase chain reaction (PCR) assay. Significant increases in salivary nitrate/nitrite were observed for all treatments (all $P < 0.05$). The PCR assay showed that water, essential oil, and povidone-iodine mouthwash had little effect, whereas *V. dispar* DNA bands were markedly inhibited after washing with chlorhexidine. These results suggest that essential oil

and povidone-iodine mouthwash have little effect on oral nitrate-reducing activity. Salivary nitrite production was not reduced by chlorhexidine, but the fainter band of *V. dispar* DNA suggests that longer daily use might blunt this nitrate-reducing activity.

Keywords: nitrate; nitrite; saliva; mouthwash; nitrate-reducing bacteria.

Introduction

Dietary nitrate, derived mainly from vegetables and drinking water, is absorbed rapidly into the bloodstream from the upper intestine and concentrated in the salivary glands by the active transport system, increasing the concentration up to 10-fold relative to that in plasma (1). Excreted nitrate is rapidly reduced to nitrite by bacterial activity, which at one time was thought to be harmful because it could be a cause of infant methemoglobinemia or participate in the formation of carcinogenic *N*-nitrosamine (2). It is now known that after intake of nitrate solution or nitrate-rich food, production of nitric oxide by intestinal bacteria, combined with pre-existing endogenous nitric oxide, causes vasodilation, thus helping to reduce blood pressure (3-5). The Dietary Approaches to Stop Hypertension (DASH) diet contains 1,222 mg nitrate per day, exceeding by 550% the World Health Organization's recommended intake of 222 mg per day for a 60-kg adult (6). Dietary nitrate also has antimicrobial effects on gut pathogens and plays a key role in host defense (7,8)

Mouthwashing is widely used to combat bacterial plaque for the treatment of halitosis and periodontitis.

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It has been reported that reduction of detrimental oral microflora through the use of chlorhexidine mouthwash drastically blunts beneficial nitrate-reducing activity, resulting in a sharp reduction of salivary nitrite, even after nitrate loading (9-11). Use of 0.2% chlorhexidine mouthwash twice a day for 7 days reduces salivary nitrite production by 90% and increases systolic and diastolic blood pressure by 2-3.5 mmHg (9).

To our knowledge, inhibition of nitrate-reducing activity by mouthwash use has been investigated mainly in the context of chlorhexidine, and there has been little information about the effects of other commercially available products. Any marked reduction of salivary nitrite concentration will likely reflect a diminished number of oral nitrate-reducing bacteria. So far, however, there has been no direct evidence that bacterial growth is inhibited by mouthwashing.

In the present study, we investigated the effects of three types of mouthwash—essential oil, povidone-iodine, and chlorhexidine—on nitrate-reducing activity and a representative oral nitrate-reducing bacterium.

Materials and Methods

Participants and protocol

Twelve healthy non-smoking university students and staff (six males and six females, aged 19 to 44 years) were enrolled in this study. They had no history of antibiotic or mouthwash use in the previous 3 months. Written informed consent was obtained from all participants, and the study protocol was approved by the Human Ethics Committee of Iwate University (No. 201407). All participants were asked to refrain from eating vegetables at dinner before the sampling day and to skip breakfast. They arrived at the laboratory prior to 7:30 a.m. for each experiment.

Before washing the oral cavity, a 1.5-mL whole saliva and bacteria sample was collected. For collection of bacteria, the dorsal surface of the tongue of each participant was swabbed 5 times using a sterile cotton wool stick. The posterior surface of the tongue is the major area responsible for nitrate reduction in the oral cavity (12). The stick was immediately placed in 1 mL of phosphate-buffered saline (PBS) to create a bacterial slurry. After washing the oral cavity with a brush for 3 min for each mouthwash treatment, each participant ate 100 g of lettuce, which is equivalent to 110 mg of nitrate (Fujinuma K et al. Ann Rep Tokyo Metr Inst Pub Health 58: 195-203, 2007), with two or three butter rolls and 150 mL of orange juice in 10 min.

The mouthwashes used in this study were as follows: 1) water (control); 2) Listerine (active ingredients:

eucalyptol 0.092%, menthol 0.042%, methyl salicylate 0.060%, and thymol 0.064%, dissolved in up to 25% ethanol; Johnson & Johnson K.K., Bangkok, Thailand); 3) Isodine (povidone-iodine 7%; Meiji Seika Pharma, Tokyo, Japan); 4) Butler CHX (chlorhexidine gluconate 0.05%; Sunstar, Osaka, Japan). In practice, each mouthwash was used according to the manufacturer's guidelines. Listerine is used directly, and 0.5 mL of Isodine and Butler CHX are diluted with 9.5 mL of water. The final concentrations are 0.35% for Isodine and 0.0025% for Butler CHX. The order of mouthwash use was randomized, and each experiment was conducted at 7-day intervals. Samples were collected at 1, 5, and 10 h after mouthwashing and nitrate loading, and immediately stored at -20°C for analysis. At the 5-h collection point (12:30), all participants ate a 200-g rice ball with a cooked egg and green tea.

Analysis of salivary nitrate and nitrite

The method used for salivary nitrite measurement was described previously (13). The whole saliva sample was diluted 100-400-fold using distilled water and filtered using an ADVANTEC Grade No. 2 filter for nitrite analysis. A standard colorimetric method involving diazotization with sulfanilamide coupled with *N*-(1-naphthyl) ethylenediamine to form an azo-dye was used, and the samples were tested spectroscopically at 540 nm. Nitrate was measured after reduction to nitrite by passing it through a cadmium-copper column.

Polymerase chain reaction (PCR) protocol and semi-quantification of bacterial DNA

The nitrate-reducing bacterium targeted in this study was *Veillonella dispar*, which has potent nitrate-reducing activity and is one of the species most frequently isolated from the surface of the tongue (12). In our pilot study involving 20 healthy adults, *V. dispar* was detected more frequently and clearly than *V. atypica*, another predominant nitrate-reducing bacterium in the oral cavity (12), via the PCR method using the same procedure as that described below (unpublished data).

For identification, the forward primer-5'-AACGCGTT-GAAATTCGTCATGAAC-3' and the reverse primer-5'-GTGTAACAAGGGAGTACGGACC-3' were used (14). The primers were synthesized by BEX Co. Ltd (Tokyo, Japan).

The PCR mixture (50 μL) was composed of 24 μL H_2O , 12 μL bacterial slurry serially diluted 10-fold with PBS (from 10^{-1} to 10^{-4}), 12 μL direct PCR kit (KAPA2G Robust HotStart ReadyMix with dye, Kapa Biosystems, Cape Town, South Africa), and 1 μL of each primer (50

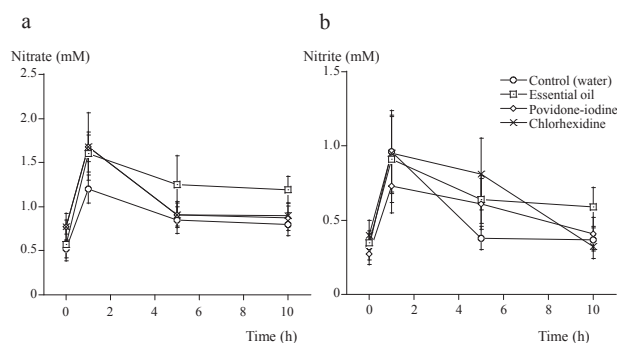


Fig. 1 Salivary nitrate and nitrite concentrations for the control (water), essential oil, povidone-iodine, and chlorhexidine treatments. Values are expressed as mean \pm SEM ($n = 12$). (a) The F -values were 10.35, 5.73, 4.80, and 7.30, respectively, which were all statistically significant, $F(3, 21, 0.01) = 3.07$. (b) The F -values were 6.28, 4.14, 3.97, and 5.58, respectively, which were also all statistically significant. All salivary nitrate/nitrite concentrations at the 1-h collection point were increased significantly from the baseline (all $P < 0.05$).

pmol/ μ L). This PCR system has high amplification efficiency even in the presence of an inhibitor, and is a useful tool for forensic samples (15).

PCR was performed in a thermal cycler (Model TP600, Takara Bio Inc., Otsu, Japan). The reaction mixture was predenatured at 95°C for 3 min, followed by 30 or 32 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s and extension at 72°C, with a final extension at 72°C for 10 min. The PCR products (12 μ L) were analyzed using electrophoresis in 1% agarose gel (Lonza, Rockland, ME, USA) on a submarine-type apparatus (Mupid-2 plus, Mupid Co., Ltd, Tokyo, Japan). Precisely 10 μ L of a 250-bp DNA ladder was used as a size marker (Takara Bio Inc., Otsu, Japan). After electrophoresis, the gel was stained with 50 μ g/100 mL ethidium bromide solution. The intensity of each PCR product relative to the 250-bp DNA ladder on the same gel was calculated using Image J (NIH, Bethesda, MD, USA) for semi-quantification of bacterial DNA.

Data analysis

Salivary nitrite and nitrate and the relative intensities of the bands were reported as means \pm SEM. Differences in the mean values within the same experiment were analyzed by one-way repeated measures ANOVA. When a significant F -value was obtained, Dunnett's test was used to compare the basal level with those of the 1-, 5-, and 10-h samples. Statistical significance was set at $P < 0.05$. All statistical analyses were carried out using Kaleidagraph 3.6 (HULINKS, Tokyo, Japan).

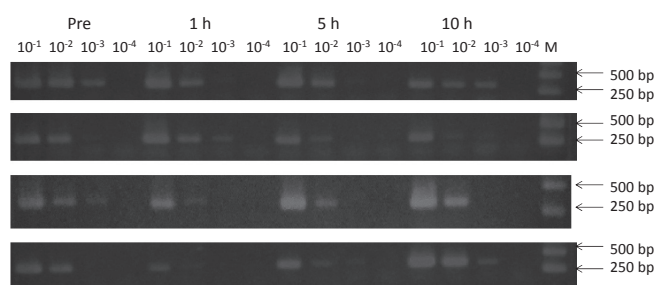


Fig. 2 Analysis of direct PCR results for *V. dispar* DNA from bacterial slurry, diluted by 10^{-1} to 10^{-4} , after mouth washing with water (control), essential oil, povidone-iodine, and chlorhexidine, respectively.

Results

Salivary nitrate and nitrite

Figure 1 shows the salivary nitrate and nitrite concentrations in each experiment. Salivary nitrate concentrations after treatment with water (control), essential oil, povidone-iodine, and chlorhexidine increased significantly from 0.52 ± 0.10 , 0.57 ± 0.18 , 0.77 ± 0.15 , and 0.45 ± 0.08 mM at the baseline to 1.20 ± 0.16 , 1.60 ± 0.21 , 1.68 ± 0.38 , and 1.15 ± 0.17 mM at 1 h, respectively (All $P < 0.05$). Salivary nitrite concentrations also increased significantly from 0.32 ± 0.09 , 0.35 ± 0.08 , 0.27 ± 0.07 , and 0.40 ± 0.10 mM at the baseline to 0.96 ± 0.28 , 0.91 ± 0.29 , 0.73 ± 0.18 and 0.95 ± 0.26 mM at 1 h, respectively (all $P < 0.05$).

PCR and agarose gel electrophoresis

One male participant was excluded from the analysis because a clear DNA band was not obtained in any of the experiments. The participants showed no increase in their salivary nitrite levels after nitrate loading.

No apparent effects of essential oil and povidone-iodine on the DNA bands were observed, but a markedly diminished band was seen at 1 h after chlorhexidine application (Fig. 2). Clear bands were difficult to observe after 10^{-3} and 10^{-4} slurry dilution; therefore, bands derived from 10^{-1} and 10^{-2} dilution were used for calculating the intensity relative to the 250-bp DNA ladder band. The intensity of the bacterial DNA band was divided by that of the 250-bp DNA ladder band.

After chlorhexidine application, there was a significant difference in the band intensity for the 10^{-2} dilution ($P <$

Table 1 Intensity of each DNA band of *V. dispar* relative to that of a 250-bp molecular weight marker

	Dilution	Baseline	1 h	5 h	10 h	F	P
Control	10 ⁻¹	1.03 ± 0.16	1.35 ± 0.21	1.37 ± 0.16	1.19 ± 0.13	1.99	0.13
	10 ⁻²	0.94 ± 0.17	0.82 ± 0.10	1.02 ± 0.16	1.00 ± 0.19	0.50	0.68
Essential oil	10 ⁻¹	0.91 ± 0.13	1.05 ± 0.14	1.30 ± 0.21	1.10 ± 0.15	1.75	0.18
	10 ⁻²	0.90 ± 0.17	0.81 ± 0.09	0.82 ± 0.15	0.70 ± 0.15	0.51	0.69
Povidone-iodine	10 ⁻¹	0.98 ± 0.29	0.90 ± 0.17	1.04 ± 0.21	1.31 ± 0.31	1.19	0.32
	10 ⁻²	0.79 ± 0.19	0.55 ± 0.17	0.68 ± 0.16	0.86 ± 0.16	2.24	0.10
Chlorhexidine	10 ⁻¹	1.19 ± 0.32	1.05 ± 0.41	1.32 ± 0.29	1.50 ± 0.23	1.54	0.22
	10 ⁻²	1.02 ± 0.29	0.35 ± 0.13**	0.48 ± 0.15*	0.86 ± 0.17	4.70	<0.01

All data are expressed as mean ± SEM (n = 11). *P < 0.05 or **P < 0.01 vs baseline.

0.05), but no significant difference was observed after the other treatments (Table 1).

Discussion

Many studies have examined the effectiveness of essential oil, povidone-iodine, and chlorhexidine mouthwashes (16-20). Chlorhexidine is regarded as the strongest and most effective agent for combating plaque and gingivitis (19,20), while the effects of essential oil and povidone-iodine are controversial. Use of essential oil mouthwash for 6 months reportedly did not change the counts of oral pathogens such as *Bacteroides*, *Streptococcus mutans*, and *Candida albicans* (16). In a 1-year follow-up study, essential oil was reported to have an uncertain effect in terms of plaque control (17). Periodontal treatment with povidone-iodine for 6 months failed to show any additional benefits compared with sterile saline ultrasonic scaling (18).

Studies of salivary nitrite production have indicated that oral nitrate-reducing activity is not inhibited markedly by mouthwashing. However, chlorhexidine at a concentration of 0.12% or 0.2% reduced salivary nitrite drastically (9-11), whereas the concentration of 0.0025% adopted in the present study was insufficient to disrupt salivary nitrite production. One possible reason is that other nitrate-reducing bacteria were not affected by chlorhexidine mouthwash. Recently, Woessner et al. (11) observed significant production of salivary nitrite after mouthwashing with essential oil and ingestion of 140 mL of beetroot juice (8.4 mM or 521 mg of nitrate), similarly to our present findings. They also reported that Cepacol (active ingredient: cetylpyridinium chloride 0.05%; activity similar to that of 0.12% chlorhexidine) inhibited the production of salivary nitrite.

In the present study, 0.05% chlorhexidine was diluted to 0.0025% based on the product manufacturer's guidelines. Therefore, the final concentration was 48-80 times weaker than the 0.12-0.2% product. In Japan, chlorhexidine use for sterilization of the oral cavity, hands, and

catheters resulted in more than 10 cases of anaphylactic shock in the 1980s (21,22). Stephens et al. (23) reported that chlorhexidine allergy has been described mainly in Japanese individuals. This may be the reason for the lower concentration of chlorhexidine mouthwash available in Japan.

In the present study, chlorhexidine mouthwash did not reduce nitrite production, although it inhibited the band of *V. dispar* DNA. The minimum inhibitory concentrations of chlorhexidine for *Staphylococcus aureus*, *Salmonella* spp., and *Escherichia coli* are 8 mg/L (0.0008%), 32 mg/L (0.0032%), and 64 mg/L (0.0064%), respectively (24). Exposure to 0.0025% chlorhexidine for 30 min suppressed the adhesion of oral *Candida* species to buccal epithelial cells by 50.89% (25). These results indicate that daily use of chlorhexidine mouthwash, even at a low concentration of 0.0025%, may affect oral microflora and inhibit nitrate-reducing activity.

Figure 2 shows the result of a PCR assay of diluted saliva from a participant who produced a significant amount of nitrite. Obvious changes were not observed after washing with water, essential oil, or povidone-iodine mouthwash. A markedly thinner DNA band was observed at 1 h after chlorhexidine washing, and then this gradually recovered. Growth of oral *Veillonella* spp. seems to have been relatively rapid. This bacterial genus is recognized to be an early colonizer of dental biofilms (26). A statistically significant difference in the intensity of the DNA band was seen after 10⁻²-diluted chlorhexidine mouthwash use (Table 1). The small number of participants and the large discrepancy in the number of bacteria among individuals may have made it difficult to obtain significant data and to identify the effects of mouthwash use on the bacterial counts.

In conclusion, essential oil and povidone-iodine mouthwash had little effect on nitrate-reducing activity and the count of *V. dispar*. Mouth washing with 0.0025% chlorhexidine did not affect salivary nitrite production, but it appeared to inhibit the growth of *V. dispar*. Longer

daily use of chlorhexidine mouthwash, even at low concentrations, may inhibit beneficial nitrate-reducing activity in the oral cavity.

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Conflict of interest

The authors have no conflict of interest to declare.

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