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Short communication

Salivary nitrate – an ecological factor in reducing oral acidity

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Human oral cavities represent a novel environment with a constant supply of concentrated nitrate. For humans, over 80% of dietary nitrate originates from fruit and vegetables. With a healthy, balanced diet, rich in fruit and vegetables, the concentration of nitrate in saliva can reach up to more than three times the European drinking water standard. The physiological function of the active excretion of salivary nitrate is unknown. Furthermore, little is known of the ecological function of oral nitrate and the effect on the oral environment during its subsequent oral microbial conversions. The objectives of the research were to investigate the effect on salivary pH coupled with oral microbial nitrate and/or nitrite reduction. Human saliva samples were incubated anaerobically in the presence of 111.0 mmol glucose (2%), with and without 1.5 mmol nitrate/nitrite, and pH and nitrate/nitrite consumption were measured during the time-course of the incubations. We found that anaerobic incubation of saliva containing a mixture of oral bacteria in the presence of nitrate/nitrite substrates and glucose resulted in a higher pH than was found in controls in the absence of nitrate/nitrite. These results suggest that the presence of these electron acceptors repressed acid fermentation, or increased alkali production, or consumed acid produced, thus reducing salivary acidity. This finding identifies salivary nitrate as a possible ecological factor in reducing oral acidity. The possibility that a symbiotic relationship between host nitrate excretion and nitrate-reducing microorganisms might help to protect against tooth decay should be explored further.

Key words: oral nitrate; pH; reduction; salivary nitrate; tooth decay

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Human oral cavities represent a novel environment with a constant supply of concentrated nitrate (5, 19, 24). Nitrate availability in other natural environments, such as the oceans, on the other hand, is limited by a series of biological processes (12). Human dietary nitrate is absorbed into the bloodstream from the stomach and small intestine, and then concentrated by a factor of 10 to 20 by the salivary glands (2, 5, 19, 24). Resultant salivary nitrate concentration ranges between 0.3 to 2.6 mmol depending on the diet (5, 19, 24). Salivary nitrate is dose-dependent on dietary nitrate (5, 19, 24) and more than 80% of dietary nitrate originates from fruits and vegetables (32). As a result, humans with a healthy, balanced diet rich in fruit and vegetables may have a constant nitrate flow into the oral cavity and its concentration reaches up to more than three times higher than that of the European drinking water standard (50 mg/l) (31). Most mammals, including humans, also synthesize nitrate (2, 6, 7). This indigenous nitrate arises from oxidation of nitric oxide (NO), which is a signal molecule with links to numerous biological functions (9). Therefore, even with a nitrate-free diet, there will be significant nitrate concentrations in plasma (2). The physiological function of active salivary nitrate excretion is as yet unknown.

Nitrate is the next most favourable electron acceptor in the environment for microorganisms that are conducting energy-yielding metabolism when oxygen becomes limited and it is reduced into different nitrogen species in the process (3, 10, 30, 33). This is crucial in the global nitrogen cycle, such as in the removal of nitrate in the marine ecosystem (12). The reduction of nitrate in anaerobic environments is dominated by two dissimilatory processes: respiratory denitrification and dissimilatory nitrate reduction to ammonium (DNRA) (29, 30). Salivary nitrate is reduced to nitrite and nitrous oxide by oral microorganisms in humans and animals (17, 19, 23, 28), indicating that nitrate respiration is operating in the oral cavity. There have been concerns regarding the harmful effect of salivary nitrite. For example, it has been associated with the

formation of nitrosamines, which are known to be carcinogenic to animals (18, 20, 27). More recently, increasing evidence suggests that salivary nitrite has beneficial effects to the host, such as antimicrobial activity, which can be enhanced under acidified conditions to prevent stomach and oral infections (19).

When oxygen becomes limited, nitrate respiration takes precedence over the next energy-inefficient, fermentation process (10, 33). Some bacterial niches in human hosts, such as the interior of dental plaques, are anaerobic (21, 22). Nitrate respiration in such niches is energetically less favourable than aerobic respiration, but more favourable than fermentative breakdown of carbohydrates. In fact, most bacteria that carry out DNRA, are also fermentative (29). For oral bacteria with both DNRA and fermentation metabolisms, the supply of nitrate in the oral cavity may activate their nitrate respiration and repress fermentation and, as a result, buffer salivary acidity. Repressing fermentation is not the only effect contributing to a saliva pH increase because of nitrate respiration. Other mechanisms relating to nitrate reduction, such as hydroxyl ion generation (8, 26) and the scavenging of organic acid (3), may further contribute to the reduction of oral acidity. Human saliva contains a high density of microorganisms detached from oral biofilms (15, 16, 21, 22). When human saliva is incubated with glucose, the pH has been found to fall as a result of fermentative metabolism by salivary bacteria (15, 16). This has been used as a conventional approach to studying factors affecting oral pH and tooth decay.

Materials and methods

Sampling

A total of 13 volunteers (six male, seven female) were selected across the age span 20–48 years. Saliva samples were collected by spitting saliva into 20-ml sterile plastic tubes without any stimulation. The total volume for each sample was between 8 and 10 ml. For experiments with no pre-incubation, the sample tubes were placed in ice-water during collection. For other experiments, samples were collected at room temperature (20 ± 3°C). Samples were stored at 4–8°C if not analysed immediately, but were never stored for more than 8 h.

Degassing and pre-incubation

Equal volumes of Milli Q water were added to each saliva sample and mixed thoroughly to reduce viscosity. Aliquots (2 ml) of the diluted saliva samples were then sub-sampled into six 40-ml, sterile, amber vials (triplicate for each treatment) with polypropylene hole caps, and sealed with PTFE/silicone septa (Supelco [Poole, UK] 27121U). The samples were then degassed with pure nitrogen through a sterile stainless needle (Fisher [Loughborough, UK] SZR-370-135X), which was pierced through the septum into the vial with the tip submerged in the sample. Gas outlet during degassing was carried out via a sterile disposable syringe needle (TERUMO [Surrey, UK] 0411080). To stop contamination during degassing, nitrogen gas was passed through a 0.2-μm filter. The degassing procedure lasted 10 min, at a rate of approximately 200 ml/min. After degassing, samples requiring pre-incubation were incubated at 30°C for 12 h.

Nitrate reduction assay

Glucose (BDH, Poole, UK) and glucose plus nitrate/nitrite [added as potassium nitrate (Sigma, Poole, UK) or potassium nitrite (Fisher), respectively] substrate solutions were prepared in the sample vials as for the sample preparation from stock solution in Milli Q water. The pH of the solution was adjusted to 7.0 by the addition of 2 mol NaOH and/or HCl. The solutions were then degassed as described previously.

Saliva samples and substrate solutions were both placed in a 37°C water for 5 min before starting the reaction. The reactions were started by piercing the septa and injecting 3-ml aliquots of substrate solution into the saliva sample vials to a final glucose concentration of 111.0 mmol (2%) and nitrate/nitrite of 1.5 mmol. Aliquots (0.8 ml) were taken by piercing the septa using a 1 ml sterile, disposable syringe needle at the time indicated.

Determination of pH and nitrate/nitrite

Sample pH was measured in a 1-ml Eppendorf tube, using a micro pH probe (VWR [Luthorworth, UK] 6621767), attached to a pH meter (JENWAY [Essex, UK] 3305). Nitrate and nitrite concentrations were determined using an ion chromatography system (Dionex, Surrey, UK), with conductivity detector and anion exchange column (AS9-HS). The mobile phase was 1.8 mmol $NaCO_3$ and 1.7 mmol $NaHCO_3$ in water, at a flow rate of 1.5 ml/min.

Aeration

Where aeration was applied, a similar method was used as for degassing, but air replaced nitrogen during the incubation. The air was supplied by an air pump (Whisper 6000, Interpret Ltd-Dorking, Surrey UK) at a rate of approximately 100 ml/min.

Statistical analysis

All statistical analysis was carried out using MINITAB-14 software. The *t*-tests were carried out using both the pH value at 3 h and slopes of pH change between treatments during incubation.

Results

pH fluctuation coupled with nitrate consumption

The pH fall decreased during incubation with nitrate (1.5 mmol) compared with controls (without nitrate) ($P < 0.001$, Paired *t*-test, $n = 13$). A significant relative pH increase in the nitrate treatment was observed compared to the control during incubation ($n = 13$; Fig. 1A). There was a very strong negative correlation between the average nitrate concentration and average relative pH increase during incubation ($R = -0.952$, $P = 0.012$, $n = 13$; Fig. 1B). Figure 1C represents an empirical cumulative distribution function (CDF) plot. It highlights the pH differences between the nitrate treatment and the control ($n = 13$) for all saliva samples and at all incubation times, indicating no pH difference for pH 7 and above for both treatments, but suggesting that the pH was generally lower in control than in the nitrate treatments during incubation. Approximately 22% of data points ($n = 195$) were less than pH 6 in the control, with only 15% of data points ($n = 195$) less than pH 6 in the nitrate treatment. Twenty per cent of data points were less than pH 5.8 in the control, with the same percentage of data less than pH 6.3 in the nitrate treatment.

There were four volunteer's samples out of 13 in which the pH fall was not reduced to a significant extent during incubation. Interestingly, nitrate was not consumed in a significant quantity during incubation for these four individuals (data not shown).

pH fluctuation coupled with nitrite consumption

Nitrous oxide has been detected in human exhaled air (23). In this study, during the reduction of nitrate, the accumulation of nitrite did not account for the loss of nitrate in terms of stoichiometry (data not shown), indicating that nitrite is further reduced during microbial nitrate reduction. Indeed,

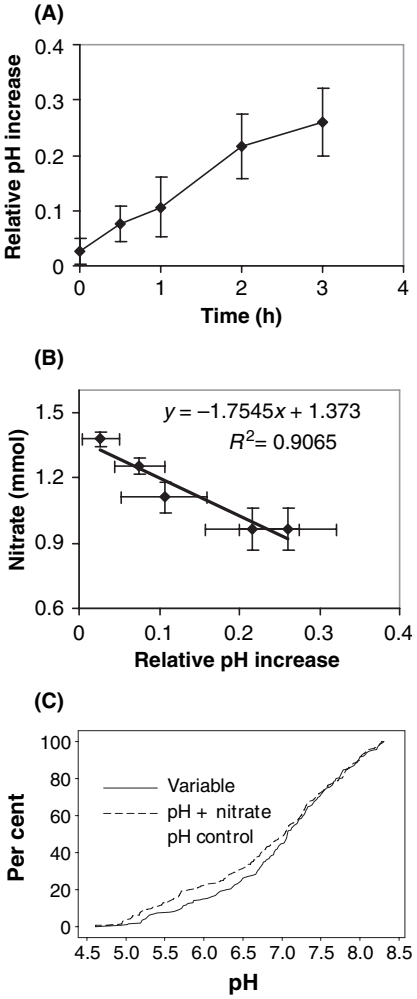

Fig. 1. Fluctuations of salivary pH and nitrate concentration during anaerobic incubation. (A) Relative pH increases in nitrate treatment during incubation ($n = 13$). The values of 'relative pH increase' are calculated from the pH values in the nitrate treatment by deducting the pH values in control (without nitrate) for all experiments of 13 samples at different times. All samples were pre-incubated and incubated anaerobically during the reaction. (B) The correlation between average nitrate concentration and relative pH increase ($n = 13$). (C) Empirical Cumulative Distribution Function (CDF) plots ($n = 13$, each line was plotted from 195 pH data-points). It calculates the proportion of data-points less than each x value (pH value), and then plots the proportion (y value) as a function of x. The y-axis scale represents the percentage of data points less than each × (pH) value, not a probability scale for a specific distribution. Bars are standard errors of the mean in all figures.

when replacing nitrate with nitrite in a primary experiment using three saliva samples, under the same conditions, the fall in salivary pH declined significantly in the presence of nitrite. Figure 2 shows the relative pH increase and nitrite concentration at 3 h ($n = 3$). There was no significant pH difference in one out of three

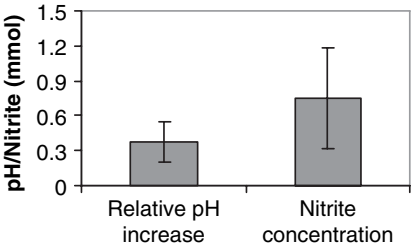

Fig. 2. Effect of nitrite reduction on pH during anaerobic incubation. All conditions are the same as described in Fig. 1, except nitrate was replaced by nitrite. Only relative pH difference and nitrite concentration at 3 h were plotted ($n = 3$).

samples in the presence of nitrite. However, in this experiment, nitrite was not consumed in significant quantities (data not shown). The remaining two samples demonstrated progressive consumption of nitrite, and the relative pH increase was coupled to nitrite consumption (data not shown).

Factors influencing nitrate consumption and pH fluctuation

When using saliva from one individual, no reduced acidity was observed when nitrate was added and saliva was incubated aerobically, without anaerobic pre-incubation, and there was no consumption of added nitrate. Even nitrate carried over from the original saliva samples, in the control experiment, was not consumed in significant quantities (Fig. 3A). The reduced salivary acidity was also observed after the addition of nitrate to washed saliva pellets, during anaerobic incubation (Fig. 3B).

Discussion

Whole human saliva usually contains 2.6 mmol ammonium (11), which is higher than the concentration required to suppress assimilatory nitrate reduction (29). This ensures salivary nitrate is used as an electron acceptor, to support anaerobic nitrate respiration in buffering salivary pH, rather than consumed by assimilatory nitrate reduction by the oral microflora. The results suggest that the function of active concentration and excretion of salivary nitrate by humans and other animals might have been selected to support the anaerobic microbial nitrate respiration that competes for carbon with processes that lead to fermentative acid production; the subsequent nitrate respiration generates hydroxyl ions and scavenges organic acids which have been produced.

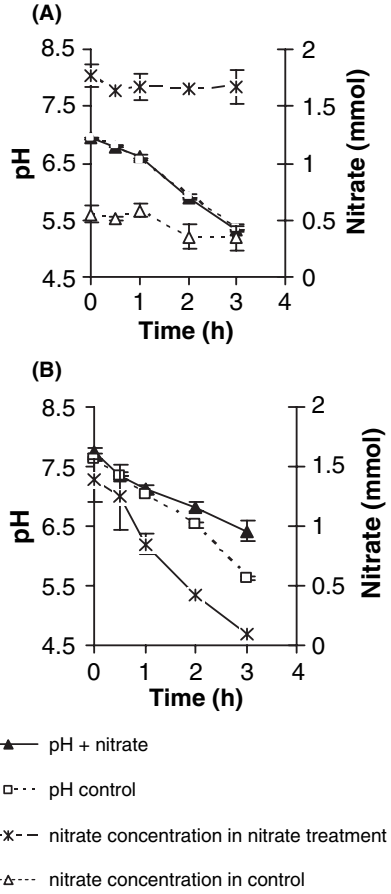

Fig. 3. Effects of aeration and saliva supernatant on pH and nitrate consumption. The saliva incubation conditions were: (A) no pre-incubation and incubated aerobically; saliva was washed three times using 1 × PBS (phosphate-buffered saline), and re-suspended to the original volume. Other procedures were the same as described in *Materials and methods* except without pre-incubation (B). Each data-point represents the means from triplicate experiments from a volunteer's saliva sample.

Experiments using saliva from one individual also supported the suggestion that the consumption of nitrate is unlikely to have been the result of assimilatory nitrate reduction because aeration inhibited nitrate consumption, and assimilatory nitrate reduction is not sensitive to oxygen (29). The washed cell experiment demonstrated that the effect of nitrate on the pH of saliva is unlikely to be linked chemically to the supernatant, and is more likely to be associated with the activity of microbial populations in the pellets. In Fig. 1C, differences are mainly apparent below pH 7. The lack of difference above pH 7 between treatments is because data for pHs above 7 mainly reflect the initial incubation conditions (all saliva samples were initially above pH 7).

The primary experiment showed that nitrite also mediated the reduction in acidity during anaerobic incubation, suggesting that nitrate was consumed via an anaerobic nitrate respiration pathway. However, the final reduction pathway needs to be further elucidated. Of course one could also expect selective enrichment of denitrifiers (or nitrate-respiring bacteria) when nitrate is available and oxygen is limited (29). Salivary nitrate might have selected an anaerobic, nitrate-respiring microbial community to compete for carbon with obligate, fermentative bacteria. One of the ecological functions of dissimilatory nitrate reduction is to scavenge fermentative products, such as organic acids (3). Salivary nitrate may support the consumption of organic acids, which have already been produced in the oral cavity, as electron donors through DNRA and the resultant pH increase in the oral cavity. The reason for the lack of nitrate reduction in the 4 out of 13 saliva samples might have been the lower microbial activity in saliva or individual differences in saliva microflora. This is not surprising because samples were collected without control, such as after brushing of teeth, and before fluid or food consumption. The same reason might be responsible for one individual result obtained in the experiment with nitrite, which did not indicate either nitrite reduction or acidity reduction. The reason for the lack of nitrate/nitrite reduction in these samples should be explored in the future. The lack of acidity and nitrate/nitrite reduction capacities in all these experiments also supports the hypothesis that the slower pH decrease is linked to microbial metabolism of nitrate or/and nitrite.

It is commonly believed that lactobacilli and streptococci are the main lactic acid producers responsible for tooth decay (21, 22). Nitrate may not affect their metabolism because most of them do not reduce nitrate. However, species of *Actinomyces* common in human sub-gingival plaque (25), are the main nitrate reducers in the human oral cavity (4), and are carbohydrate-fermenting bacteria (14). For these bacteria, suppression of acid production could be theoretically expected when nitrate is available.

In the oral cavity, streptococci ferment carbohydrates to produce lactic acid, which is a principal fermentation substrate used by the veillonellae (16). It has been shown that veillonellae from the human oral cavity can reduce nitrate (4). Lactic acid is also produced in the rumen (1). The importance of lactate-fermenting bacteria with nitrate-reducing capacity in protecting from lactate accumulation in rumen (referred to as lactic rumen acidosis) has been postulated. The mechanism is based on supplying nitrate to enhance the use of lactate by the nitrate-reducing *Selenomonas ruminantium* (1). In fact, lactate can stimulate nitrate and nitrite reduction to a greater extent than glucose in these bacteria (13). It will be very interesting to investigate the effect of salivary nitrate on the metabolic communication between lactic-acid-producing communities and lactic-acid-utilizing communities, such as occurs in the rumen. The mechanism of nitrate-reduction-related oral acid reduction should be further explored.

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References

1. Asanuma N, Hino T. Ability to utilize lactate and related enzymes of a ruminal bacterium, *Selenomonas ruminantium*. Anim Sci J 2005: **76**: 347–352.
2. Benjamin N, McKnight G. Metabolism of nitrate in human-implications for nitrate intake. In: Wilson WS, Ball AS, Hinton RH, ed. Managing risks of nitrates to humans and the environment. London: Royal Society of Chemistry, 1999: 281–294.
3. Cole JA. Physiology, biochemistry and genetics of nitrate dissimilation to ammonia. In: Revsbech NP, Sorensen J, ed. Denitrification in soil and sediment. New York: Plenum Press, 1990: 57–76.
4. Doel JJ, Benjamin N, Hector MP, Rogers M, Allaker RP. Evaluation of bacterial nitrate reduction in the human oral cavity. Eur J Oral Sci 2005: **113**: 14–19.
5. Duncan C, Dougall H, Johnston P et al. Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. Nature Med 1995: **1**: 546–551.
6. Green LC, Ruiz de Luzuriaga K, Wagner DA et al. Nitrate biosynthesis in man. Proc Natl Acad Sci USA 1981: **78**: 7764–7768.
7. Green LC, Tannenbaum SR, Goldman P. Nitrate synthesis in the germfree and conventional rat. Science 1981: **212**: 56–88.
8. Grguric G, Wetmore SS, Fournier RW. Biological denitrification in a closed seawater system. Chemosph 2000: **40**: 549–555.
9. Gryglewski RJ, Minus P, ed. Nitric oxide: basic research and clinical application. Amsterdam-Berlin-Oxford-Tokyo-Washington: IOS Press, 2001.
10. Gunsalus RP. Anaerobic respiration. In: Lederberg J, ed. Encyclopedia of Microbiology, Vol. 2, 2nd edn. San Diego: Academic Press, 2000: 180–188.
11. Huizenga JR, Vissink A, Kuipers EJ, Gips CH. *Helicobacter pylori* and ammonia concentrations of whole, parotid and submandibular/sublingual saliva. Clin Oral Investig 1999: **3**: 84–87.
12. Hulth S, Aller RC, Canfield DE et al. Nitrogen removal in marine environments: recent findings and future research challenges. Marin Chem 2005: **94**: 125–145.
13. Iwamoto M, Asanuma N, Hino T. Effects of energy substrates on nitrate reduction and nitrate reductase activity in a ruminal bacterium, *Selenomonas ruminantium*. Anaerobe 2001: **7**: 315–321.
14. Kalfas S, Edwardsson S. Effect of culture medium on acid production from sorbitol by oral bacteria. Acta Odontol Scand 1990: **48**: 217–222.
15. Kleinberg I. A mixed-bacteria ecological approach to understanding the role of the oral bacteria in dental caries causation: an alternative to *Streptococcus mutans* and the specific-plaque hypothesis. Crit Rev Oral Biol Med 2002: **13**: 108–125.
16. Kolenbrander PE. Oral microbial communities: biofilms, interactions, and genetic systems. Annu Rev Microbiol 2000: **54**: 413–437.
17. Li H, Duncan C, Townend J et al. Nitrate-reducing bacteria on rat tongues. Appl Environ Microbiol 1997: **63**: 203–210.
18. Lijinsky W, Epstein SS. Nitrosamines as environmental carcinogens. Nature 1970: **225**: 21–23.
19. Lundberg JO, Weitzberg E, Cole JA, Benjamin N. Nitrate, bacteria and human health. Nat Rev Microbiol 2004: **2**: 593–602.
20. Magee PN. Toxicity of nitrosamines: their possible human health hazards. Food and Cosmet Toxicol 1971: **9**: 207–218.
21. Marsh P, Marsh PD. Oral ecology and its impact on oral microbial diversity. In: Kuramitsu HK, Ellen RP, ed. Oral bacterial ecology: the molecular basis. Wymondham: Horizon Scientific, 2000: 11–65.
22. Marsh PD. Are dental diseases examples of ecological catastrophes? Microbiology 2003: **149**: 279–294.
23. Mitsui T, Kondo T. Effects of mouth cleansing on the levels of exhaled nitrous oxide in young and older adults. Sci Total Environ 1998: **224**: 177–180.
24. Pannala AS, Mani AR, Spencer JP et al. The effect of dietary nitrate on salivary, plasma, and urinary nitrate metabolism in humans. Free Radic Biol Med 2003: **34**: 576–884.
25. Paster BJ, Boches SK, Galvin JL et al. Bacterial diversity in human subgingival plaque. J Bacteriol 2001: **183**: 3770–3783.
26. Rijn JV, Tal Y, Schreier HJ. Denitrification in recirculating systems: theory and applications. Agricul Engine 2006: **34**: 364–376.
27. Spiegelhalder B, Eisenbrand G, Pressmann R. Influence of dietary nitrate on nitrite content of human saliva: possible relevance to in vivo formation of *N*-nitroso-compounds. Food Cosmet Toxicol 1976: **14**: 545–548.

28. Tannenbaum SR, Weisman M, Fett D. The effect of nitrate intake on nitrite formation in human saliva. Food Cosmet Toxicol 1976: **14**: 549–552.
29. Tiedje JM. Ecology of denitrification and dissimilatory nitrate reduction to ammonia. In: Zehnder AJB, ed. Biology of anaerobic microorganisms. New York: Wiley, 1988: 79–244.
30. Unden G, Becker S, Bongaerts J, Schirawski J, Six S. Oxygen regulated gene expression in facultative anaerobic bacteria. Anton van Leeuwenhoek 1994: **66**: 3–23.
31. World Health Organization. European standards for drinking water, 2nd edn. Geneva: WHO, 1970: 33.
32. White JW Jr. Relative significance of dietary sources of nitrate and nitrite. J Agric Food Chem 1975: **23**: 886–891.
33. Zehnder AJB, Stumm W. Geochemistry and biogeochemistry of anaerobic habitats. In: Zehnder AJB, ed. Biology of anaerobic microorganisms. New York: Wiley, 1988: 1–38.