

Effects of a lactoperoxidase-system-containing toothpaste on dental plaque and whole saliva in vivo

Varpuleena Kirstilä, Marianne Lenander-Lumikari and Jorma Tenovuo
Department of Cariology, Institute of Dentistry, University of Turku, Turku, Finland

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The effects of a lactoperoxidase-system-containing toothpaste, Biotene, on saliva and dental plaque were studied. In a double-blind crossover study 20 healthy volunteers used an experimental (comprising the complete peroxidase system) or a placebo (without lactoperoxidase, KSCN, and glucose oxidase) toothpaste twice daily for 2 weeks separated by a 2-week washout period. At base lines and at the end of both test periods saliva and plaque samples were collected, and plaque pH changes were monitored. Saliva was analyzed for hypothiocyanite (HOSCN/OSCN⁻) and thiocyanate (SCN⁻) concentrations and salivary peroxidase activity. The amount of total streptococci, mutans streptococci, lactobacilli, and total anaerobic flora was determined both in saliva and in plaque samples. The accumulation and the acidogenicity of plaque were also quantitated. A 2-week daily use of Biotene had no effect on salivary flow rate, peroxidase activity, HOSCN/OSCN⁻, SCN⁻, or any of the monitored bacterial counts compared with the placebo toothpaste. The accumulation of dental plaque was not affected by the lactoperoxidase-system-containing toothpaste. The acidogenicity of plaque did not change significantly, nor did the two test dentifrices differ in their ability to inhibit the plaque pH drop caused by sucrose in subjects with normal salivary flow rate. □ *Dental plaque; dentifrice; lactoperoxidase; mutans streptococci; saliva*

Jorma Tenovuo, Department of Cariology, Institute of Dentistry, University of Turku, Lemminkäisenkatu 2, FIN-20520 Turku, Finland

A large number of in vitro studies show that the peroxidase-thiocyanate (SCN⁻)-H₂O₂ system is antimicrobial against many oral bacteria (1), fungi (2), and viruses (3). In vivo the antimicrobial agent generated by the peroxidase system is hypothiocyanite ion (OSCN⁻), which is in acid-base equilibrium (pK 5.3) with hypothiocyanous acid (HOSCN) (4). The role of salivary HOSCN/OSCN⁻ in vivo is less clear, obviously because many antimicrobial systems—immune and non-immune—act in the mouth simultaneously, also interacting in many ways (5). However, evidence exists that HOSCN/OSCN⁻ inhibit glucose uptake by cariogenic bacteria (6), acid production by dental plaque (7), and even the initial phases of dental caries (8).

Because of the obvious potential of the peroxidase system to inhibit oral diseases, attempts have been made to activate this system in vivo (9). The limiting component

for the production of HOSCN/OSCN⁻ is H₂O₂ (10), and therefore the system can be activated in vivo by supplementing saliva with appropriate amounts of H₂O₂, which—added either as a mouthrinse or by means of peroxide-producing enzymes (such as glucose oxidase)—significantly increases the amount of HOSCN/OSCN⁻ in whole saliva (11–13). Recently, we reported observations on a peroxidase-system-containing toothpaste (Biotene), which significantly increased HOSCN/OSCN⁻ levels in human saliva both in vitro and in vivo (14).

Previous attempts to enhance the activity of oral peroxidase systems have been based on the addition to H₂O₂-generating enzymes and KSCN (for example, Zendium (15)). However, studies with toothpastes other than Biotene have not reported any increase in salivary HOSCN/OSCN⁻ levels, and the clinical effects have also been controversial (5).

Since Biotene during twice daily use for 4 weeks did not affect the levels of mutans streptococci, lactobacilli, total streptococci, or total microbial flora in whole saliva (14), we have now extended the studies to analyze the possible effects on dental plaque. The number of these bacteria and the accumulation rate and acidogenicity of dental plaque were assessed using Biotene toothpaste with and without (placebo) the peroxidase system components. The acidogenicity of plaque was quantitated by analyzing the glucose-stimulated lactic acid production and by monitoring sucrose-exposed plaque pH in situ immediately after the use of the two test dentifrices.

Materials and Methods

Subjects and study design

The study was a double-blind crossover design. Each test period was 14 days, separated by a washout period of at least 14 days. Twenty healthy volunteers (7 males, 13 females), aged 16–55 years (mean, 34.5 years), took part in the investigation. The criteria for participating were high levels (10^5 – 10^6 colony-forming limits (CFU)/ml) of salivary mutans streptococci as measured with the Dentocult SM Strip Mutans method (Orion Diagnostica, Espoo, Finland) before the trial.

At the base lines and at the end of both test periods similar clinical procedures were performed, consisting of saliva and plaque sampling and plaque pH monitoring. After base-line measurements all participants were given instructions to brush with the experimental toothpaste (dentifrice A or B) twice daily, in the morning and in the evening, for 2 weeks. They were advised to use the same amount of toothpaste as they did usually. This amount was later calculated to be 0.67 ± 0.35 g. Brushing time was not observed. The subjects were asked to refrain from using any other oral hygiene or antimicrobial products and from using other fluoride- or xylitol-containing products during the whole experiment. During the washout period the subjects returned to their normal oral hygiene methods, using any toothpaste

except the test ones. After the second baseline recordings the other experimental dentifrice was given to the subjects for 2 weeks' use.

The subjects were requested to refrain from using any oral hygiene for 48 h before each clinical evaluation, and they were asked not to eat, drink, or smoke for at least 1 h before sampling and pH measurements. The study procedures took place at the same time of day for each subject.

Test dentifrices

The two experimental products, coded dentifrice A and B, were kind gifts from Biopole Co., Brussels, Belgium. The dentifrices were delivered in identical plain white tubes to ensure that neither the examiner nor the subjects were aware of the contents of the tube. Both toothpastes contained sodium monofluorophosphate (0.15% F^-), xylitol (1%), and a nonionic surfactant, Araisolve 200, which is a polyoxyethylene 20 isohexadecyl ether. The pH of both toothpastes was 6.5. The code was opened after all the data had been processed. Toothpaste B, the normal formula, contained in addition lactoperoxidase (bovine milk; 454 ABTS enzyme units/mg, equivalent to 0.02%), KSCN, and a dextrose-glucose oxidase system for H_2O_2 generation (14). Toothpaste A (placebo) was devoid of lactoperoxidase, KSCN, and glucose oxidase but contained dextrose, xylitol, and fluoride.

In plaque pH monitoring experiments dentifrice A or B was used as slurry. Three milliliters sterile water was added to 0.7 g toothpaste and mixed vigorously. The slurry was prepared immediately before rinsing.

Collection and treatment of saliva and plaque samples

Paraffin-stimulated whole saliva was collected from each subject for 5 min, and the secretion rate was determined as milliliters per minute. Immediately after the saliva collection, 100 μ l of fresh and uncentrifuged saliva was used for the hypothiocyanite (HOSCN/OSCN $^-$) assay. For microbiologic analysis a 100- μ l aliquot was transferred to a

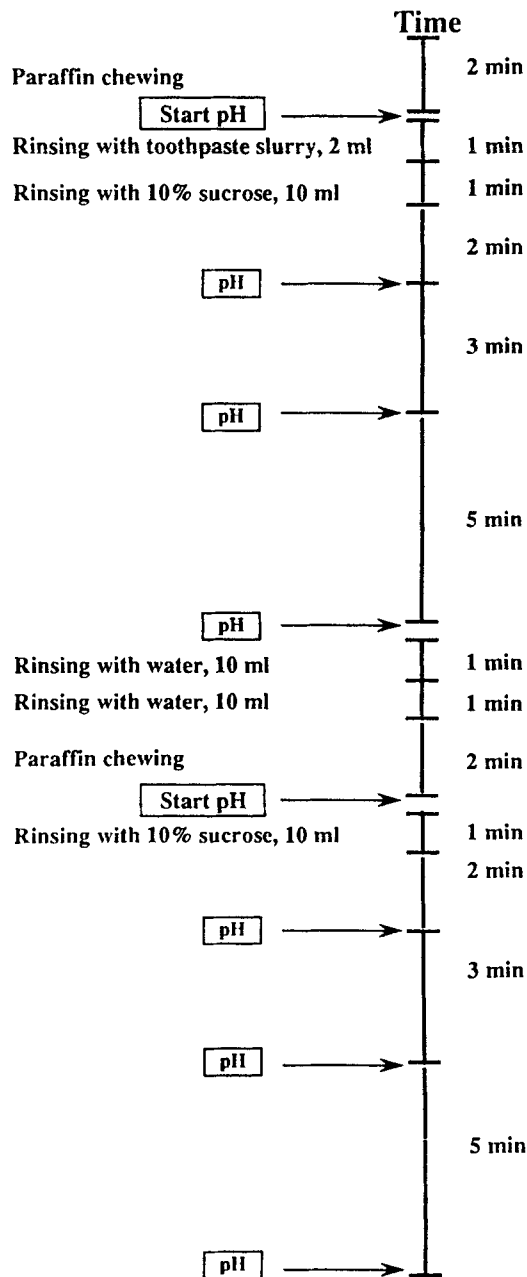


Fig. 1. Scheme for monitoring pH changes in dental plaque induced by sucrose (10%) with and without prerinsing with a toothpaste slurry.

tube containing 1 ml of tryptic soy broth (TSB; Oxoid, Basingstoke, UK), supple-

mented with 20% glycerol. The TSB tubes were stored at -20°C until analyzed.

The rest of the collected saliva was centrifuged at $18,000\text{ g}$ (Superspeed RC-2B centrifuge; Sorvall, Norwalk, Conn., USA) for 10 min at $+4^{\circ}\text{C}$. Portions of centrifuged saliva needed for the analysis of thiocyanate (SCN^-) and the total peroxidase activity were stored frozen at -20°C until analyzed. The saliva samples were kept on ice during handling.

After saliva sampling, supragingival plaque was collected from all tooth surfaces with a sterile curette for approximately 5 min. No plaque was collected from the two interdental sites that had been chosen for pH measurements. The wet weight of the plaque was determined immediately. The sample was then transferred to a tube containing 2 ml phosphate-buffered saline (PBS) (Orion Diagnostica) and sterile glass beads. The tube was vortexed for 30 sec, and a $100\text{-}\mu\text{l}$ aliquot was withdrawn and transferred into a tube containing 1 ml of TSB supplemented with 20% glycerol. The TSB tubes were stored frozen at -20°C until microbiologic analysis.

The rest of the plaque suspension was supplemented with $100\text{ }\mu\text{l}$ 2 M glucose (final concentration, 0.1 M) to induce lactic acid (LA) production. The suspension was incubated in a shaking water-bath at 37°C for 10 min. LA production was terminated by centrifugation at $18,000\text{ g}$ (Sigma model 201 m; Sigma Ltd, Germany) for 10 min at $+4^{\circ}\text{C}$, and the supernatant was separated and stored at -20°C until analyzed.

Plaque pH determinations

The plaque pH monitoring experiments were performed with a palladium touch electrode (Beetronde MEPH-3L; W-P Instruments, New Haven, Conn., USA) connected to an Orion Research (Cambridge, Mass., USA) model 701-A millivolt meter. The touch electrode consisted of a metal-metal oxide wire loop, 0.1 mm in diameter and 2 mm long, mounted in a tapered base. The proper function of the electrode was checked before each measurement, using pH 4.01 and 7.0 buffer solutions, and the same elec-

Table 1. Effects of a 2-week daily use of the experimental toothpastes on the flow rate and the hypothiocyanite-generating peroxidase system components in saliva

	Toothpaste A (placebo)		Toothpaste B (Biotene)	
	Base line, mean $\pm$ SD	2 weeks, mean $\pm$ SD	Base line, mean $\pm$ SD	2 weeks, mean $\pm$ SD
Flow rate (ml/min)	2.0 $\pm$ 1.0	1.9 $\pm$ 0.9	2.0 $\pm$ 0.8	1.8 $\pm$ 0.9
Hypothiocyanite (μ M)	47.8 $\pm$ 17.1	56.7 $\pm$ 23.0	48.6 $\pm$ 19.5	49.1 $\pm$ 31.8
Total salivary peroxidase (mU)	0.88 $\pm$ 0.21	0.82 $\pm$ 0.29	0.80 $\pm$ 0.33	0.79 $\pm$ 0.28
Thiocyanate (mM)	1.09 $\pm$ 0.55	1.09 $\pm$ 0.55	0.97 $\pm$ 0.49	1.04 $\pm$ 0.62

No significant differences were observed (Student's paired *t* test); *n* = 20 in all assays.

trode was used in all recordings throughout the study. The electrode was calibrated at the start of each measurement and after each plaque pH determination by using the buffer solutions mentioned above. The millivolt readings were converted to pH values by means of the standard curves. The two interdental sites chosen for monitoring in each individual were those in the upper jaw with best visible plaque accumulation and no restorations. The hand-held electrode was inserted into the interdental space just apical to the contact point.

The pH monitoring experiments were performed in a standardized manner, following the scheme shown in Fig. 1. The effects of the test products on the changes in plaque pH were monitored by comparing the pH decrease in plaque caused by 10% sucrose with the pH changes in plaque when pririnsed with toothpaste A or B (Fig. 1).

Chemical assays

Concentrations of hypothiocyanite (HOSCN/OSCN⁻), thiocyanate (SCN⁻), and the total peroxidase activity were determined in all saliva samples. HOSCN/OSCN⁻ concentrations were analyzed by reaction with the colored anionic monomer of 5,5-dithiobis-(2-nitro-benzoic acid) (Nbs)₂ as described by Aune & Thomas (16) and modified by Pruitt et al. (17). The thiocyanate ions were quantified by the ferric nitrate method described by Betts & Dainton (18).

Salivary peroxidase activity was measured at +22°C by following the rate of oxidation

of 5-thio-2-nitrobenzoic acid (Nbs) to (Nbs)₂ by OSCN⁻ ions generated during the oxidation of SCN⁻ by salivary peroxidase or myeloperoxidase (19, 20).

Lactic acid production in plaque was quantitated using the L-Lactic acid Kit (Mannheim Boehringer, Germany) (21) and a double-beam spectrophotometer (Shimadzu Corp., Kyoto, Japan).

Microbiologic assays

The saliva and plaque samples before and after 14 days' use of dentifrice A or B were analyzed for mutans streptococci (MS), lactobacilli, total streptococci, and the total anaerobic flora. The TSB tubes were thawed and vortexed thoroughly for 1 min. After serial tenfold dilutions the bacteria were plated as follows: total streptococci and MS on mitis salivarius (Difco Laboratories, Detroit, Mich., USA) and mitis salivarius bacitracin agar plates, respectively. The mitis salivarius agar plates were supplemented with 20% sucrose (BDH, Chemicals Ltd., Poole, UK) and incubated aerobically for 2 days at 37°C. The mitis salivarius bacitracin agar plates were supplemented both with sucrose and with 0.5 μ g/ml bacitracin (Sigma Chemical Co.), and MS were incubated for 3 days in a 7% CO₂ atmosphere at 37°C. Lactobacilli were cultivated on Rogosa SL agar plates (Difco Laboratories) and incubated anaerobically for 3 days at 37°C. The total flora was determined by plating samples on blood agar plates containing 5% bovine blood (Orion Diagnos-

Table 2. Effects of a 2-week daily use of the experimental toothpastes on plaque accumulation and lactic acid production

	Toothpaste A (placebo)		Toothpaste B (Biotene)	
	Base line, mean $\pm$ SD	2 weeks, mean $\pm$ SD	Base line, mean $\pm$ SD	2 weeks, mean $\pm$ SD
Amount of plaque (mg)	31.9 $\pm$ 15.5	28.5 $\pm$ 14.7	27.4 $\pm$ 10.4	25.0 $\pm$ 12.6
Lactic acid (μ g/mg)	1.55 $\pm$ 0.61	1.58 $\pm$ 0.98	1.63 $\pm$ 0.59	1.90 $\pm$ 0.94

No significant differences were observed (Student's paired *t* test); *n* = 20 in all assays.

Table 3. Sucrose (10%)-induced pH drop in dental plaque with and without prerinse with the experimental toothpastes; pH was monitored before and after a 2-week daily use of the toothpastes

	Plaque pH			
	Base line		2 weeks	
	0 min, mean $\pm$ SD	10 min, mean $\pm$ SD	0 min, mean $\pm$ SD	10 min, mean $\pm$ SD
Toothpaste A (placebo)				
Toothpaste + sucrose	6.6 $\pm$ 0.3	5.5 $\pm$ 0.6	6.5 $\pm$ 0.4	5.5 $\pm$ 0.6
Sucrose	6.6 $\pm$ 0.3	5.3 $\pm$ 0.6	6.5 $\pm$ 0.4	5.5 $\pm$ 0.8
Toothpaste B (Biotene)				
Toothpaste + sucrose	6.6 $\pm$ 0.3	5.3 $\pm$ 0.6	6.6 $\pm$ 0.4	5.1 $\pm$ 0.6
Sucrose	6.6 $\pm$ 0.3	5.3 $\pm$ 0.7	6.6 $\pm$ 0.4	5.2 $\pm$ 0.6

No significant differences were observed (Student's paired *t* test); *n* = 20 in all measurements.

tica) and incubated anaerobically for 2 days at 37°C. After appropriate incubation times the number of colony-forming units (CFU/ml saliva) was determined.

Results

A 2-week daily use of a lactoperoxidase-system-containing toothpaste (Biotene) had no effect on salivary flow rate, total salivary peroxidase activity, or the levels of SCN⁻ in paraffin-stimulated whole saliva (Table 1). No increase in the amount of the peroxidase-system-generated HOSCN/OSCN⁻ in saliva could be discerned after 2 weeks' use of Biotene compared with the toothpaste (placebo) without the peroxidase system components (Table 1).

The accumulation of dental plaque was not

affected by Biotene (Table 2). The acidogenicity of plaque quantitated by the glucose-stimulated lactic acid production did not change significantly when a peroxidase-system-containing toothpaste was used (Table 2), nor were there any differences between the two test dentifrices in their capacity to inhibit the plaque pH drop caused by sucrose (Table 3). Biotene toothpaste had no notable effect on the number of oral microorganisms: mutans streptococci, total streptococci, lactobacilli, and total counts of anaerobic flora in saliva or plaque did not change significantly during the 2-week study periods (Table 4).

Discussion

Our study shows that in subjects with normal salivary secretion rate the daily use of Bio-

Table 4. Effects of a 2-week daily use of the experimental toothpastes on the numbers of bacteria in saliva and dental plaque

	Toothpaste A (placebo)		Toothpaste B (Biotene)	
	Base line, mean $\pm$ SD	2 weeks, mean $\pm$ SD	Base line, mean $\pm$ SD	2 weeks, mean $\pm$ SD
Saliva				
Mutans streptococci*	5.1 $\pm$ 0.9	5.2 $\pm$ 0.9	5.2 $\pm$ 1.0	5.5 $\pm$ 1.1
Lactobacilli	3.5 $\pm$ 2.1	3.8 $\pm$ 1.8	3.6 $\pm$ 1.8	4.0 $\pm$ 1.7
Total streptococci	7.6 $\pm$ 0.3	7.7 $\pm$ 0.4	7.6 $\pm$ 0.4	7.7 $\pm$ 0.4
Total anaerobes	8.2 $\pm$ 0.4	8.2 $\pm$ 0.3	8.2 $\pm$ 0.3	8.2 $\pm$ 0.4
Plaque				
Mutans streptococci	4.3 $\pm$ 1.8	4.6 $\pm$ 1.6	4.1 $\pm$ 2.0	4.2 $\pm$ 2.2
Lactobacilli	2.2 $\pm$ 2.0	1.9 $\pm$ 2.0	2.1 $\pm$ 2.1	2.1 $\pm$ 1.9
Total streptococci	7.4 $\pm$ 0.4	7.5 $\pm$ 0.5	7.2 $\pm$ 1.7	7.2 $\pm$ 0.7
Total anaerobes	7.8 $\pm$ 0.4	8.0 $\pm$ 0.4	8.0 $\pm$ 0.4	7.9 $\pm$ 0.5

* Log CFU/ml; $n = 20$ in all experiments. No significant differences were observed (Student's paired t test).

tene toothpaste does not significantly affect the accumulation or acidogenicity of dental plaque. In line with our previous studies of salivary microorganisms (14), the numbers of plaque-derived mutans streptococci, total streptococci, lactobacilli, and total anaerobes did not change significantly during the experimental periods. This confirms earlier observations that peroxidase-system-generated HOSCN/OSCN⁻ is usually not bactericidal but rather a bacteriostatic defense system (1, 7, 22), which in physiologic concentrations does not affect the accumulation of dental plaque (23, 24).

In contrast to our previous study (14), we did not observe any significant increase in salivary HOSCN/OSCN⁻ levels after 2 weeks' use of Biotene. This difference may result from the fact that the base-line HOSCN/OSCN⁻ levels were substantially higher among the subjects of the present study. Furthermore, the saliva samples were now collected after a 48-h period with no oral hygiene, compared with only 1 h in the previous study (14). Any generated HOSCN/OSCN⁻ is likely to react rapidly with the accumulated plaque (7).

Since fluoride (0.15%) and xylitol (1%) concentrations were identical in the test and the placebo toothpastes, they are unlikely to have affected in any way the comparison of the two dentifrices.

Several studies have, however, indicated that oral HOSCN/OSCN⁻ may inhibit the glucose uptake and acid production by plaque bacteria (7, 25), in particular by mutans streptococci (6, 26, 27). Since a peroxidase-system-containing toothpaste (Biotene) can increase the HOSCN/OSCN⁻ levels in saliva both in vitro and in vivo (14), it may affect the sucrose-stimulated acid production by dental plaque. Our findings do not, however, support this assumption. On the basis of these and of our previous in vivo studies (14), it can be concluded that the oral peroxidase systems among individuals with normal salivary flow rate does not lead to notable inhibition of plaque accumulation or plaque acidogenicity or reduction of the oral levels of cariogenic bacteria.

Under acidic conditions (pH 5.0) HOSCN/OSCN⁻ effectively inhibits the growth of mutans streptococci and lactobacilli in human whole saliva, but no effect is seen at pH ≥ 6.0 (13). The pH of Biotene is 6.5, which may partly explain why no reduction in the numbers of these bacteria was detected in this study. The antimetabolic effect of HOSCN/OSCN⁻ on bacterial acid production is also most powerful at low pH (5.0–5.5) (27–29), which contributes to the fact that no inhibition of plaque acidogenesis was detected in the present study. Thus, if technically possible, a lower pH of Biotene

could be more powerful in vivo against cariogenic microorganisms.

It should be emphasized that Biotene has originally been developed to partly restore the antimicrobial capacity of human saliva in patients with hyposalivation or xerostomia (5). This idea seems sound: to add a physiologic (4) salivary antimicrobial system to a mouth that lacks saliva-mediated protection. So far, very little in vivo evidence exists that this system really helps to protect oral tissues in the absence of saliva from harmful microbes and/or from peroxide toxicity. Preliminary studies have reported that Biotene toothpaste improves gingival health in irradiated head and neck cancer patients when compared with the placebo dentifrice (30) and reduces periodontal probing depth and pocket bleeding in xerostomic patients when compared with a normal fluoride toothpaste (van den Eynde et al., personal communication). The function of the salivary peroxidase system is impaired in patients undergoing chemotherapy (31), who might benefit from restoration of the salivary defense to prevent frequent oral complications (32).

Biotene is commercially available also in the form of a mouthrinse (a nonalcoholic rinse that comprises also lysozyme and lactoferrin) and a long-lasting moisturizing gel (Oralbalance) for lubrication and protection of dry oral mucosal surfaces. The gel comprises the peroxidase system components but no other antimicrobial substances. The mouthrinse has notably decreased in vivo the total microbial flora in the mouth (33), but, to our knowledge, no clinical studies exist of the efficacy of the peroxidase-system-containing gel.

Interestingly, endogenous peroxidase system activation has proved to be effective in the control of microbial growth in bovine milk (34) and of several food-borne pathogens, such as *Campylobacter* (35), *Listeria* (36), and *Staphylococcus aureus* (37). Accordingly, activation or restoration of the endogenous peroxidase systems in the human mouth is one example of attempts to enhance natural defense systems against infectious diseases. Although this does not seem to be of great importance in healthy

subjects, it is more evident that patients with hyposalivation or xerostomia could benefit from restoring saliva's own defense systems.

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