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A synergistic chlorhexidine/chitosan combination for improved antiplaque strategies

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Background: The minor efficacy of chlorhexidine (CHX) on other cariogenic bacteria than mutans streptococci such as *Streptococcus sanguinis* may contribute to uneffective antiplaque strategies.

Methods and Results: In addition to CHX (0.1%) as positive control and saline as negative control, two chitosan derivatives (0.2%) and their CHX combinations were applied to planktonic and attached sanguinis streptococci for 2 min. In a preclinical biofilm model, the bacteria suspended in human sterile saliva were allowed to attach to human enamel slides for 60 min under flow conditions mimicking human salivation. The efficacy of the test agents on streptococci was screened by the following parameters: vitality status, colony-forming units (CFU)/ml and cell density on enamel. The first combination reduced the bacterial vitality to ~0% and yielded a strong CFU reduction of 2–3 $\log_{10}$ units, much stronger than CHX alone. Furthermore, the first chitosan derivative showed a significant decrease of the surface coverage with these treated streptococci after attachment to enamel.

Conclusions: Based on these results, a new CHX formulation would be beneficial unifying the bioadhesive properties of chitosan with the antibacterial activity of CHX synergistically resulting in a superior antiplaque effect than CHX alone.

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An important approach in the prevention of caries and periodontal disease is the reduction of the supragingival dental plaque. This specific form of biofilm can be negatively affected by chemical plaque control. The antiplaque activity of chlorhexidine (CHX), representing the current gold standard for antibacterial oral agents, is known to be decreased by inhibition mechanisms of the active site groups of the bisguanide caused by salivary or subgingival sulcus proteins (1–3). The antimicrobial activity of CHX on *Streptococcus sanguinis* (4) as an early colonizer of human teeth is poor compared to its effect on *S. mutans* (5, 6).

Chitosan, a partially deacetylation product of chitin, is a widely distributed polycationic biopolymer exhibiting various promising biological activities. These include antimicrobial and antifungal activities next to biogradable and biocompatible properties (7). The precise antibacterial mechanism of chitosan is still unknown but different mechanisms have been proposed. Chitosan possesses reactive positively charged amino groups that are able to interact with the negatively charged bacterial cell membranes resulting in the leakage of proteinaceous and other intracellular constituents and alteration of cell permeability (8). A further useful property is the bioadhesive nature, the ability of good retention on oral surfaces (9, 10) and thus the suitability to act as vehicle for the release of effective oral therapeutical agents (10, 11). *Streptococcus sanguinis* is known to be a major human plaque-forming strain with cariogenic potential (12) and was therefore chosen as indicator strain for antiplaque effects.

The aim of this pilot study was, as a first step, to evaluate the impact of new formulations consisting of CHX with two bioadhesive chitosan compounds on two different life forms (planktonic and sessile) of *S. sanguinis*.

Material and methods

A preclinical flow chamber system allowed the separated treatment of planktonic and surface-attached *S. sanguinis* cells with antiseptic/antiadhesive agents (13). The test strain *S. sanguinis*, biotype I, serological group H (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was acquired from dental caries.

Figure 1 illustrates the study design. Two kinds of experiments were conducted: (i) treatment of planktonic streptococci (Fig. 1a) and (ii) treatment of streptococci attached to enamel (Fig. 1b).

Treatment of planktonic streptococci

In the first experimental approach, planktonic streptococci were treated for 2 min with one of the following:

1 NaCl (0.9%, saline, negative control)
2 CHX (chlorhexidine, 0.1%, w/v, positive control) (Synopharm, Barbüttel, Germany)
3 Chit 1 (chitosan, 0.2%) (Tinocare CP, Ciba Specialty Chemicals, Basle, Switzerland)
4 Chit 2 (chitosan, 0.2%) (CL 113, Biopolymer AS, Drammen, Norway)
5 comb 1 (combination of Chit 1 and CHX)
6 comb 2 (combination of Chit 2 and CHX).

Prior to each experiment, a fresh mixture of chlorhexidine, chitosans and of the chitosan/chlorhexidine combinations were prepared. After washing with saline, the bacteria were suspended in sterile human saliva and exposed to enamel slides inserted into the flow chamber. After 60 min, the bacterial viability was monitored in the salivary suspension and on enamel slides: colony-forming units (CFU/ml) of planktonic treated cells (Fig. 2), percentage of vital streptococci treated in the planktonic life form (%VS, Fig. 3a), percentage of vital streptococci treated in the planktonic life form and then allowed to attach (%VS_{a1}, Fig. 3b), and for the cell density on the substratum surface: total bacterial cell counts attached to enamel (BC_{a1}/mm^2, Fig. 4a).

Treatment of streptococci attached to enamel

In the second experiment, native streptococci suspended in sterile human saliva were exposed to enamel slides in the flow chamber. After 60 min, the enamel-bound bacteria were treated with the same six agents for 2 min and then monitored for bacterial viability: percentage of vital attached treated streptococci (%VS_{a2}, Fig. 3c), and local cell density on enamel: total bacterial cell counts attached to enamel (BC_{a2}/mm^2, Fig. 4b). The conditions of the salivary suspension were kept constant during the experimental periods. The microscopical analysis of the bacterial vitality using the fluorescent-based Life/Dead BacLight viability Kit (Molecular Probes, Europe BV, Leiden, the Netherlands) was described earlier (14).

Statistics

The BC and CFU values were log transformed. Mean and mean-based

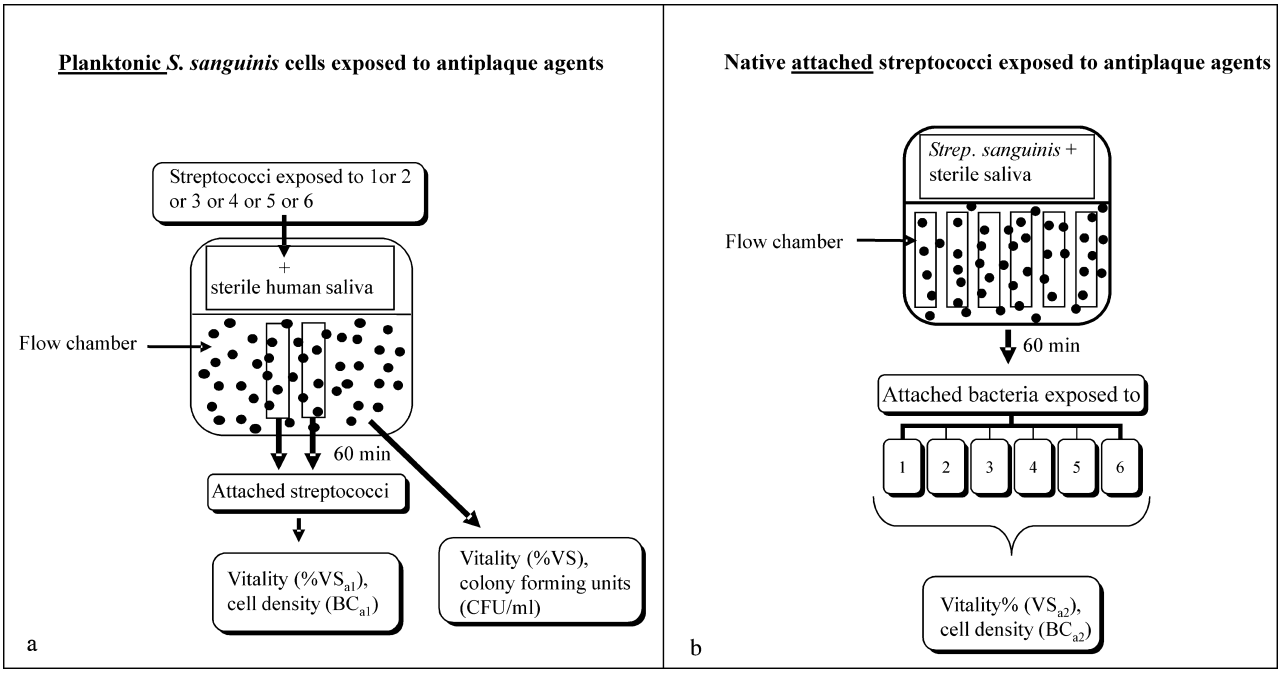

Fig. 1. Study design. (a) Planktonic *Streptococcus sanguinis* cells exposed to the antiplaque agents 1–6. (b) Attached streptococci exposed to the antiplaque agents 1–6. 1, NaCl (negative control); 2, CHX (positive control); 3, Chit 1; 4, Chit 2; 5, comb 1 (combination of Chit 1 and CHX); 6, comb 2 (combination of Chit 2 and CHX). CHX, chlorhexidine; Chit, chitosan.

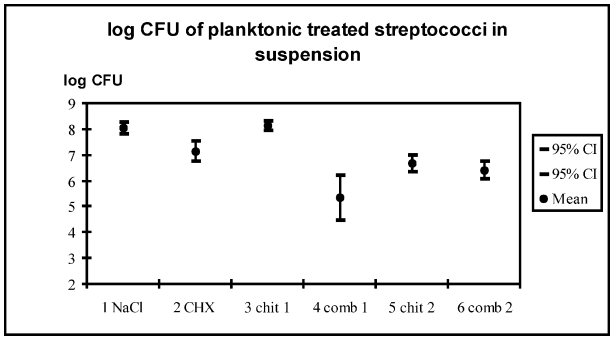

Fig. 2. Log colony-forming units (CFU) of planktonic treated streptococci after 60-min experimental period (log CFU/ml, outlined in Fig. 1a). 1, NaCl (negative control); 2, CHX (positive control); 3, Chit 1; 4, Chit 2; 5, comb 1 (combination of Chit 1 and CHX); 6, comb 2 (combination of Chit 2 and CHX). CHX, chlorhexidine; Chit, chitosan; CI, confidence interval.

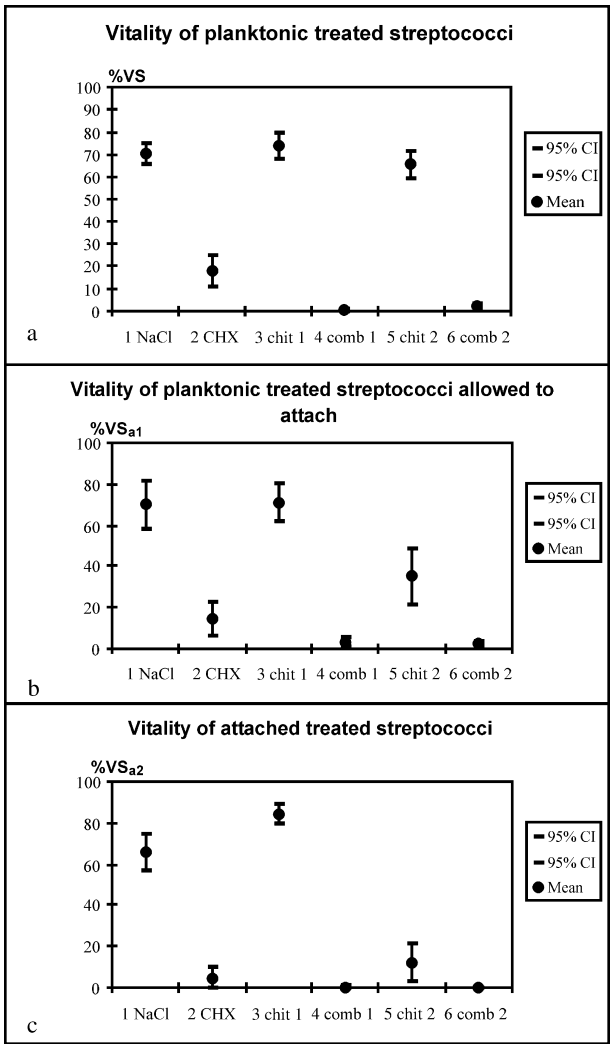

Fig. 3. (a) Vitality proportions of planktonic treated streptococci (%VS, outlined in Fig. 1a). (b) Vitality proportions of planktonic treated streptococci allowed to attach to enamel ($\%VS_{a1}$, outlined in Fig. 1a). (c) Vitality proportions of attached treated streptococci ($\%VS_{a2}$, outlined in Fig. 1b). 1, NaCl (negative control); 2, CHX (positive control); 3, Chit 1; 4, Chit 2; 5, comb 1 (combination of Chit 1 and CHX); 6, comb 2 (combination of Chit 2 and CHX). CHX, chlorhexidine; Chit, chitosan; CI, confidence interval.

95% confidence intervals (CI) were determined for log CFU, %VS, $\%VS_{a1}$, $\%VS_{a2}$, log BC_{a1} and log BC_{a2}. Significant differences were indicated when the confidence intervals do not overlap. Ten series were performed for each test agent.

Results

Treatment of planktonic streptococci

With regard to the cultural viability (CFU/ml) of the treated planktonic streptococci (Fig. 2), the comb 1 showed significantly the strongest antibacterial effect by reducing the numbers of CFU at a range of 2–3 $\log_{10}$ units followed by comb 2 in comparison to CHX treatment. The antibacterial effect of Chit 2 was stronger than that of the single substances CHX and Chit 1 (Fig. 2). The antivital effect of the test agents on planktonic cells suspended in saliva (%VS) decreased in the order comb 1 > comb 2 > CHX. Chit 2, NaCl and Chit 1 had nearly no antivital effect (Fig. 3a). The vitality of planktonic treated cells followed by their attachment to enamel ($\%VS_{a1}$) was reduced in the order comb 2 > comb 1 > CHX > Chit 2. NaCl and Chit 1 had no antivital effect (Fig. 3b). Concerning the cell density on enamel (log BC_{a1}) (Fig. 4a), all test agents caused reduced bacterial cell counts on the enamel slides compared to those of the negative control NaCl.

Treatment of attached streptococci

Figure 3(c) shows the antivital effect ($\%VS_{a2}$) of the test agents on the cells in the declining order comb 1 = comb 2 > CHX > Chit 2. NaCl and Chit 1 had no antivital effect. The cell density of streptococci treated in the attached life form (log BC_{a2}) was mostly affected by Chit 1 exposure than that treated in the planktonic life form, followed by Chit 2. The bacterial treatment with the two Chit/CHX combinations or CHX did not reveal any detaching activity compared to the negative control (Fig. 4b).

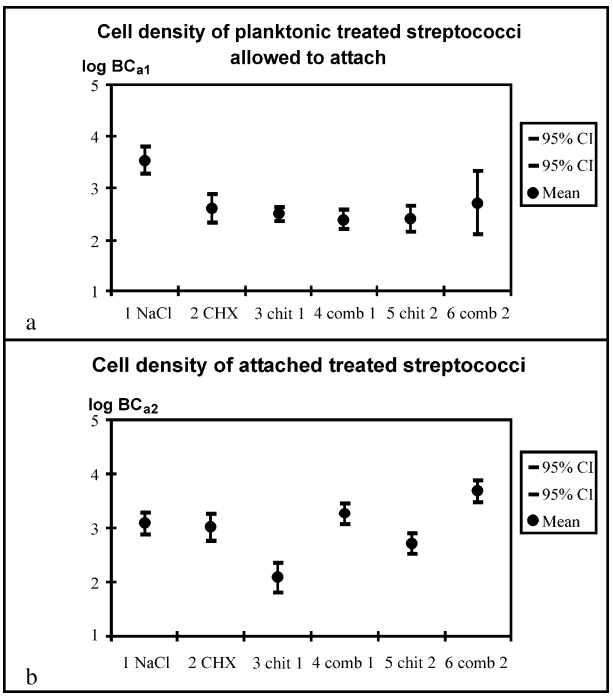

Fig. 4. (a) Log of total bacterial cell counts of planktonic treated streptococci allowed to attach to enamel (BC_{a1}/mm^2, outlined in Fig. 1a). (b) Log of total bacterial cell counts of attached treated streptococci (BC_{a2}/mm^2, outlined in Fig. 1b). 1, NaCl (negative control); 2, CHX (positive control); 3, Chit 1; 4, Chit 2; 5, comb 1 (combination of Chit 1 and CHX); 6, comb 2 (combination of Chit 2 and CHX). CHX, chlorhexidine; Chit, chitosan; CI, confidence interval.

Comparison of the effects of the test agents on planktonic and attached streptococci

Chit 1 alone showed strong detaching activity among the test agents but no antivital effect on both bacterial life forms. CHX exposure caused a higher vitality reduction of attached cells ($\%VS_{a2} = 5\%$) than of planktonic cells ($\%VS_{a1} = 14\%$, $\%VS = 19\%$). Both Chit/CHX combinations affected the vitality of planktonic and sessile bacteria stronger than the separate ingredients.

Discussion

The widely used antiplaque agent CHX is known to have a significant suppressive effect on mutans streptococci but it fails to decrease the total viable numbers of *Streptococcus sanguinis* or lactobacilli even when applied in a concentrated formulation as chlorhexidine varnish (1%, 3%, 40%) (15, 16). This confirms the results of Emilson (6), which demonstrate the reduced susceptibility of *S. sanguinis* to CHX. In consequence, in oral cavities of individuals with high caries risk, the relative proportions of mutans streptococci will decrease following CHX treatment with a common concentration of 0.1–0.2%, but the level of *S. sanguinis* as well as that of lactobacilli contributing to caries development may persist nevertheless. This proceeding may lead to unsuccessful anticaries therapy. Apart from the antibacterial effect of chemotherapeutics, their influence on the microbial adhesion to tooth surfaces plays a key role for plaque formation and caries development. The study of Tarsi *et al.* (17) showed the ability of the natural polysaccharide chitosan to prevent the adherence of *Streptococcus mutans* to hydroxyapatite. This effect was attributed to the properties of chitosan, e.g. stimulating ordered regeneration of oral soft tissues, preventing the deleterious effect of organic acids and impairing bacteria. A recent study established a close relationship between the amount of adsorbed chitosan on bacterial cell surfaces and its inhibition efficiency verified by transmission electron micrography (18). The idea of the present study was to combine the favourable properties of two chitosan derivatives with the antibacterial standard agent CHX and to screen these formulations for their effect on both life forms of *S. sanguinis* in a preclinical biofilm model. The concentration of chitosan used in the present study (0.2%) corresponds to the minimal inhibitory concentration of chitosan for oral streptococci evaluated by Tarsi (19). The mode of interaction between chitosan and the outer cell membranes of bacteria seems to be related to the concentration. At a lower concentration (< 0.2 mg/ml) chitosan probably binds to the negatively charged bacterial surface to cause agglutination, whereas at higher concentrations the large number of positive charges leads to a net positive charge and thus keeps bacteria in suspension (8).

Concerning the results of the present study, the Chit/CHX combinations improved the weak antibacterial CHX activity on planktonic *S. sanguinis* cells and enhanced its moderate antivital activity on these streptococci in the planktonic and attached life form. The strong antibacterial activity (particularly of comb 1) on suspended streptococci by reducing the numbers of CFU/ml revealed a synergistic way of action of CHX in the presence of chitosan promoting the disintegration of the bacterial cells. Furthermore, Chit 1 seemed to have inhibitory or detaching effects on the adherence of the test strain, shown by a lower cell density after its application. These findings support the proposal that chitosan could be a promising candidate for the therapeutical interference of dental plaque formation by interactions of their positive charged free amino groups with anionic parts of the bacterial cell walls and their ability of bacterial aggregation (20, 21).

So far, only few studies deal with the effect of formulations composed of Chit and CHX on oral microorganisms. Giunchedi *et al.* (22) reported the

inhibition of the adhesion of *Candida albicans* to human buccal cells by Chit. Using chitosan microspheres as controlled drug delivery systems combined with CHX as a model drug, the antimicrobial activity of CHX was improved and the release of the drug in the oral cavity was prolonged (11, 22). The oral application of Chit and CHX in films or gels (1–2%) was also followed by prolonged retention times on the oral mucosa and drug release from the gels (9, 20). As yet, no serious side effects of chitosan application have been reported (23). The LD_{50} of chitosan in mice was determined to be greater than 16 g/kg (24).

The promising outcome of the present results was gained involving a single strain based biofilm model. Therefore, further studies will be of special interest to determine the most effective ratios of Chit/CHX concentrations, to extend the selection of cariogenic microorganisms and to include *in vivo* conditions in order to elucidate the underlying mechanisms and thus help to optimise new antiplaque formulations.

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