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Susceptibility of planktonic versus attached *Streptococcus sanguinis* cells to chlorhexidine

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Abstract The effect of chlorhexidine (CHX) on the viability of *Streptococcus sanguinis* was investigated in a preclinical biofilm model separately on cells in the planktonic or attached life form. Saliva-coated human enamel and glass slides were exposed to the streptococci suspended in sterile saliva for 30 min and 60 min in the flow chamber system. The CHX exposition was performed in two parts: pretreatment of the planktonic bacteria before their attachment to enamel or glass, and treatment of bacteria already attached to enamel. The susceptibility measured by vitality percentages was determined by fluorescence microscopy using vital/dead cells. After CHX pretreatment of planktonic cells, the mean values of the vitality percentages after adhesion were 14–18% (enamel) and 24–25% (glass). In contrast, the mean vitality percentages of untreated attached streptococci reached 70–75% (enamel) and 68% (glass). The vitality percentages of CHX-exposed bacteria dropped markedly to 2–5%, whereas those of untreated attached cells remained at 65–66%. The exposure of initially attached streptococci to CHX resulted in greater reduction of bacterial viability than with the planktonic counterparts. This preclinical biofilm model allows the investigation of various bacterial life forms and can furthermore be used to select efficient antiplaque therapeutics which might be beneficial for clinical plaque control.

Keywords Chlorhexidine · Flow chamber system · Initial biofilm · *Streptococcus sanguinis* · Viability

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Introduction

The development of caries and periodontal disease is based on the accumulation of dental plaque on tooth surfaces. Dental plaque is a specific form of biofilm caused by salivary coating of oral surfaces [12] and microbial deposition as a result of the dynamic balance between microbial attachment processes and mechanical forces of detachment in the oral cavity [6, 7, 24]. These complex adhesion interactions impede analysis of the effects of antiseptics on different microbiological life forms separately in situ. Therefore, various multispecies- or single species-based in vitro models have been developed for investigating biofilm formation and the efficacy of antiplaque therapeutics [13, 22, 26, 33]. The present study was based on the application of a single species flow chamber system, which allowed for short exposure of planktonic and initially deposited bacteria to selected agents under controlled environmental conditions [29]. By means of this adhesion model, separate investigation of the susceptibility of planktonic and surface-attached *Streptococcus sanguinis* cells to chlorhexidine (CHX) was possible.

Streptococcus sanguinis [28], one of the early colonizers of human teeth, is known to be a major plaque-forming strain with cariogenic potential [32]. The susceptibility to chlorhexidine (CHX) of *S. sanguinis* grown in biofilms is reported to depend on various variables, e.g., age of the biofilm [21], structure of the extracellular matrix [32], and bacterial growth rate [3]. The susceptibility to antibacterial agents of bacteria living in mature biofilms was established to be much lower than that of corresponding planktonic cells [8]. On the other hand, it was recently shown in vitro that initially surface-coated streptococci were more affected by antiseptics than the planktonic counterparts [4]. Whereas the relative proportion of viable mutans streptococci decreased following CHX treatment in humans, *S. sanguinis* showed reduced susceptibility to bisbiguanide, especially when grown in older biofilms [10, 11]. In contrast, the exposure of planktonic *S. sanguinis* bacteria to CHX resulted in an

extensive $\log_{10}$ reduction of colony-forming units (CFU) of about 4.4 [32].

In several adhesion studies, the substratum glass was preferred because of its transparency and ease of sample evaluation [1, 5, 20]. From the thermodynamic point of view, glass and enamel were reported to behave similarly in studies of single strain adhesion [23]. In the present study, human enamel slides were used as a representative oral surface substratum for supragingival biofilm, in spite of the distinctive interfering autofluorescence of enamel, in order to compare bacterial adhesion to glass and enamel before and after CHX treatment.

The specific aim of the present study was to investigate the susceptibility of *S. sanguinis* cells to CHX in planktonic vs initially enamel-attached life forms separately in a preclinical biofilm model.

Materials and methods

Study design

The study design is summarized in Fig. 1. The first part assessed the susceptibility of planktonic *S. sanguinis* cells to CHX by exposing them to either 0.1% (w/v) CHX for 2 min (test group 1) or 0.9% sodium chloride (NaCl) for 2 min (control group 1). Subsequently, the bacteria were suspended in sterile human saliva and then allowed to attach to enamel and glass slides in the flow chamber system for 30 min and 60 min (n=10). Then one slide each of enamel and glass were assayed for the percentage of vital streptococci by labeling the attached cells with fluorescent stains. The effect of CHX on planktonic streptococci living in the environmental suspension of the flow chamber system was estimated at the beginning (t=0 min) and end of the experiments (t=60 min) comparing three viability parameters: percentage of vital streptococci in suspension (%VS_s), total bacterial count of the suspension (BC/ml), and CFU/ml, as shown in Table 1 (n=10).

The second part of the study investigated the susceptibility to CHX of initial bacterial coating only on enamel surfaces. At 30 min and 60 min, two enamel slides were removed. One was exposed to 0.1% CHX for 2 min (test group 2, n=10). As an untreated control, the other slide was rinsed with 0.9% saline for 2 min (control group 2, n=10). Each experiment was performed ten times.

Flow chamber system

The flow chamber system was described in detail in a previous report [29]. This flow chamber (no. 1301) (Minucells, Bad Abbach,

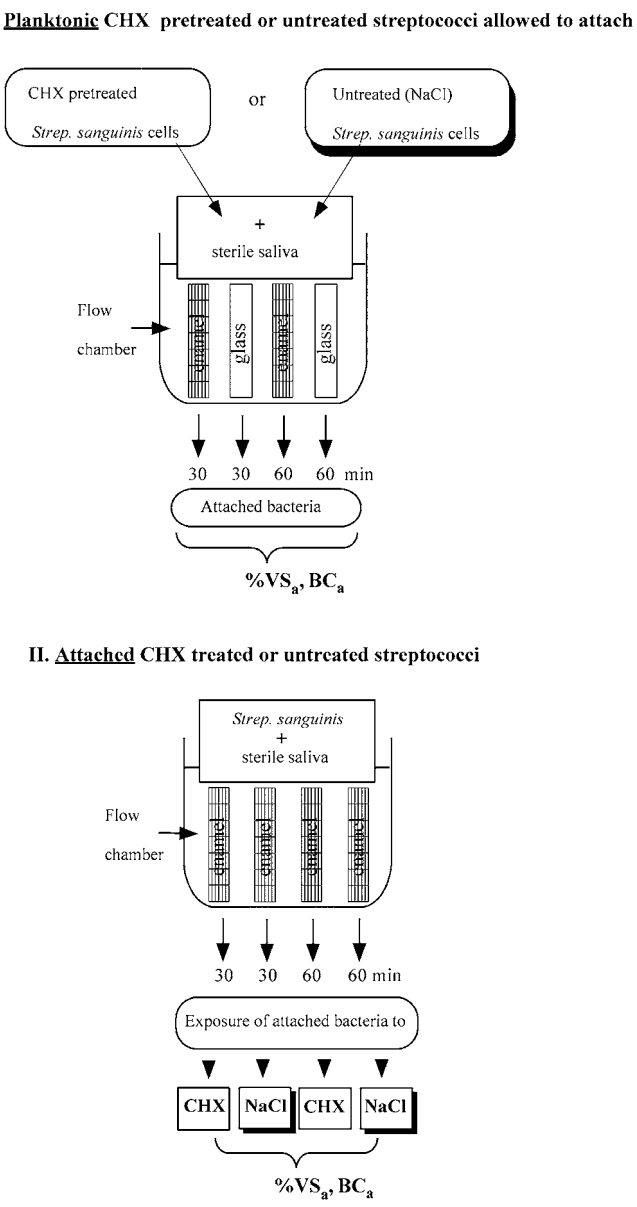

Fig. 1 Study design. NaCl: 0.9%, exposure for 2 min. CHX treatment: 0.1% (w/v) for 2 min. *%VS$_a$* percentage of vital streptococci attached

Table 1 Suspension data expressed by the mean and 95% confidence interval values of %VS_a (percentage of vital streptococci attached), log BC ml^{-1} (total bacterial counts), and log CFU ml^{-1} (colony forming units) at the beginning (0 min) and end of the experiments (60 min). N=10 for each experiment

For planktonic *S. sanguinis* cells[a]	0 min	60 min
CHX-pretreated bacteria (test group 1)		
%VS_s	15.0 (10.6, 20.4)	18.0 (11.2, 24.8)
log BC ml^{-1}	7.9 (7.6, 8.2)	7.8 (7.3, 8.2)
log CFU ml^{-1}	7.3 (7.1, 7.6)	7.1 (6.8, 7.5)
Untreated bacteria (control group 1)		
%VS_s	75.0 (68.9, 81.1)	70.5 (65.9, 75.1)
log BC ml^{-1}	8.4 (8.4, 8.5)	8.4 (8.4, 8.5)
log CFU ml^{-1}	8.0 (7.7, 8.3)	8.1 (7.8, 8.3)
Attached *S. sanguinis* cells[b]		
%VS_s	73.5 (67.7, 79.4)	67.5 (59.6, 75.5)
log BC ml^{-1}	8.1 (7.8, 8.5)	8.1 (7.7, 8.4)

[a] CHX-pretreated or untreated (NaCl) planktonic streptococci allowed to attach
[b] Native attached streptococci exposed to CHX or NaCl

Germany) contained six parallel-mounted substrata plates. The suspension medium consisted of *S. sanguinis* cells (10^8/ml) in sterile human saliva. A peristaltic pump guaranteed constant circulation of the suspension medium adjusted to 0.8 ml/min, corresponding to the average salivary flow rate.

Saliva

About 25 ml of whole saliva was collected from two healthy volunteers by paraffin stimulation 2 h after a meal. After sonification and separation of debris with a 70-µm filter (Becton Dickinson, Heidelberg, Germany), the saliva sample was centrifuged at 25,524 *G* for 30 min at +3°C (Biofuge 22 R) (Heraeus, Hanau, Germany). The supernatant was filtered by two Millex low-binding protein filters (0.45 µm and 0.22 µm) (Millipore, Eschborn, Germany) connected in series. The filtrate was tested for growth of micro-organisms and stored at +6°C up to 2 days. Before use, the sterile saliva was adjusted to a pH level of about 7.1–7.2 by supplementing the phosphate buffer (Na_2HPO_4 and KH_2PO_4, 0.067 mol/l).

Adhesion substrata

Enamel slides were prepared from freshly extracted, intact human third molars. They were sectioned longitudinally during irrigation with water, fixed to glass slides mechanically, and polished with a grinding machine. The surfaces were processed to surface roughness (R_a) of 0.29±0.08 µm for enamel (n=80) and 0.32±0.12 µm for glass (n=40), according to the magnitude of natural enamel-enamel contact areas of 0–1 µm [31] measured by the Perthometer S6P (Mahr, Göttingen, Germany) (Table 1). All adhesion substrata were exposed to sterile saliva for 10 min to create a salivary coating.

Micro-organisms and culture conditions

An inoculum of *S. sanguinis* cells (no. 260068) (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was grown in Schaedler broth for 24 h at 37°C. The bacteria were then transferred to fresh Schaedler broth again (passage 1). This procedure was performed up to five times. Only bacteria grown from passages 1–5 were used for the experimental runs to reduce the possibility of phenotypic variations increasing with the frequency of subcultivation [27]. Grown for 17 h, 16 ml of the bacterial suspension was sonicated and harvested by centrifugation (8,240 *G*, 5 min) (Biofuge Fresco) (Heraeus). After washing with 1 ml of physiological saline, the bacteria were suspended in pretreated buffered saliva to a density of 10^8 cells/ml. The salivary suspension functioned as a resting cell medium for the planktonic streptococci. The total bacterial cell counts in the salivary streptococci suspension were enumerated by dark-field microscopy (objective ×25, magnification ×500) (Orthoplan) (Leica, Bensheim, Germany) with a standard bacteria counting chamber (counting volume 0.8 µl). Each sample was estimated twice, and the mean was calculated.

Detection of microbial viability

The actual vitality status of planktonic micro-organisms and those adhering to substrata could be evaluated applying a dual fluorescent staining method (Live/Dead BacLight Bacterial Viability Kit) (Molecular Probes, Eugene, Ore., USA) in addition to the plate count method [9]. Syto 9 indicated green fluorescence by binding to nucleic acids of vital and dead micro-organisms; propidium iodide induced red fluorescence by intercalation with nucleic acids of dead bacteria.

Staining of planktonic bacteria (%VSs)

Five hundred microliters of bacterial saliva suspension were stained for 15 min. The pellet was analysed by fluorescence microscopy using two filters: blue excitation at 450–490 nm (FITC) and green excitation at 546 nm (rhodamine). The percentages of suspended vital streptococci (FITC excitation) and dead cells (rhodamine excitation) were estimated by means of a counting grid (25 squares) integrated in one ocular. The percentage of vital cells was defined as %VS=100% minus % dead cells.

Staining of attached bacteria (%VSa)

The substratum was removed from the flow chamber system, carefully dipped into distilled water, and decontaminated on one side. The other side was incubated with 7 µl of staining solution for 15 min at room temperature in the dark and subsequently coated with a cover slide. Microscopic evaluation included the counting of vital/dead cells in ten visual fields randomly distributed on the substratum. The percentage of attached vital streptococci ($\%VS_a$) was determined by relating the vital counts to the total numbers of vital and dead cells.

Cultural method

Bacterial samples were plated onto Schaedler agar plates serving as controls when comparing microbial viability assessed by fluorescent labeling and conventional cultivation [9]. Aliquots of 20 µl were plated twice in three dilutions (10^{-4}, 10^{-5}, 10^{-6}) and incubated anaerobically (Anaerocult) (Merck, Darmstadt, Germany) at 37°C for 48 h. Colony-forming units were enumerated and given as counts/ml of the initial suspension.

Chlorhexidine treatment

Planktonic S. sanguinis cells

Streptococcus sanguinis cells grown in Schaedler broth for ~17 h were washed in 0.9% saline. After exposure to 0.1% CHX for 2 min, the bacteria were washed again with 0.9% saline and suspended in human sterile saliva used as an environmental salivary suspension.

Enamel-associated S. sanguinis cells

After 30-min and 60-min exposure to the environmental suspension in the flow chamber, the enamel discs were removed and rinsed in 0.1% CHX or 0.9% saline for 2 min. The samples were carefully dipped into distilled water and stained for fluorescence analysis as described above.

Statistics

The BC and CFU values were log transformed. Mean and mean-based 95% confidence intervals (CI) were determined for $\%VS_a$, $\%VS_s$, log BC/ml, and log CFU/ml. Each experiment was performed ten times.

Results

All 80 samples of streptococci attached to autofluorescing enamel could be evaluated by fluorescence microscopy. The resting cell conditions of the environmental suspension in the flow chamber monitored by the parameters $\%VS_s$, log BC/ml, and log CFU/ml (Table 1) showed constant values during the experimental periods of 60 min.

Table 2 CHX-pretreated (test group 1) and untreated (NaCl, control group 1) planktonic streptococci allowed to attach to enamel/glass surfaces for 30 min and 60 min. Values represent the mean values and 95% confidence intervals. *N*=10 for each experiment. *%VS$_a$* percentage of vital streptococci attached

Planktonic *S. sanguinis* cells	30 min	60 min
CHX-pretreated bacteria (test group 1) %VS$_a$		
Enamel	17.5 (8.1, 26.8)	14.3 (6.1, 22.5)
Glass	23.6 (15.6, 31.6)	24.7 (16.7, 32.7)
Untreated bacteria (control group 1) %VS$_a$		
Enamel	75.4 (66.9, 83.9)	70.2 (58.5, 81.9)
Glass	68.2 (60.3, 76.1)	67.7 (58.9, 76.4)

Table 3 Percentages of CHX-treated (test group 2) and untreated (NaCl, control group 2) streptococci attached (%VS$_a$) after 30-min and 60-min exposure times. Values represent the means and 95% confidence intervals. *N*=10 for each experiment

Attached *S. sanguinis* cells	30 min	60 min
CHX-pretreated bacteria, enamel (test group 2) %VS$_a$	2.0 (–0.3, 4.4)	4.7 (–0.5, 9.8)
Untreated bacteria, enamel (test group 2) %VS$_a$	65.3 (59.4, 71.2)	66.1 (57.5, 74.6)

The susceptibility of planktonic CHX-pretreated and untreated bacteria which were then allowed to attach to enamel was compared to that of streptococci attached to glass. The treatment of planktonic streptococci with CHX caused a reduction in $\log_{10}$ CFU/ml values of ~1 (Table 1). Before the CHX treatment, more streptococci survived on enamel than on glass, whereas after exposition to CHX, the vitality percentages of enamel-associated cells were lower than those attached to glass (test group 1).

The mean values of untreated bacteria (control group 1) attached to saliva-coated enamel surfaces were in the same range as those deposited onto glass slides, as shown in Table 2.

In comparison to control group 2 (NaCl), the treatment of attached streptococci with CHX (test group 2) was followed by a strong reduction in bacterial viability to a level near zero (Table 3). With regard to all experimental runs, the exposure time of substrata for bacterial adhesion (30 min or 60 min) did not cause distinctive differences in the corresponding bacterial vitality percentages.

Discussion

The present study focuses on the susceptibility to CHX of free-living vs sessile *S. sanguinis* cells attached to saliva-coated enamel during the initial stage of bacterial adhesion. At both stages, the bacteria were measured in the flow chamber system serving as preclinical biofilm model. Besides the transparent substratum glass used as a reference material, human enamel slides were also processed and naturally characterized by a distinctive autofluorescence for the given wavelength spectrum (300–600 nm) when examined by fluorescence microscopy [14]. The preparation technique of grinding thin enamel discs in combination with the brilliant stains Syto 9 and propidium iodide enabled differentiation between viable and dead cells, in addition to the plate count method. The main advantage of the methods used in the present study was the local detection of streptococci in situ without destruction of the biofilm structure by detachment procedures, as shown in various other studies [16, 18, 22, 32]. The detachment may result in a substantial conversion of sessile to planktonic microbial life forms, followed by possible phenotypic changes in oral bacteria [26]. Therefore, the fluorescent-based characterization of surface-bound micro-organisms supplies additional information about the microbial physiological state and local distribution, independently of the culturability of these bacteria.

Chlorhexidine was selected for the antimicrobial treatment because it is the best characterized and most effective chemical antiplaque agent known today [2, 17]. The concentration selected corresponds to that used clinically for plaque control.

The results of the present study show clear differences between planktonic and solid surface-associated cells of *S. sanguinis* regarding their susceptibility to CHX. The exposure of suspended bacteria to CHX for 2 min caused a minor $\log_{10}$ CFU/ml reduction of less than 1. These findings contrast with another report of a CHX-mediated $\log_{10}$ CFU/ml reduction of about 4 [32]. For comparability, the different concentrations of CHX have to be considered. Wilson et al. [32] applied 0.2% CHX instead of the 0.1% in the present investigation. In this work, with regard to the effect of CHX on the streptococci already attached, a strong reduction in bacterial viability with values close to zero was evident in comparison to the control group. This stands in contrast to the moderately reduced vitality proportion of attached bacteria resulting from CHX-pretreated planktonic cells.

The results of Burgemeister et al. [4] correspond to our findings of higher susceptibility of attached *S. sanguinis* cells to CHX during the initial phase of coating in the present study. A reduced susceptibility of *S. sanguinis* within the biofilm matrix as reported by several authors [19, 21, 22, 33] may be related to the stage of biofilm formation, i.e., to the mature late phase of single species or multispecies biofilms. During early biofilm formation involving single species, the vitality of native adhering streptococci was shown to be low in a previous report [30]. The cellular susceptibility of these initial micro-organisms

may possibly be intensified by treatment with a strong antiseptic such as CHX known to possess a considerable affinity to all oral surfaces, including enamel [25].

It is acknowledged that the mode of action of CHX in plaque inhibition in vivo occurs in two phases. The first effect (immediate bactericidal action during application) is followed by a second one characterized by prolonged bacteriostatic action resulting from CHX adsorption to the biofilm-coated enamel surface [15]. The high substantivity of CHX may favor the killing of initially deposited bacteria arranged as a cellular monolayer film. In contrast, CHX had only a superficial effect on mature multilayer biofilm [34].

In conclusion, the susceptibility of enamel-bound *S. sanguinis* cells to CHX using a flow chamber system was higher than that of the planktonic counterparts during initial phases of biofilm formation. The in vitro model used here can also be applied as a preclinical screening system to evaluate new antiplaque agents with regard to their efficacy on oral bacteria in different life forms separately. These results add to the current knowledge of how antiseptics work and therefore will help in the search for future antiplaque therapeutics for clinical application in vivo.

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