Biofilm and dental caries : application of the constant depth film fermentor
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Chapter 4

Constant Depth Film Fermentor for Studying Chlorhexidine and Fluoride Effects on Biofilms
Caries-Preventive Agents
Effects on Biofilms

Abstract

Caries-preventive agents were studied for their effects on biofilms and caries development in dentin. To this end, *Streptococcus mutans* biofilms were grown in dentin grooves in the Constant Depth Film Fermentor (CDFF) split model for 20 days. Growth medium and 30 min 2% sucrose were pulsed 4 times/day alternately. Between days 6 and 12, 0.05% chlorhexidine only or combined with 135 ppm fluoride was pulsed 2 x 5 min/day to either side of the CDFF, respectively. Samples were removed from the CDFF at various days. The biofilms were assessed for viability (by plating) and acidogenicity (by capillary electrophoresis). The dentin specimens were analyzed for mineral loss and lesion depth by transversal microradiography. The results showed that viability and lactic acid production of biofilm cells decreased during the treatments and recovered when the treatments were discontinued, although this recovery took at least 4 days to start. The profiles of these parameters were similar between the two treatment groups during the experiment. Lesion formation in the dentin grooves did not change consistently with the change in viability and lactic acid production, particularly in the chlorhexidine only group: when given the treatments the lesion size still increased. The addition of fluoride to the antimicrobial treatments resulted in remineralization at the entrance of the grooves but not at the bottom. In conclusion, this study showed that the CDFF biofilm model is suitable to investigate the effects of antimicrobial agents on viability and acidogenicity of the biofilms and the resultant caries development in dentin.

Introduction

Chlorhexidine has been widely used as an antimicrobial agent since it was first introduced in dentistry thirty years ago [Emilson, 1994]. As a cation agent, it binds to negatively charged bacterial cell walls and thereby disrupts membrane integrity. At low concentrations, the action of chlorhexidine is bacteriostatic, and at higher concentrations it is bactericidal [Denton, 2000]. Being tested in bacterial suspensions, chlorhexidine was found to have a broad spectrum antibacterial activity. In particular, it was effective against the highly cariogenic microorganism *Streptococcus mutans* [Emilson, 1977]. The range of minimum inhibitory concentration of chlorhexidine
was 0.0001% - 0.002% [Sreenivasan and Gaffar, 2002]. Since the properties of bacterial cells in biofilms were shown to be different from those in suspensions [Costerton et al., 1995], chlorhexidine effects were also studied in biofilm models. These results indicated that biofilm cells were 10 to 100 times more resistant to chlorhexidine than planktonic cells [Gilbert et al., 1998; Kinniment et al., 1996b]. Moreover, one study showed that the viability of Streptococcus sanguis biofilm was only 2 log units reduced one day after 0.05% or 0.2% chlorhexidine treatment and gradually recovered although the treatment continued [Pratten et al., 1998a]. Given these discrepancies, it is important to further study the efficacy of antimicrobials on biofilm cells. Caries is a process involving acid production in the biofilms and lesion formation in the dental hard tissue. However, in none of the biofilm studies acid production in the biofilms or lesion formation has been measured.

Like chlorhexidine, fluoride has antimicrobial properties. The mechanism of action of these two agents is different. The former functions by attaching to cell walls and the latter by diffusion as HF into the cell, resulting in acidification of the cell interior and inhibition of various enzymes [ten Cate and van Loveren, 1999]. Combination of the mechanism of the two antimicrobial agents is expected to make the agents more effective than either alone. Studies with bacterial cell suspensions did show that the combination treatment could further reduce the acid production and inhibit the growth of the cells [McDermid et al., 1985; Meurman, 1988]. Whether this synergistic effect could be observed in bacterial cells grown in biofilms is unknown.

We have shown that demineralization and remineralization can be studied in a biofilm-hard tissue model when the Constant Depth Film Fermentor (CDFF) is properly controlled [Deng et al., 2004b; Deng and ten Cate, 2004]. Viability and acidogenicity of biofilm cells and the resulting lesion formation in dentin can be studied simultaneously in this model. Moreover, the split model design not only allowed two conditions or treatments to be applied in one experiment, but also ensured similar baseline values for the treatments [Deng et al., 2004b].

Our purpose was to study the output parameters during and after periodic application of chlorhexidine in the CDFF. These parameters included viability and acidogenicity of Streptococcus mutans biofilms and lesion formation in dentin. A combination of fluoride and chlorhexidine was also applied to test the additional effects of fluoride.
Caries-Preventive Agents
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Materials and Methods

Split Constant Depth Film Fermentor

A Constant Depth Film Fermentor (CDFF) (University of Wales, Cardiff), described by Wilson [1999], was used to grow biofilms. The motor of the CDFF was modified to allow the turntable not only to rotate 360° (as originally designed), but also to oscillate over 180°, splitting the CDFF into two independent sides. With this set-up, each side has its own ports to deliver the medium and its own polytetrafluoroethylene (PTFE) scraper bar to scrape the liquid over the specimens. Different treatments could be applied to the two sides. When it rotates 360°, the rotating turntable can contain 15 PTFE pans; when it oscillated over 180°, only 11 pans, 5 in one and 6 in the other side, can be included in order to avoid carry-over. Each sampling pan has 5 cylindrical holes (5.0 mm in diameter), where dentin specimens are placed on top of PTFE plugs.

Preparation of Specimens

Sixty dentin discs (1.5 mm thick and 5 mm diameter) were prepared from freshly obtained bovine incisors. They were covered with bonding agent (Scotchbond, multi purpose, 3M), then three parallel grooves were sawn perpendicular to the outer surface with a diamond coated wire (Well type A3-4; W. Ebner, Mannheim, Germany) [Lagerweij et al., 1996a]. All grooves were about 330 μm wide and 650 μm deep. The surfaces of the dentin discs were placed flush with the surface of PTFE pans.

Inoculation and Operation of the CDFF

The bacterium used in this study was Streptococcus mutans C180-2 [De Stoppelaar et al., 1967]. The procedure of inoculation and operation of the CDFF was the same as in a previous study [Deng et al., 2004a]. Briefly, 100 ml sterile human saliva was pumped into the CDFF for 3.5 h, followed by the S. mutans culture for 8 h. Then sterile growth medium or 2% (w/v) sucrose solution was dripped into the system at a rate of 0.5 ml/min. The sucrose was applied 4 times per day for periods of 30 min. The growth medium contained 3.7 g/L BHI medium (Difco, Laboratories, Detroit, Michigan, USA) with 0.9 mM phosphate, 1.5 mM CaCl₂ and 25 mM PIPES buffer. pH was adjusted to 7.0 by the addition of 5 M KOH.
Chapter 4

The Regime of the Treatments

Two different treatments, 0.05% chlorhexidine digluconate (CHX) and a mixed solution of 0.05% CHX and 135 ppm fluoride solution (as NaF), were given to each side of the CDFF, respectively. The regime of the treatment is described below (figure 1).

![Diagram showing the regime of treatments with phases and treatment processes](image)

**Fig. 1.** The regime of the treatments. ♦ refers to sampling days.

During the first 5 days after inoculation (phase 1), the turntable of the CDFF rotated 360° and no treatment was given.

From day 6 to day 12 (phase 2), the treatment solutions were pulsed immediately after a sucrose pulse with the turntable oscillating over 180°. The frequency of the pulsing was twice daily for 5 minutes at a rate of 1 ml/min, while the supply of growth medium and sucrose was stopped. On days 5, 7, 9 and 12, a sample pan from each group was removed from the CDFF immediately after a 30-min sucrose pulse. This was at 360 min after the respective treatments.

From day 13 until day 20 (phase 3), the treatments were stopped while the turntable still oscillated over 180° and the biofilms at each side were grown under the growth medium and sucrose pulse. On days 16 and 20, sample pans were removed from the CDFF for further analysis.

All sample pans were removed with a sterile stainless steel tool. The dentin specimens were removed aseptically from the pans for the determination of biofilm viability, acid production, mineral loss and lesion depth of dentin specimens.
Assessment of CDFF Biofilm Viability

For each dentin specimen, biofilms from one groove were checked for viability and from the other two grooves they were pooled and analyzed for acid content. Biofilms were removed by repeated scraping with 3 consecutive sterile paper points (size #20, QDENT; NDO Leeflang, the Netherlands). This method was discussed in detail by Deng et al [2004a]. All three paper points were placed into a sterile vial containing 1ml Cysteine Peptone Water solution (5 g yeast extract, 1 g peptone, 8.5 g NaCl, 0.5 g L-cysteine hydrochloride and 100 ml glycerol per liter, adjusted to pH 7.3). Samples were dispersed by sonication on ice for 30 sec at an amplitude of 40 W (Vibra cell™, Sonics & Materials INC, USA). Serially diluted samples were plated onto BHI agar. The plates were incubated anaerobically at 37°C for 3 days.

Assessment of Acid Production

For acid analysis the biofilms were removed by paper points in the abovementioned way and transferred into vials containing 50 µl Milli-Q water on ice. The vials were placed in 80°C water bath for 5 minutes and centrifuged for 15 min at 13,000 rpm under 4°C (Heraeus centrifuge, Dijkstra bv, the Netherlands). The supernatants were analyzed for organic acid analysis by capillary electrophoresis. From the pellets the protein contents were determined by Bradford’s method [Damen et al., 2002].

Assessment of Mineral Loss and Lesion Depth

After the biofilms were removed, the dentin specimens were sectioned and three sections per specimen were radiographed and analyzed with the transversal microradiography (TMR) procedure [Lagerweij et al., 1996a]. Scans were made of both walls of each groove at 50 µm (‘entrance’) and at the deepest part (‘bottom’) of the groove. The mineral loss, expressed as integrated mineral loss (IML) and the lesion depth (LD) were calculated (TMR software 1.25e, Inspektor Research Systems, Amsterdam, The Netherlands). IML and LD values at various positions of the groove per specimen were averaged from corresponding values of scans and sections.

Statistics

All statistical analyses were performed with SPSS (Version 9.0). The effects of various treatments on viability, acid production, mineral loss and lesion depth were
analyzed by two-way ANOVA. When the location of the groove was included as parameter, the data were analyzed by repeated-measurement ANOVA.

Results

Viability of *S. mutans* Biofilms

The viability of biofilm cells during the 3 phases is shown in figure 2. Five days after the inoculation, the number of viable cells reached $4.8 \times 10^8 \pm 5.8 \times 10^7$ CFU/groove. This number decreased gradually after the start of the treatments. At the end of the phase 2, viability had dropped more than 3 log units. In phase 3, the viability remained low the first 4 days and during the next 4 days it recovered to 10% of the values at the start of the treatment phase. There were no significant differences in the killing and the recovery of the biofilm cells between the two treatments.

![Fig. 2. Viability of *S. mutans* biofilms over time under 0.05% CHX (○) versus 0.05% CHX + 135 ppm F⁻ (□). All treatments were pulsed twice daily from day 6 until day 12 (n = 5).](image)

Acid Production of *S. mutans* Biofilms

After a 30-min sucrose pulse lactic acid was the main acid produced by *S. mutans* biofilms in the CDFF. The change of lactic acid over time is illustrated in figure 3. In phase 1, the lactic acid production of the biofilm was $3.33 \pm 0.49$ nmol/µg protein. Similar to the viability of the biofilms, after starting the treatments, acid production
decreased, reaching the detection limit (0.21 nmol/μg protein) at the end of phase 2. Acid production remained at this low level the first 4 days in phase 3 and recovered to 9% (the chlorhexidine group) or 14% (the mixture group) of the baseline values at the end of phase 3. The profiles of lactic acid production throughout the experiment were similar for the two experimental groups, except on day 7, one day after the start of the treatments, when the reduction of the lactic acid concentration was significantly smaller in the mixture group than in the chlorhexidine group.

**Fig. 3.** Lactic acid production of *S. mutans* biofilms over time under 0.05% CHX (O) versus 0.05% CHX + 135 ppm F⁻ (□). All treatments were pulsed twice daily from day 6 until day 12 (n = 5).

**Lesion Formation in the Dentin Grooves**

Figure 4 gives the IML and LD values at the entrance and the bottom of the dentin grooves on days 5, 12 and 20, which corresponded to the end of phase 1, 2 and 3, respectively.

For both treatment groups, subsurface lesions had formed homogeneously along the wall of the grooves at the end of phase 1. In the chlorhexidine group, the IML values along the wall of the grooves increased significantly during phase 2 and remained at these levels during phase 3. The increase of the IML values was more severe (*p < 0.05*) at the bottom of the grooves than at the entrance. In the mixture group, the IML values decreased significantly at the entrance, but did not change at the bottom of the grooves during phase 2. These values again increased slightly at the entrance, and increased significantly at the bottom of the grooves during phase 3.
Different from the IML values, the LD values increased significantly under both treatments. This increase was more severe at the bottom than at the entrance of the grooves and more in the mixture group than in the chlorhexidine group.

![Fig. 4. Intergrated mineral loss (IML) and lesion depth (LD) values at the entrance and the bottom of the grooves on day 5 (□), day 12 (■) and day 20 (▲). CHX refers to 0.05% CHX treatment; CHX+F refers to 0.05% CHX + 135 ppm F treatment. All treatments were pulsed twice daily from day 6 until day 12. * refers to the significant difference among the groups (n = 5)](image)

**Discussion**

In this CDFF biofilm study, the antimicrobial effects of chlorhexidine on viability and acidogenicity of biofilms were demonstrated. A decrease in lactic acid production and viability of the biofilm cells was observed during the treatments and a recovery of these two parameters was seen when treatments were discontinued. During the course of the experiments the change of lesion formation in dentin followed the changes of viability and acid production, though delayed.
In contrast to other biofilm models used for testing antimicrobial agents [Guggenheim et al., 2001; Pratten et al., 1998a], with this CDFF model it was possible to follow all three parameters, which are potential indicators for the caries process. Different from a study reported by Pratten et al. [1998a], where a small reduction and quick recovery in viability during the chlorhexidine treatment were seen for S. sanguis biofilm, our study showed about 4 log units reduction in viability of S. mutans biofilms during a 0.05% chlorhexidine treatment and a delayed recovery after stopping the treatment. Compared to our study, Pratten and coworkers used a poor nutrient-containing growth medium, only 1 min chlorhexidine treatment and different bacterial species. This suggests that the susceptibility of biofilms to antimicrobials is affected by the growth conditions of the biofilms, the application time of antimicrobials and the bacterial species. More work on this topic seems to be indicated.

Various clinical and in situ studies on chlorhexidine have shown a significant reduction of mutans streptococci counts in saliva and plaque, but no change in caries scores [Dasanayake et al., 2002; van Strijp et al., 1997]. In our study, we also found a reduction in viability and lactic acid production of the biofilms, but an increase in lesion formation in dentin, especially during the chlorhexidine only treatment. We noted that, as for clinical trials, acid production and viability measures the properties of the biofilm cells at the time of sampling, while lesion formation reflects the changes of the mineral content in dentin cumulated up to the sampling time. Therefore, these three parameters evaluate the efficacy of antimicrobials on caries formation from different perspectives.

When compared to the chlorhexidine only group, the influence of fluoride on lesion formation was evident. This effect was stronger at the entrance than at the bottom of the grooves. This phenomenon was also reported from our previous CDFF biofilm study [Deng et al., 2004b]. As explanation we proposed that fluoride affected the dissolution of dental hard tissue, leading to less neutralization of acids and consequently a deeper penetration of acids into the grooves. In fact, a limited effectiveness of caries-preventive agents at the bottom of the groove was found not only in the combination of fluoride and chlorhexidine group but also in the chlorhexidine only group. This would indicate that the retention site such as the bottom of the groove in this study or fissures in vivo might be difficult to protect from caries by antimicrobial agents.
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We found no synergistic effects of chlorhexidine and fluoride on either the viability or acidogenicity of the biofilm. This observation was different from reports with planktonic cells [McDermid et al., 1985; Meurman, 1988], presumably due to differences in bacterial properties under the two growth conditions. In a clinical regrowth study, the combination treatment was shown to be less effective than chlorhexidine only. It was assumed that the inclusion of fluoride might reduce chlorhexidine availability or adsorption to the surfaces of the teeth [Mendieta et al., 1994].

Compared to the Pratten et al. [1998a] study, the recovery of both the viability and acidogenicity of S. mutans cells was significantly slower in the present study. Irrespective the treatment type, there was a lag phase of at least four days after stopping the treatment. As to the reasons for this lag phase, we can only speculate: first, the retention of chlorhexidine in the dentin grooves and slow clearance, as compared to biofilms at a smooth surface, might repress the growth of S. mutans cells [Gjermo et al., 1974]. Second, chlorhexidine has been shown to possess a postantibiotic effect (PAE) [Fuursted et al., 1997]. PAE was described as a delayed response of pathogens (e.g. Escherichia coli), even when the antibiotic had been neutralized.

In the present study, 0.05% chlorhexidine instead of the commonly used clinical concentration of 0.2% was chosen. This choice was based on an earlier study, where 0.2% CHX was shown to reduce the viability and acid production to values under the detection limit after a 10-min treatment (data not shown). Since a purpose of the current study was to test the changes in viability, acidogenicity of the biofilms and lesion formation in the dentin induced by caries-preventive agents, a lower concentration of chlorhexidine was applied.

In conclusion, this study shows that the CDFF biofilm model is suitable to investigate the effects of antimicrobial agents on viability and acidogenicity of biofilms and lesion formation in dentin. No synergistic effect of the combination of fluoride and chlorhexidine was observed for the biofilm parameters though additional effects could be seen in dentin. A delayed recovery of biofilm cells was seen after the antimicrobial treatment had been stopped.

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