Microbicidal efficacy of thiocyanate hydrogen peroxide after adding lactoperoxidase under saliva loading in the quantitative suspension test

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Objective: As shown in the quantitative suspension test adding lactoperoxidase to a thiocyanate (SCN\(^{-}\)) hydrogen peroxide (H\(_{2}\)O\(_{2}\)) combination over the physiological saliva level has significant positive antimicrobial effects to a level of totally killing Streptococcus mutans, Streptococcus sanguinis, and Candida albicans. The aim of this study was to evaluate this positive effect under human saliva loading.

Methods: The bactericidal and fungicidal effect of lactoperoxidase was evaluated in a quantitative suspension test by using two test mixtures of a 2.0% thiocyanate and 1.2% hydrogen peroxide solution, one without (Group A) and one with (Group B) lactoperoxidase under saliva loading. Following the quantitative suspension tests (EN-13727/EN-13694), the growth of surviving bacteria and fungi in a nutrient broth was measured. The exposure times were restricted to 1, 3, 5, and 15 min. All statistical analyses were carried out with SPSS 11.5.

Results: In the quantitative suspension test, the combination of thiocyanate and hydrogen peroxide showed relatively low antimicrobial effectiveness on S. mutans, S. sanguinis, and C. albicans in the presence of human saliva at measured time points in comparison to the mixture with lactoperoxidase, which showed a high bactericidal activity within 15 min (S. mutans and S. sanguinis) and fungicidal activity within 5 min (C. albicans).

Conclusion: The antimicrobial effectiveness of the tested thiocyanate hydrogen peroxide combination was increased significantly by adding lactoperoxidase in the quantitative suspension test under human saliva loading.

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1. Introduction

The lactoperoxidase–thiocyanate–hydrogen peroxide system, one of saliva’s defence systems, has been shown to inhibit bacterial\(^{2,3}\) and fungal viability.\(^{6}\) In addition, antiviral activity was found.\(^{7,9}\) Therefore, stimulating or supporting this innate host defence system by adding parts of the lactoperoxidase–thiocyanate–hydrogen peroxide system or the complete system to dental hygiene products could be interesting as an adjuvant to mechanical plaque control. However, the antimicrobial effects of the lactoperoxidase–thiocyanate–
hydrogen peroxide system-based toothpastes or mouth rinses are controversial: whilst some authors found an antimicrobial effect in clinical usage,1,3,5 others could not.1,5,13

Lactoperoxidase (LPO) enzymes catalyse the oxidation of the salivary thiocyanate ion (SCN⁻) by hydrogen peroxide (H₂O₂) to OSCN⁻ and the corresponding acid hypothiocyanous acid (HOSCN), O₂SCN⁻ and possibly O₃SCN⁻,14 which have more antimicrobial effectiveness than does SCN⁻ or H₂O₂ alone. A study by Adolph et al. showed that the lactoperoxidase-thiocyanate-hydrogen peroxide system’s antimicrobial efficiency could be enhanced by better concentration ratios of system components.15 Pruit et al., for example, saw the main limiting component for the production of the oxidation products of SCN⁻ in whole saliva to be the H₂O₂ concentration.6 The peroxidase system in vivo was enhanced by adding small amounts of H₂O₂-generating enzymes to toothpastes.17,18 Mansson-Rahemuttela et al. enhanced the lactoperoxidase-thiocyanate-hydrogen peroxide system by increasing not only H₂O₂ but also SCN⁻ concentration.19 In addition, Rosin et al. showed that increasing H₂O₂ and SCN⁻ over their physiological saliva levels reduced plaque and gingivitis significantly compared to baseline values and a placebo.20 A new dentifrice based on these results shares the same effects regarding plaque and gingivitis prevention as it did a benchmark product containing triclosan.21 Overall, an unsolved question was, why do toothpastes or mouth rinses containing parts of lactoperoxidase-thiocyanate-hydrogen peroxide system, or even the complete system, not show adequate antimicrobial activity in healthy persons?

Thus, the question arose, is it possible to increase above their physiological levels the antimicrobial effectiveness by adding not just thiocyanate and hydrogen peroxide but also LPO to oxidise as much as possible of the SCN anions to become an effective antimicrobial agent?

In a pilot study, the influence of LPO on a thiocyanate/hydrogen peroxide suspension was evaluated in a standardised quantitative suspension test based on the European Norms (EN) 1040 and 1275.22 Contrary to the thiocyanate/hydrogen suspension without LPO (reduction factor (RF) < 1 at all measured time points), the suspension with added LPO showed high antimicrobial effectiveness. RF > 5, which led to complete killing of the microbes, was reached after 3 min for Candida albicans, after 5 min for Streptococcus mutans, and after 15 min for Streptococcus gordonii.

Therefore, we hypothesised that it might be possible to retain these observed antimicrobial activity by adding to saliva thiocyanate, hydrogen peroxide, and LPO above their physiological levels. Based on our previous results and the demonstrated potential of the lactoperoxidase-thiocyanate-hydrogen peroxide system22, the current study was conducted under human saliva loading to confirm the potential of the lactoperoxidase-thiocyanate-hydrogen peroxide system as an adjuvant to mechanical plaque control.

2. Materials and methods

2.1. Saliva collection

Every donor gave informed consent to provide saliva for research purposes. Unstimulated saliva was collected anonymously and immediately pooled on ice. Donors were 20 healthy individuals (caries- and gingivitis-free, nonsmokers, mean age 29.4 years, 12 males and 8 females) who had refrained from eating for a minimum of 2 h prior to contributing of saliva (mean 4 ml in 12 min ± 6.15 min). The suspension test started directly after saliva collection. Colony forming units of the untreated saliva were determined to evaluate possible influence on the results.

The study was performed based on European Norms (EN) 13727 and 13624 for bactericidal and fungicidal tests of antisepsics under saliva loading23,24 by means of three key microbes: S. mutans, which, along with a few other bacteria, plays a major role in forming dental biofilm and tooth decay; S. sanguinis, which belongs to one of the early colonisers and is recognised as a key player in the bacterial colonisation of tooth surfaces; and C. albicans, which plays a key role in developing oral candidiasis based on bacterial shift caused by an antibacterical substance.

2.2. Standardised quantitative suspension test

Just before beginning the quantitative suspension test, unreacted H₂O₂ in the test solution was removed by catalase. A 6.9 ml test solution (with and without added LPO) was thoroughly vortexed with 0.1 ml bacterial (S. mutans or S. sanguinis) or fungal (C. albicans) suspension (overnight culture, 1.5–5.0 × 10⁸ cfu/ml or 1.5–5.0 × 10⁷ cfu/ml) and 3 ml saliva and stored at 37 °C. After 1–3, 5, and 15 min contact time, the test mixture was again thoroughly vortexed, and 1 ml was transferred into 9 ml neutraliser (polysorbat 80 30 g/l, lecithin 3 g/l, l-histidin 1 g/l, sodium thiosulfate 5 g/l, aqua bidestillata ad 1000 ml). After 5 min ± 10 s neutralisation time, 1.0 ml of the neutralised test suspension was mixed with 9.0 ml of dilution solution, and 0.1 ml of this final solution was spread on tryptone soya agar (TSA, Oxoid, Germany) plates. After 42–48 h incubation at 36 °C (±1 °C), macroscopically visible colonies were counted on the plates. The arithmetic means of the duplicates were calculated, preferring plates with 15–300 cfu as recommended by European Standards.23,24 Every trial was carried out independently seven times, and the arithmetic means with the corresponding standard deviations were calculated.

2.3. Test preparations

Before the experiment was carried out, all components were prepared as follows.

Test organisms. Preservation and culture of the test organisms (S. mutans, ATCC 35668, S. sanguinis, ATCC 10556, and C. albicans, ATCC 10233) were carried out corresponding largely to EN 13777 and EN 13624 (adjusted number of cells in the suspension: 1.5 × 10⁵–5.0 × 10⁶ cfu/ml for bacteria and 1.5 × 10⁷–5.0 × 10⁸ cfu/ml for fungi).23,24

Solutions of test mixtures. Buffer adjusted to pH 5.3: 7 parts 0.2 M K₂HPO₄, 1 part 0.2 M KH₂PO₄, 0.1 M solution (2%, w/v; 0.34 M); 2.8 g NaSCN/100 ml freshly glass-distilled water, H₂O₂ solution (1.2%, w/v; 0.34 M): 33.6 g carbamide peroxide (CH₃N₂O₄·H₂O)·100 ml glass-distilled water (prepared immediately before the trial); buffer-LPO solution: 5.0 mg LPO (Sigma-Aldrich Chemie Co., Taufkirchen, Germany) different
Table 1 - Reduction factors of the thiocyanate hydrogen peroxide combination without and with added LPO to S. mutans under saliva loading in the suspension test at different time points.

<table>
<thead>
<tr>
<th>n = 7</th>
<th>Group A without added LPO</th>
<th>Group B with added LPO</th>
<th>A vs. B&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reduction factor</td>
<td>Comparisons within A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Reduction factor</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>1 vs. 3</td>
<td>3 vs. 5</td>
</tr>
<tr>
<td>1</td>
<td>1.33 ± 0.22</td>
<td>0.722</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.35 ± 0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.43 ± 0.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1.48 ± 0.30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Wilcoxon test with a significant level of <0.05.

<sup>b</sup> Mann-Whitney U test with a significance level of <0.001.

|       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |

The microbicidal kills were expressed as their decimal logarithms. The reduction factor was calculated as follows: \( \log_{10}(RF) = \log_{10}(CFU) - \log_{10}(CFU_{a}) \), where \( CFU \) = number of CFU per ml control medium (water of standardised hardness), and \( CFU_{a} = number of CFU per ml test A or B medium.

The comparisons at the contact time points between groups A and B (with and without added LPO) were performed with the Mann-Whitney U test and within groups with the Wilcoxon test. All statistical analyses were carried out with SPSS 11.5.

2.4. Statistical analysis

The fungicidal efficacy of the thiocyanate hydrogen peroxide combination without LPO increased with time but only at a low level (RF mean value < 1.5). Thus, the combination without added LPO was not sufficiently effective. The addition of LPO (Group B) showed a distinct fungicidal reduction (RF 4.95) after 15 min, which was statistically significantly different from the RF of Group A (Table 1 and Graphic 1).

3.1. S. mutans

The bactericidal effectiveness of the thiocyanate hydrogen peroxide combination without LPO increased with time but only at a low level (RF mean value < 2) with practically no bactericidal effectiveness (Table 2 and Graphic 2). After adding LPO (Group B), an effective bactericidal efficacy was achieved after 15 min (RF 5.54 ± 0.81). The comparison between groups A and B showed a statistically significant difference in favour of Group B (with added LPO) after 15 min.

3.3. C. albicans

The fungicidal efficacy of the thiocyanate hydrogen peroxide combination without LPO (Group A) increased with time but...
Table 3 – Reduction factors of the test thiocyanate hydrogen peroxide combination without and with added LPO to C. albicans under saliva loading in the suspension test at different time points.

<table>
<thead>
<tr>
<th>Time [min]</th>
<th>Group A without added LPO</th>
<th>Group B with added LPO</th>
<th>A vs. B (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reduction factor</td>
<td>Comparisons within A (^*)</td>
<td>Reduction factor</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>p</td>
<td>p</td>
</tr>
<tr>
<td>1</td>
<td>0.08 ± 0.15</td>
<td>0.051</td>
<td>p</td>
</tr>
<tr>
<td>3</td>
<td>0.20 ± 0.12</td>
<td>0.021</td>
<td>p</td>
</tr>
<tr>
<td>5</td>
<td>0.66 ± 0.34</td>
<td>0.008</td>
<td>p</td>
</tr>
<tr>
<td>15</td>
<td>2.34 ± 0.59</td>
<td>5.67 ± 0.13</td>
<td>p</td>
</tr>
</tbody>
</table>

\(^a\) Wilcoxon test with a significant level of <0.05.
\(^b\) Mann-Whitney U test with a significance level of <0.001.
\(^c\) Complete killing of all cells in test suspension.

only at a low level (RF mean value < 2.3) with practically no bactericidal effectiveness (Table 3 and Graphic 3). The thiocyanate hydrogen peroxide combination with added LPO (Group B) showed an effective bactericidal reduction after 3 min (RF 4.08 ± 1.12), which increased to 5.04 ± 0.80 and 5.67 ± 0.13 after 5 and 15 min, respectively. The comparison between groups A and B showed a statistically significant difference in favour of B (with added LPO) at all time points after 3 min.

![Graphic 1 – Reduction factors of the thiocyanate hydrogen peroxide combination without and with added LPO to S. mutans under saliva loading in the suspension test at different time points.](image1)

![Graphic 2 – Reduction factors of the thiocyanate hydrogen peroxide combination without and with added LPO to S. sanguinis under saliva loading in the suspension test at different time points.](image2)

4. Discussion

In our previous study, we showed that the applied quantitative suspension tests were useful for evaluating the effect of LPO on the lactoperoxidase-thiocyanate-hydrogen peroxide system’s antimicrobial effectiveness. To study the effect with human native saliva, unstimulated saliva from 20 healthy (caries- and gingivitis-free) individuals was pooled and not treated or sterilised. The study was carried out directly after saliva collection.

The pooled saliva colony forming units ranged from $3.2 \times 10^6$ to $3.5 \times 10^7$ with a mean value of $1.19 \times 10^7$. Considering the number of tested microbes in comparison to the whole species number of the salivae, the saliva microbes had no relevant influence on the results.

Besides the concentration of their components, the efficiency of the lactoperoxidase-thiocyanate-hydrogen peroxide system depended on its exposure time and pH value. Therefore, to determine when the lactoperoxidase-thiocyanate-hydrogen peroxide system or the oxidation products reached its initial highest bacterial and fungicidal effectiveness, the tests were carried out at exposure times of 1, 3, 5, and 15 min. In the previous study, the suspensions with LPO reached their total killing time after 5 min for S. mutans, after 15 min for S. sanguinis, and after 3 min for C. albicans.

We assumed that the demonstrated antimicrobial effect was based on the lactoperoxidase-thiocyanate-hydrogen peroxide system’s products such as OSCN and higher oxidation derivatives. This assumption was supported by the suspension tests of our previous study. The single components (SCN⁻, LPO) and combinations (LPO + SCN⁻, LPO + H₂O₂) showed no clinically relevant effects (RF ≤ 0.3) at all time points. Only the single component H₂O₂ showed a reduction factor of 1.5 after 15 min. The antimicrobial effect of H₂O₂ is well known.

The lactoperoxidase-thiocyanate-hydrogen peroxide system is bacteriostatic, inhibiting the glycolysis of oral bacteria around saliva’s average pH level, which is mostly neutral over the day. However, Kersten et al. showed that the system can shift to be bactericidal around pH 5.0. Possible reasons are reaction products of OSCN⁻ or shifting of OSCN⁻ to HOSCNO⁻ by a low pH value in favour of HOSCNO⁻. Unlike OSCN⁻, HOSCNO⁻ has no charge, which facilitates the penetration through the lipophilic bacterial cell membrane or even through biofilms and raises the antimicrobial effectiveness of the lactoperoxidase-thiocyanate-hydrogen peroxide system. Therefore, all tests were conducted at the pH of 5.3 of HOSCNO⁻/OSCN⁻. S. mutans causes a drop in plaque pH < 5.5 by metabolising carbohydrates, excreting large amounts of lactic acid, and resulting in the demineralisation of tooth hard substances. Under these conditions, a defence system is critical. In contrast to other reactive oxygen substances such as H₂O₂, which will be totally consumed, hypothiocyante can be generated by the lactoperoxidase-thiocyanate-hydrogen peroxide system continuously.

The suspension tests without added LPO (controls, Group A) at all time points showed no clinically relevant antimicrobial effectiveness (highest RF: S. mutans 1.5, S. sanguinis 1.9, and C. albicans 2.3). In comparison, however, to the results of the previous study (without saliva loading: RF < 1), the reduction factors are increased. We assumed that the increase in the reduction factor after 15 min in the control group could be the result of small amounts of OSCN⁻ produced by native oral peroxidases present in the supplemented saliva.

The concentration level of salivary LPO is around 1.9 µg/ml-2.0 µg/ml (activity: 6 ± 3 units/ml), and the concentration level of MPO (myeloperoxidase) ranges from 0.7 to 3.6 µg/ml (activity: 24 ± 14 units/ml). In contrast to LPO, MPO can oxidise not only SCN⁻ but also chloride (Cl⁻) to hypochlorite (OCl⁻), which is a very strong antibacterial agent. In addition, OCl⁻ reacts nonenzymatically with SCN⁻ to create OSCN⁻. Thus, this is a second pathway for OSCN⁻ production.

Moreover, increased RF values in the control group could be explained by antibacterial components such as enzymes (e.g., amylase, lysozyme, lactoferrin) or antibodies (e.g., IgA, IgG, IgM) present in saliva. For example, the iron-binding glyco-
protein lactoferrin, scavenging essential Fe³⁺ from bacteria, can be found in saliva and crevicular fluid¹⁷ and acts bacteriostatically and bactericidal.³⁷,³⁸

Proteins, glycoproteins, and carbon hydrates are known for having a positive effect on H₂O₂-producing microbial flora.²⁰,²¹ Furthermore, glucose and lactate can react with glucooxidase and lactatoxidase to build hydrogen peroxide.¹⁵,¹⁷,¹⁸

The results of the test groups confirmed the large effect of the LPO enzymes on antibacterial and antifungal effectiveness of the lactoperoxidase-thiocyanate-hydrogen peroxide system shown in the previous study,²² even under saliva loading. Compared with the previous study,²² the reduction factors for the S. mutans and S. sanguinis test groups increased slower and were slightly lower. The total antimicrobial effectiveness [complete killing of the microbe] was reached at 15 min for S. mutans and S. sanguinis and at 5 min for C. albicans in comparison of the previous study (complete killing of S. mutans after 5 min and C. albicans after 3 min). The reason could be several saliva components, which influence the activity of the peroxidase–H₂O₂–SCN⁻ system. For example, proteins can reduce OSCI to SCN⁻ by reacting with third and to create dihydroxys.⁵⁴,⁵⁵

It is known that LPO is strongly absorbed by saliva sediment,⁶⁰,⁶¹ which can decrease enzyme activity and lead to the reduction of the lactoperoxidase-thiocyanate-hydrogen peroxide system's antibacterial effectiveness. Adding phosphate, for example, releases bound peroxidase and recovers the saliva's LPO activity.⁶⁶

Saliva components can have an increasing or decreasing effect on the antimicrobial effectiveness of the lactoperoxidase-thiocyanate-hydrogen peroxide system.

In comparison to the previous study²² (without saliva loading), the antimicrobial effect was reduced; however, it was still bactericidal and fungicidal on test organisms in a clinically relevant range. Thus, it seems that the system has sufficient capacity to be adapted to different tasks in preventing and curing oral diseases.

Until now, adverse effects of the lactoperoxidase–thiocyanate–hydrogen peroxide system have not been known; however, the OSCN-generation pathway includes forming radicals.⁶⁸ A massive generation of free radicals can disturb the biological balance between oxidative and antioxidative substances. The generated OSCN is a reactive oxygen substance. Free radicals as well as reactive oxygen substances can lead to adverse effects if they are in excess. Therefore, it is essential to consider the balance between generated radicals and scavengers (oxidative and antioxidative substances) by the redox buffer of saliva.⁶⁹

In conclusion, the relatively low antimicrobial effectiveness of the thiocyanate hydrogen peroxide combination on S. mutans, S. sanguinis, and C. albicans in the presence of human saliva could be increased significantly by adding lactoperoxidase enzymes in the quantitative suspension test under human saliva loading. Considering the potential advantages of using components of human saliva's antimicrobial peroxidase defence system to prevent oral diseases by reducing the risk of bacterial shift and other side effects, the concentration of the single components, reaction time, and conditions of the lactoperoxidase-thiocyanate-hydrogen peroxide system should be optimised in subsequent studies.

Acknowledgement

This study was supported by McNeil Consumer Healthcare GmbH (Germany).

Funding: This study was supported by McNeil Consumer Healthcare GmbH (Germany).

Competing interests: Axel Kramer holds a patent regarding usage of the lactoperoxidase system's components in oral health care products. The remaining authors declare they have no conflicts of interest.

Ethical approval: Not required.

References


