ORAL MICROBIOLOGY

The sensitivity of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* to different (pseudo)halide-peroxidase combinations compared with mutans streptococci

RIIKKA IHALIN, VUOKKO LOIMARANTA, MARIANNE LENANDER-LUMIKARI and JORMA TENOVUO

Institute of Dentistry, Turku Immunology Centre and TuBS, University of Turku, Turku, Finland

Previous studies have shown that the peroxidase system with iodide is particularly effective against *Actinobacillus actinomycetemcomitans*. In the present study, the effects of iodide, chloride and thiocyanate in combinations with lactoperoxidase (LP) and myeloperoxidase (MP) on the viability of *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Streptococcus mutans* and *S. rattus* were analysed. Bacteria were incubated in buffer solution containing peroxidase, substrate(s) and H₂O₂ (all in oral physiological concentrations), and plated after 0, 0.5 and 1 h. The oxidation product of iodide was the most bactericidal against all the bacteria tested. The effect was significantly weaker on mutans streptococci. Physiological concentrations of thiocyanate abolished the effects of LP-H₂O₂-iodide and MP-H₂O₂-iodide/chloride combinations. Thiocyanate-peroxidase systems have already been used in oral hygiene products. The incorporation of iodide into these products could make them much more potent against periodontal pathogens, and also help to prevent transmission of these pathogens from person to person *via* saliva.

Introduction

Human oral fluids, saliva and gingival crevicular fluid, contain peroxidase enzymes which are part of the innate host defence system. Salivary peroxidase (SP) is secreted by major salivary glands and is found only in saliva, whereas myeloperoxidase (MP) originates from polymorphonuclear leucocytes (PMNLs) and filters into saliva from gingival crevicular fluid. The amount of MP in gingival crevicular fluid, and thereafter also in saliva, depends on the periodontal status [1]. In periodontally healthy persons, the amount of MP in whole saliva is *c.* 3.6 µg/ml, which is twice the amount of SP (1.9 µg/ml) [2]. The complete peroxidase system consists of three components: a peroxidase enzyme, hydrogen peroxide (H₂O₂) and an oxidisable substrate such as a halide or a pseudohalide. Both SP and MP can oxidise thiocyanate (SCN⁻) and iodide (I⁻), whereas chloride (Cl⁻) is oxidised only by

MP. In gingival crevicular fluid, Cl⁻ is most abundant (*c.* 90 mM) and the amount of I⁻ (4.3, SD 1.9 µM) is only about one-tenth of the SCN⁻ concentration (37 SD 24 µM) [3, 4]. In stimulated whole saliva, the Cl⁻ concentration is in the range 10–56 mM and the I⁻ concentration is *c.* 10 SD 7 µM [5]. The salivary concentrations of SCN⁻ vary and depend, for example, on diet and smoking habits. The normal range of salivary SCN⁻ for non-smokers is 0.5–2 mM, but concentrations as high as 6 mM can be found in saliva from smokers [6, 7]. The peroxidase systems have been shown to inhibit bacterial [8–14], fungal [15, 16] and viral [17–19] viability.

Actinobacillus actinomycetemcomitans and *Porphyromonas gingivalis* are among the main pathogens in periodontitis. *Fusobacterium nucleatum* has been proposed as the major cause of initial periodontal irritation and it usually maintains its proportion in the periodontal flora when gingivitis progresses to periodontitis [20]. The complete lactoperoxidase (LP) system has been used in some oral hygiene products, mainly to prevent caries. Although the use of such products elevates the amount of hypothiocyanous acid/hypothiocyanite ion (HOSCN/OSCN⁻) in saliva [21],

Received 6 Dec. 1999; revised version accepted 19 May 2000.

Corresponding author: Dr. R. Ihalin (e-mail: riikka.ihalin@utu.fi).

their effects on the levels of *Streptococcus mutans*, lactobacilli or the total oral flora *in vivo* have not been significant [21–23]. However, there is some evidence of their favourable influences on the condition of the gingiva in patients who suffer from oral dryness [22].

The effect of peroxidase systems on *A. actinomycetemcomitans* has been studied extensively [10, 13, 24, 25]. An earlier study demonstrated a superior effect of the oxidation product of I^- compared with the other (pseudo)halides on *A. actinomycetemcomitans* [10]. However, *A. actinomycetemcomitans* is only one of the pathogens in periodontitis. Therefore, this study focused on *P. gingivalis* and *F. nucleatum*. These results were compared with previous and current findings on the susceptibility of *A. actinomycetemcomitans*, *S. mutans* and *S. rattus* to the peroxidase system. These results will help further development of antimicrobial oral hygiene products.

Materials and methods

Bacteria and growth conditions

A clinical isolate of *P. gingivalis* TUPg007 (kindly provided by Dr K. Kari, University of Helsinki, Finland) and *F. nucleatum* ATCC 10953 were grown on Brucella Blood Agar (Becton Dickinson, Cockeysville, MD, USA) with frozen sheep blood 5% and non-selective CVE-plates containing trypticase (Becton Dickinson) 1%, yeast extract (Difco, Detroit, Michigan, USA) 0.5%, NaCl 0.5%, glucose 0.2%, L-tryptophan 0.02%, Bacto-agar (Difco) 1.5%, frozen sheep blood 5%, respectively. Before each experiment, *S. mutans* ATCC 25175 and *S. rattus* ATCC 19645 were grown on Blood Agar (Gibco BRL, Paisley) with frozen sheep blood 5%. After incubation, *S. mutans* and *S. rattus* were plated on MS-plates containing Mitis-salivarius agar (Difco) 9% and potassium tellurite 15 mg/L. An anaerobic atmosphere (CO_2 10%, N_2 80%, H_2 10%) and a temperature of 37°C were used for growth of the anaerobic bacteria (*P. gingivalis* and *F. nucleatum*) and a CO_2 -rich atmosphere (CO_2 7%, O_2 19%, N_2 74%) at 37°C for *S. mutans* and *S. rattus*. Before each experiment, *F. nucleatum*, *S. mutans* and *S. rattus* were grown for 4 days and *P. gingivalis* for 6 days. The bacteria were suspended in the test media to OD_{600} : 0.90 SD 0.01 for *P. gingivalis*, *S. mutans* and *S. rattus*, and 0.97 SD 0.02 for *F. nucleatum*, corresponding to $c. 6.3 \times 10^8$, 1.7×10^9 , 1.7×10^9 and 1.1×10^8 cfu/ml, respectively.

Media and chemical assays

Solution I [10] was used in the experiments with I^- and SCN^- . It contained 9 mM Na_2HPO_4 , 24 mM KH_2PO_4 , 1.5 mM $MgSO_4$ and 67 mM Na_2SO_4 . When Cl^- was used as a substrate, Solution II (9 mM Na_2HPO_4 , 24 mM KH_2PO_4 , 1.5 mM $MgSO_4$) was used. The pH of the media was adjusted to 6.5.

LP and MP activities were analysed by Nbs-SCN assay [26]. In the assay, peroxidases in the sample oxidise 4.0 mM SCN^- in the presence of 100 μM H_2O_2 to produce hypothiocyanite ions ($OSCN^-$). $OSCN^-$ ions oxidise 5-thio-2-nitrobenzoic acid (TNB), which is produced from 5,5'-dinitrobis-(2-nitrobenzoic acid) (DTNB) by reduction with 2-mercaptoethanol. The TNB concentration was adjusted to $c. 50 \mu M$ with 2-mercaptoethanol. The absorbance of the reaction mixture was determined at 412 nm before and 15 s after the addition of H_2O_2 , and the amount of oxidation product produced was counted from the decrease in absorbance: 1 mU corresponds to 1 μM oxidising product of TNB produced in 1 min. Stock solutions of LP and MP were prepared and the activity was determined. The peroxidase stock solutions were divided into tubes and kept frozen ($-20^\circ C$) until used. Both LP and MP sustained their activities when stored at $-20^\circ C$ in phosphate buffer.

Determination of the effective H_2O_2 concentration

P. gingivalis was chosen as a test bacterium because of its greater sensitivity to the peroxidase systems than *F. nucleatum*, as observed in preliminary studies. The H_2O_2 concentrations were varied 0–40 μM and 0–10 μM in tests with LP- SCN^- (1 mM) and LP- I^- (5 μM) combinations, respectively. The LP concentration was 5 $\mu g/ml$. The bacteria were incubated for 30 min with LP and either SCN^- or I^- and the appropriate concentration of H_2O_2 , then plated on Brucella blood agar and numbers of cfu were determined as described previously [10].

Effects of the peroxidase systems on the viability of bacteria

The antibacterial effects of different (pseudo)halide combinations of I^- , Cl^- and SCN^- as substrates of both LP and MP systems were examined as described in detail by Ihalin *et al.* [10]. Bovine LP, RZ = 0.74 (Sigma) and human MP, RZ = 0.76 (a generous gift from the late Professor B. Månsson-Rahemtulla, Birmingham, AL, USA) were used at a final concentration of 5 μg (22 mU)/ml and 4 μg (22 mU)/ml, respectively. The I^- concentration was 5 μM , the SCN^- concentration was either 50 μM or 1 mM and the H_2O_2 concentration was 1.25 μM or 5 μM . After incubation of bacteria, and the appropriate components of the peroxidase system, on a shaking water bath at 37°C for 1 h, 5 μl of a reducing reagent, dithiotreitol (DTT), were added to a final concentration of 1 mM to abolish the oxidation. Samples were taken immediately before the addition of H_2O_2 (0 min) and after incubation for 30 and 60 min. Ten-fold dilutions of the samples were made in peptone water containing DTT – tryptone (Difco) 2.5%, thiotone E peptone (Becton Dickinson) 2.5%, NaCl 5%, DTT 0.02% – in all experiments with anaerobic bacteria. In experiments with *S. mutans* and *S. rattus*, DTT was excluded from the dilution medium.

After each experiment, dilutions were plated to enumerate the number of cfu and the growth conditions were as described previously. The incubation time for *P. gingivalis* was 7 days, for *F. nucleatum* 4 days and for *S. mutans* and *S. rattus* 3 days. Each experiment was repeated three times and the means of log₁₀ cfu, as well as standard deviations (SD), were counted. A control without H₂O₂ was included in every experiment. The effects of the peroxidase systems on the bacteria in the presence of DTT were examined as described previously [10] and every substrate (I⁻, Cl⁻, SCN⁻) was used separately.

Statistical analysis

The reduction of the number of bacteria (log₁₀ cfu/ml) was analysed by Student's paired two-tailed *t* test. Differences in log₁₀ cfu between the test systems and the controls after incubation for 0, 30 and 60 min were analysed and *p* values <0.05 were considered to be statistically significant.

Results

Effects of different H₂O₂ concentrations in LP systems

The effects of both LP-SCN⁻ and LP-I⁻ systems on the viability of *P. gingivalis* were dose-dependent with respect to H₂O₂. The LP-I⁻ system with 2.5 µM H₂O₂ killed all the bacteria within 30 min, whereas only a slight decrease in log₁₀ cfu/ml (from 7.8 to 6.6) was detected with the same amount of H₂O₂ in the LP-SCN⁻ system. Even a H₂O₂ concentration as high as 40 µM with LP and SCN⁻ did not totally inhibit the

growth of *P. gingivalis*; after incubation for 30 min the log₁₀ cfu/ml was still 5.4 SD 0.1.

Effects of the myeloperoxidase systems

The effects of MP systems with different (pseudo) halide combinations were tested with the gram-negative anaerobic bacteria, *P. gingivalis* and *F. nucleatum*. Both bacteria showed similar susceptibility and the oxidation product of I⁻ was found to be the most effective antimicrobial agent, followed by Cl⁻ and SCN⁻ (Table 1). The addition of SCN⁻ diminished the effect of the MP-I⁻ system to the same level that was achieved with SCN⁻ as the only substrate. When all these three substrates were combined, the bactericidal effect of the MP system was similar to the effect of the MP-SCN⁻ system. The concentrations of substrates were: 5 µM I⁻; 90 mM Cl⁻; 50 µM SCN⁻ and 1.25 µM H₂O₂. Moreover, the addition of SCN⁻ abolished the bactericidal effect of the MP-H₂O₂ (5 µM)-Cl⁻ (90 mM) system (data not shown). The control reaction with 5 µM I⁻, 90 mM Cl⁻ and MP 5 µg/ml without H₂O₂ reduced the viability of *F. nucleatum* more than the other controls with different (pseudo)halide combinations; therefore, this control is shown separately in Table 1.

Effects of the lactoperoxidase systems

The effects of the LP systems were tested on both gram-negative anaerobes (*P. gingivalis* and *F. nucleatum*) and gram-positive aerobes (*S. mutans* and *S. rattus*). The concentrations of substrates were 5 µM I⁻, 1 mM SCN⁻ and 1.25 µM H₂O₂. The oxidation product of I⁻ was most effective against all bacteria tested.

Table 1. Effects of myeloperoxidase (4 µg (22 mU)/ml) systems with different substrates on the viability of *P. gingivalis* and *F. nucleatum* after incubation for 0 and 60 min at 37°C, pH 6.5

Halide	Mean (SD) log ₁₀ cfu/ml*			
	<i>P. gingivalis</i>		<i>F. nucleatum</i>	
	0 min	60 min	0 min	60 min
I ⁻	7.2 (0.5)	1.6 (1.2) p < 0.0001	6.5 (0.4)	2.4 (1.4) p = 0.00091
Cl ⁻	7.6 (0.1)	3.8 (0.6) p = 0.0011	6.9 (0.1)	3.6 (0.6) p = 0.012
SCN ⁻	7.2 (0.3)	5.8 (0.3) p = 0.0084	6.8 (0.2)	6.2 (0.4) p = 0.021
I ⁻ +SCN ⁻	7.0 (0.2)	5.7 (0.1) p = 0.077	6.7 (0.3)	6.5 (0.6) p = 0.41
I ⁻ +Cl ⁻	7.6 (0.2)	2.2 (1.6) p = 0.016	6.8 (0.1)	3.5 (0.6) p = 0.18
I ⁻ +Cl ⁻ +SCN ⁻	7.5 (0.1)	5.6 (0.4) p = 0.033	6.9 (0.0)	6.4 (0.2) p = 0.86
Control†	7.4 (0.3)	6.4 (0.3)	6.8 (0.3)	6.5 (0.3)
Control I ⁻ +Cl ⁻ ‡	- - -	- - -	6.9 (0.1)	4.4 (0.6)

The substrate concentrations were: 5 µM I⁻; 90 mM Cl⁻; 50 µM SCN⁻ and 1.25 µM H₂O₂.

*Values are shown as means (SD) of three-to-nine different experiments with *p* values of Student's paired two-tailed *t* test.

†Mean (SD) of all combination control reactions (i.e., with no H₂O₂) except the control I⁻ + Cl⁻ (*F. nucleatum*).

‡Control reaction including: 5 µM I⁻, 90 mM Cl⁻ and MP 5 µg/ml with no H₂O₂.

The anaerobic gram-negative bacteria (*P. gingivalis* and *F. nucleatum*) showed similar susceptibility to all the (pseudo)halide-LP systems tested, as in experiments with MP (data not shown). The gram-positive bacteria (*S. mutans* and *S. rattus*) were more resistant to LP systems and, with *S. rattus*, there was no statistically significant inhibitory effect of I^- oxidation. The effect of the oxidation product of I^- on *S. mutans* seemed to be transient (Table 2). However, when the concentration of H_2O_2 was increased from $1.25 \mu M$ to $5 \mu M$, *S. mutans* could not recover from the effect of the LP- H_2O_2 - I^- system (Table 3).

The experiments with *P. gingivalis* and *F. nucleatum* were also done with $50 \mu M$ SCN^- , but the results did not differ from those in experiments with $1 mM$ SCN^- (data not shown).

Effects of peroxidase systems with DTT on the viability of bacteria

With all (pseudo)halides and *P. gingivalis* and *F. nucleatum*, the bactericidal effect of the peroxidase systems was abolished after adding DTT. The decrease

of $\log_{10}$ cfu/ml was inhibited both when DTT was added at the beginning (0 min) or after incubation for 30 min. No recovery of the number of cfu was observed after incubation for 30 min with DTT (Fig. 1).

Discussion

Gram-negative obligatory or facultatively anaerobic oral bacteria are located mainly in gingival crevices. However, they are able to transmit within a family from parent to child and between spouses [27–29], and saliva is thought to be the route in these transmissions. Peroxidase (SP and MP) systems are part of the innate host defence in human saliva [30] and they affect the viability of various bacteria including the gram-negative bacteria *A. actinomycetemcomitans* [10, 13, 24, 25], *P. gingivalis* [31] and *F. nucleatum* [8] *in vitro*. A recent study showed that in LP, which is structurally and catalytically close to SP [32], and MP systems, the oxidation product of I^- was most effective against *A. actinomycetemcomitans*, and that the addition of physiological concentrations of SCN^- abolished this inhibitory effect [10]. As there are several pathogens in

Table 2. Effects of lactoperoxidase ($5 \mu g$ (22 mU)/ml) systems with different substrates on the viability of *S. mutans* and *S. rattus* after incubation for 0, 30 and 60 min at $37^\circ C$, pH 6.5

Halide	Mean (SD) $\log_{10}$ cfu/ml*					
	<i>S. mutans</i>			<i>S. rattus</i>		
	0 min	30 min	60 min	0 min	30 min	60 min
I^-	7.8 (0.0)	5.1 (0.5) p = 0.018	6.2 (0.6) p = 0.082	8.1 (0.1)	7.3 (0.4) p = 0.065	6.8 (0.8) p = 0.13
SCN^-	7.8 (0.1)	7.9 (0.2) p = 0.29	7.5 (0.0) p = 0.57	8.0 (0.0)	8.0 (0.0) p = 0.58	7.9 (0.1) p = 0.48
$I^- + SCN^-$	7.7 (0.1)	7.6 (0.1) p = 0.66	7.6 (0.1) p = 0.95	8.1 (0.0)	8.1 (0.1) p = 0.54	7.9 (0.1) p = 0.43
Control†	7.9 (0.1)	7.6 (0.2)	7.6 (0.1)	8.1 (0.1)	8.0 (0.1)	8.0 (0.1)

The substrate concentrations were: $5 \mu M$ I^- ; $1 mM$ SCN^- and $1.25 \mu M$ H_2O_2 .

*Values are shown as means (SD) of three different experiments with p values of Student's paired two-tailed *t* test.

†Mean (SD) of all combination control reactions (i.e., with no H_2O_2).

Table 3. Effects of lactoperoxidase ($5 \mu g$ (22 mU)/ml) systems with different substrates on the viability of *S. mutans* and *S. rattus* after incubation for 0, 30 and 60 min at $37^\circ C$, pH 6.5

Halide	Mean (SD) $\log_{10}$ cfu/ml*					
	<i>S. mutans</i>			<i>S. rattus</i>		
	0 min	30 min	60 min	0 min	30 min	60 min
I^-	7.8 (0.3)	3.3 (0.8) p = 0.0024	2.0 (2.0) p = 0.040	8.1 (0.1)	4.0 (0.3) p = 0.0024	3.6 (0.1) p = 0.00038
SCN^-	7.8 (0.2)	7.6 (0.5) p = 0.77	7.4 (0.6) p = 0.32	8.2 (0.1)	8.1 (0.0) p = 0.77	8.0 (0.1) p = 0.16
$I^- + SCN^-$	7.8 (0.3)	7.6 (0.6) p = 0.96	7.6 (0.4) p = 0.37	8.0 (0.1)	8.0 (0.0) p = 0.29	8.0 (0.0) p = 0.62
Control†	7.8 (0.3)	7.6 (0.4)	7.7 (0.3)	8.1 (0.0)	8.1 (0.1)	8.0 (0.1)

The substrate concentrations were: $5 \mu M$ I^- ; $1 mM$ SCN^- and $5 \mu M$ H_2O_2 .

*Values are shown as means (SD) of three different experiments with p values of Student's paired two-tailed *t* test.

†Mean (SD) of all combination control reactions (i.e., with no H_2O_2).

Fig. 1. Effects of LP-I⁻ (5 μ M)-H₂O₂ (1.25 μ M) system with 1 mM DTT on the viability of (a) *P. gingivalis* and (b) *F. nucleatum* at 37°C. DTT was added at 0 or 30 min. In control experiments, DTT was not added (No DTT). Samples were withdrawn at 0 min (□), 30 min (▨) and 60 min (■). Data are shown as values for one experiment. The detection limit was log₁₀ 2 cfu/ml. MP/Cl⁻ and LP/SCN⁻ systems gave similar results (data not shown).

periodontitis, the present study investigated the effects of LP and MP systems with different (pseudo)halide combination on *P. gingivalis* and *F. nucleatum*. These effects were compared with gram-positive control bacteria, *S. mutans* and *S. rattus*. Physiological concentrations of (pseudo)halides and peroxidases were used. Previous studies on *A. actinomycetemcomitans* and *S. mutans* have shown that these halide concentrations, in combination with LP/MP and H₂O₂, cause significant antibacterial effect [10, 14], and that the peroxidase activities rapidly produce OSCN⁻ from SCN⁻ in the presence of H₂O₂ [33]. H₂O₂ concentration is not measurable in saliva, because it is actively produced and depleted in biochemical reactions. However, its concentration in whole saliva has been estimated to be in the range 8 – 13 μ M [34]. The H₂O₂ concentrations used in this study were somewhat lower than the estimated physiological range, but they were adjusted to cause statistically significant inhibition of bacterial viability with the most efficient antimicrobial peroxidase-halide combination.

In this study, the oxidation product of I⁻ (with LP and MP) was again found to be the most effective antimicrobial agent, followed by the oxidation products of Cl⁻ (with MP) and SCN⁻ (with LP and MP) against all bacteria tested. *P. gingivalis* and *F. nucleatum* showed similar susceptibility to all peroxidase systems tested. The results support the findings that *P. gingivalis* is susceptible to the LP-SCN⁻ system in the presence of H₂O₂ [31]. However, the amount of H₂O₂ used with LP/MP did not produce enough OSCN⁻ to kill *F. nucleatum* effectively in comparison with a previous study in which OSCN⁻ concentrations above 8 μ M killed >75% of bacteria [8]. Both gram-negative bacterial species were more sensitive to the MP-H₂O₂-Cl⁻ system than *A. actinomycetemcomitans* [10].

The effects of oxidised I⁻ and SCN⁻ were significantly weaker on *S. mutans* and *S. rattus* than on the gram-negative bacteria. The oxidation product of I⁻ (with LP), unlike that of SCN⁻, decreased the log₁₀ cfu of *S. mutans*/ml significantly. At low H₂O₂ concentration, the effect seemed to be only transient but *S. mutans* numbers did not recover when the concentration of H₂O₂ was increased. It has been shown that the peroxidase-H₂O₂ (126 μ M)-I⁻ (14 μ M) system kills *S. mutans* at pH 7.2 [35]. However, the H₂O₂ concentration was 25 times higher than the concentration used in the present study. It has been shown with various bacteria that the effects of the complete peroxidase systems are dose-dependent with respect to H₂O₂ [10, 14]. *S. rattus* was even more resistant to the LP-H₂O₂-I⁻ system than *S. mutans*, and no statistically significant inhibition was observed.

The H₂O₂ concentration used in this study was obviously too low to affect the viability of *S. mutans* and *S. rattus* in the LP-H₂O₂-SCN⁻ system at pH 6.5. These results agree with the findings that LP-SCN⁻ (1 mM) system, even with concentrations as high as 80 μ M H₂O₂, is not bactericidal against *S. mutans* at pH 7 after incubation for 1 h [36]. Thus, it is concluded that oral streptococci, *S. mutans* and *S. rattus*, are much more resistant to the antibacterial activity of salivary peroxidase systems than gram-negative anaerobes.

It was shown recently that SCN⁻ is the best electron donor for the MP compound I (oxidised by H₂O₂), followed by I⁻ and Cl⁻ [37]. When physiological amounts of SCN⁻ and Cl⁻ are combined, even with a 10³-fold excess of Cl⁻, MP converts 50% of H₂O₂ into HOSCN instead of HOCl [38]. This may explain why physiological amounts of SCN⁻ abolished the anti-

bacterial effect of the MP/LP-H₂O₂-I⁻ and MP-H₂O₂-Cl⁻ systems with all bacteria tested, whereas Cl⁻ had no effect on the MP-H₂O₂-I⁻ system. Similar results have also been reported with the MP-H₂O₂-Cl⁻ system against *S. mutans* [14]. Thus, SCN⁻ most probably is the preferred substrate of SP.

There is intra-species variability in the susceptibility of various bacteria to the LP-H₂O₂-SCN⁻ system [8]. In this study, only one strain of each bacterium was investigated; hence, the susceptibilities of different strains of *P. gingivalis* and *F. nucleatum* require further study. Surprisingly, the *F. nucleatum* control with MP, I⁻, and Cl⁻, without H₂O₂, decreased the viability of the bacterium in a statistically significant manner. As the addition of DTT totally abolished this effect and the addition of H₂O₂-degrading catalase only partly, the presence of some oxidising substance other than H₂O₂ can be hypothesised. Moreover, similar controls with only I⁻ or Cl⁻ had no effect on viability.

MPO-produced HOCl is able to chlorinate ammonia and amines to yield chloramines, which act as carriers of the oxidised Cl⁻. Chloramines differ physically and chemically from HOCl and, therefore, their biological effects may be significantly different from those of HOCl [39]. Saliva and gingival crevicular fluid contain a wide variety of proteins and glycoproteins, which can act like amines, modifying the effects of peroxidase-H₂O₂-halide combinations *in vivo*. Preliminary studies indicate that the antibacterial effects of the peroxidase-H₂O₂-halide combinations investigated decrease in saliva.

It is concluded that peroxidase-catalysed oxidation products of I⁻ are effective against the tested anaerobic bacteria *in vitro*, as shown for various other bacteria and fungi [9, 10, 40, 41]. In fact, the effect is considerably stronger than that of the LP-H₂O₂-SCN⁻ system, which is used in some oral hygiene products [21, 42]. Therefore, the addition of I⁻ to saliva might improve the bactericidal properties of this system and also prevent the transmission of periodontal bacteria *via* saliva. However, as was shown also in this study, the presence of physiological concentrations of SCN⁻ significantly diminishes the antibacterial properties of the LP/MP-H₂O₂-I⁻ system [10, 40]. To circumvent the problem of competitive substrates, the amount of I⁻ may be increased further. Furthermore, the presence of different proteins in saliva may also affect the bactericidal activity of the peroxidase systems *in vivo*. The present study investigated the bacterial sensitivities in buffer solution and, thus, the effects of the peroxidase systems in saliva need further studies.

We acknowledge the late Professor Britta Månsson-Rahemtulla for fruitful discussions and for providing human myeloperoxidase. This study was supported by grants from the Academy of Finland, the Finnish Dental Society, Turku University Hospital, the Institute of Dentistry of Turku University and the Hilkka Brusiin Foundation.

References

1. Cao CF, Smith QT. Crevicular fluid myeloperoxidase at healthy, gingivitis and periodontitis sites. *J Clin Periodontol* 1989; **16**: 17–20.
2. Thomas EL, Jefferson MM, Joyner RE, Cook GS, King CC. Leukocyte myeloperoxidase and salivary lactoperoxidase: identification and quantitation in human mixed saliva. *J Dent Res* 1994; **73**: 544–555.
3. Anttonen T, Tenovuo J. Crevicular thiocyanate and iodide ions: cofactors of the antimicrobial peroxidase system in leucocytes. *Proc Finn Dent Soc* 1981; **77**: 318–323.
4. Weinstein E, Mandel ID, Salkind A, Oshrain HI, Pappas GD. Studies of gingival fluid. *Periodontics* 1967; **5**: 161–166.
5. Ferguson DB. Salivary electrolytes. In: Tenovuo J (ed) Human saliva: clinical chemistry and microbiology, vol I. Boca Raton, FL, CRC Press. 1989: 75–99.
6. Lamberts BL, Pruitt KM, Pederson ED, Golding MP. Comparison of salivary peroxidase system components in caries-free and caries-active naval recruits. *Caries Res* 1984; **18**: 488–494.
7. Tenovuo J, Mäkinen KK. Concentration of thiocyanate and ionizable iodine in saliva of smokers and nonsmokers. *J Dent Res* 1976; **55**: 661–663.
8. Courtois P, Majerus P, Labbé M, Vanden Abbeele A, Yourassowsky E, Pourtois M. Susceptibility of anaerobic microorganisms to hypothiocyanite produced by lactoperoxidase. *Acta Stomatol Belg* 1992; **89**: 155–162.
9. Courtois P, Vanden Abbeele A, Amrani N, Pourtois M. *Streptococcus sanguis* survival rates in the presence of lactoperoxidase-produced OSCN⁻ and OI⁻. *Med Sci Res* 1995; **23**: 195–197.
10. Ihalin R, Loimaranta V, Lenander-Lumikari M, Tenovuo J. The effects of different (pseudo)halide substrates on peroxidase-mediated killing of *Actinobacillus actinomycetemcomitans*. *J Periodont Res* 1998; **33**: 421–427.
11. Lumikari M, Soukka T, Nurmio S, Tenovuo J. Inhibition of the growth of *Streptococcus mutans*, *Streptococcus sobrinus* and *Lactobacillus casei* by oral peroxidase systems in human saliva. *Arch Oral Biol* 1991; **36**: 155–160.
12. Månsson-Rahemtulla B, Baldone DC, Pruitt KM, Rahemtulla F. Effects of variations in pH and hypothiocyanite concentrations on *S. mutans* glucose metabolism. *J Dent Res* 1987; **66**: 486–491.
13. Miyasaki KT, Wilson ME, Genco RJ. Killing of *Actinobacillus actinomycetemcomitans* by the human neutrophil myeloperoxidase-hydrogen peroxide-chloride system. *Infect Immun* 1986; **53**: 161–165.
14. Tenovuo J, Anttila O, Lumikari M, Sievers G. Antimicrobial effect of myeloperoxidase against *Streptococcus mutans*. *Oral Microbiol Immunol* 1988; **3**: 68–71.
15. Lenander-Lumikari M. Inhibition of *Candida albicans* by the peroxidase/SCN⁻/H₂O₂ system. *Oral Microbiol Immunol* 1992; **7**: 315–320.
16. MacCarthy KG, Dahl MV. Inhibition of growth of *Trichophyton rubrum* by the myeloperoxidase-hydrogen peroxide-chloride system. *J Invest Dermatol* 1989; **92**: 639–641.
17. Chase MJ, Klebanoff SJ. Viricidal effect of stimulated human mononuclear phagocytes on human immunodeficiency virus type 1. *Proc Natl Acad Sci USA* 1992; **89**: 5582–5585.
18. Mikola H, Waris M, Tenovuo J. Inhibition of herpes simplex virus type 1, respiratory syncytial virus and echovirus type 11 by peroxidase-generated hypothiocyanite. *Antiviral Res* 1995; **26**: 161–171.
19. Yamamoto K, Miyoshi-Koshio T, Utsuki Y, Mizuno S, Suzuki K. Virucidal activity and viral protein modification by myeloperoxidase: a candidate for defense factor of human polymorphonuclear leukocytes against influenza virus infection. *J Infect Dis* 1991; **164**: 8–14.
20. Moore WEC, Moore LVH. The bacteria of periodontal diseases. *Periodontol* 2000 1994; **5**: 66–77.
21. Lenander-Lumikari M, Tenovuo J, Mikola H. Effects of a lactoperoxidase system-containing toothpaste on levels of hypothiocyanite and bacteria in saliva. *Caries Res* 1993; **27**: 285–291.
22. Bánóczy J, Dombi C, Czeglédly A, Sari K. A clinical study with lactoperoxidase-containing gel and toothpaste in patients with dry mouth syndrome. *J Clin Dent* 1994; **5**: 65–69.

23. Kirstilä V, Lenander-Lumikari M, Tenovu J. Effects of a lactoperoxidase-system-containing toothpaste on dental plaque and whole saliva *in vivo*. *Acta Odontol Scand* 1994; **52**: 346–353.
24. Clark RA, Leidal KG, Taichman NS. Oxidative inactivation of *Actinobacillus actinomycetemcomitans* leukotoxin by the neutrophil myeloperoxidase system. *Infect Immun* 1986; **53**: 252–256.
25. Miyasaki KT, Zambon JJ, Jones CA, Wilson ME. Role of high-avidity binding of human neutrophil myeloperoxidase in the killing of *Actinobacillus actinomycetemcomitans*. *Infect Immun* 1987; **55**: 1029–1036.
26. Månsson-Rahemtulla B, Baldone DC, Pruitt KM, Rahemtulla F. Specific assays for peroxidases in human saliva. *Arch Oral Biol* 1986; **31**: 661–668.
27. Asikainen S, Chen C, Slots J. Likelihood of transmitting *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in families with periodontitis. *Oral Microbiol Immunol* 1996; **11**: 387–394.
28. van Steenberghe TJ, Bosch-Tijhof CJ, Petit MD, Van der Velden U. Intra-familial transmission and distribution of *Prevotella intermedia* and *Prevotella nigrescens*. *J Periodont Res* 1997; **32**: 345–350.
29. Watson MR, Bretz WA, Loesche WJ. Presence of *Treponema denticola* and *Porphyromonas gingivalis* in children correlated with periodontal disease of their parents. *J Dent Res* 1994; **73**: 1636–1640.
30. Tenovu J. Antimicrobial function of human saliva – how important is it for oral health? *Acta Odontol Scand* 1998; **56**: 250–256.
31. Fadel M, Courtois P. Effect of peroxidase-generated hypothiocyanite on the survival rate of *Porphyromonas gingivalis*. *Med Sci Res* 1999; **77**: 667–669.
32. Månsson-Rahemtulla B, Rahemtulla F, Baldone DC, Pruitt KM, Hjerpe A. Purification and characterization of human salivary peroxidase. *Biochemistry* 1988; **27**: 233–239.
33. Lenander-Lumikari M, Månsson-Rahemtulla B, Rahemtulla F. Lysozyme enhances the inhibitory effects of the peroxidase system on glucose metabolism of *Streptococcus mutans*. *J Dent Res* 1992; **71**: 484–490.
34. Pruitt KM, Tenovu J, Månsson-Rahemtulla B, Harrington P, Baldone DC. Is thiocyanate peroxidation at equilibrium *in vivo*? *Biochim Biophys Acta* 1986; **870**: 385–391.
35. Kersten HW, Moorer WR, Wever R. Thiocyanate as a cofactor in myeloperoxidase activity against *Streptococcus mutans*. *J Dent Res* 1981; **60**: 831–837.
36. Thomas EL, Milligan TW, Joyner RE, Jefferson MM. Antibacterial activity of hydrogen peroxide and the lactoperoxidase-hydrogen peroxide-thiocyanate system against oral streptococci. *Infect Immun* 1994; **62**: 529–535.
37. Furtmüller PG, Burner U, Obinger C. Reaction of myeloperoxidase compound I with chloride, bromide, iodide, and thiocyanate. *Biochemistry* 1998; **37**: 17923–17930.
38. van Dalen CJ, Whitehouse MW, Winterbourn CC, Kettle AJ. Thiocyanate and chloride as competing substrates for myeloperoxidase. *Biochem J* 1997; **327**: 487–492.
39. Thomas EL, Learn DB. Myeloperoxidase-catalyzed oxidation of chloride and other halides: the role of chloramines. In: Everse J, Everse KE, Grisham MB (eds) *Peroxidases in chemistry and biology*, vol I. Boca Raton, FL, CRC Press. 1991: 83–103.
40. Majerus PMC, Courtois PAP. Susceptibility of *Candida albicans* to peroxidase-catalyzed oxidation products of thiocyanate, iodide and bromide. *J Biol Buccale* 1992; **20**: 241–245.
41. Vanden Abbeele A, De Meel H, Courtois P, Pourtois M. Influence of a hypoiodite mouth-wash on dental plaque formation *in vivo*. *Bull Group Int Rech Sci Stomatol Odontol* 1996; **39**: 57–61.
42. van Steenberghe D, Van den Eynde E, Jacobs R, Quirynen M. Effect of a lactoperoxidase containing toothpaste in radiation-induced xerostomia. *Int Dent J* 1994; **44**: 133–138.