Effect of Sodium Fluoride, Ampicillin and Chlorhexidine on
Streptococcus mutans Biofilm Detachment

Running title: Streptococcus mutans biofilm detachment

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Abstract

We examined the effect of three clinically used antimicrobials on *Streptococcus mutans* UA159 biofilms detachment under flow conditions. Sodium fluoride (NaF) and chlorhexidine at sub-minimum inhibitory concentration (MIC) levels promoted biofilm detachment and inhibited detachment when concentrations were higher than MIC, and reduced detached cells viability only at high concentration. Ampicillin at all concentrations tested inhibited detachment and reduced percentage of viable biofilm-detached cells. All the three antimicrobials treatment reduced live/dead cells ratio of biofilm.

Key Words

biofilm detachment; *Streptococcus mutans*
Microbial biofilms have been associated with many chronic infections in humans (3, 6). Regardless of locations, biofilms release cells into surrounding environment (16), which contribute to bacterial survival, new sites colonization and disease transmission (10, 15, 25). It has been reported that environment conditions like nutrient and oxygen tension affect biofilms detachment of various species (1, 9, 16, 26, 27). Re-attachment of Neisseria subflava and Aggregatibacter actinomycetemcomitans biofilm-detached cells has been noted (14). Several studies indicate that C. albicans biofilm detachment extent and detached cells viability are dependent on antimicrobials types (21) and detached cells gain enhanced adherence abilities (26). Given the breadth of detrimental effects caused by biofilms and biofilm-detached cells, there have been significant efforts to develop and find agents controlling biofilm detachment and decreasing pathogenicity and viability of biofilm-detached cells (13, 17).

Streptococcus mutans is one of the principal inhabitants of cariogenic dental biofilm (18), which are controlled mainly through broad-spectrum antibiotics or nonspecific mechanical removal. NaF, ampicillin and chlorhexidine are three different types of clinically used antimicrobials (4, 11, 24). We hypothesized that the three antimicrobials affect the detachment of S. mutans biofilm. And we investigated the effects of NaF, ampicillin and chlorhexidine on S. mutans biofilm detachment extent and detached cells viability as well as biofilm structure alterations.

S. mutans UA159 was grown in brain heart infusion (BHI) overnight anaerobically (10% H2, 5% CO2, and 85% N2; Forma Scientific, Inc, Marietta, OH) at 37°C. Cells were harvested by centrifugation (5,000 rpm) at 4°C and resuspended in BHI-1% sucrose to a concentration of 10^5 colony forming unit per milliliter (cfu/ml) (5). Twenty-five milliliters of S. mutans suspension was poured into a 100-mm-diameter petri dish containing four polystyrene (PLS) blocks (22mm x 30mm x 1mm; VWR scientific) for 48 hr to develop biofilms. The medium was replaced by fresh medium at 24hr. Biofilms colonized PLS blocks were washed twice with phosphate-buffered saline (PBS, 50 mM, pH 6.8) and transferred to a flask connected to a peristaltic pump to allow continuous flow (22). Fresh BHI-1% sucrose with NaF (250 ug/ml, 500 ug/ml, 1,000 ug/ml, 2,000 ug/ml), ampicillin (0.04 ug/ml, 0.08 ug/ml, 0.16 ug/ml, 0.32 ug/ml) or chlorhexidine (0.31 ug/ml, 0.63 ug/ml, 1 ug/ml, 2 ug/ml) was continuously pumped into the flask at a
constant flow rate (0.5 ml/min). The control contained BHI-1% sucrose without antimicrobials. The flowthrough was collected during 1hr, 3 hr and 6 hr and the detached cells were collected by centrifugation as detailed previously.

Studies have described the detached cells to have several virulence traits distinct from planktonic cells (22). Whether the \textit{S. mutans} biofilm-detached cells inherit antimicrobials resistance from mutidrug-resistant biofilm cells has not been investigated. MIC and minimum bactericidal concentration (MBC) of NaF, ampicillin and chlorhexidine against \textit{S. mutans} UA159 planktonic and biofilm-detached cells were determined (19). NaF inhibited \textit{in vitro} growth of \textit{S. mutans} UA159 planktonic cells (MIC =600 ug/ml) in BHI and had an MBC of 2,500 ug/ml. Compared to planktonic cells, biofilm-detached cells were two times more resistant to NaF and no bactericidal concentration was detected up to 2,500 ug/ml. The two cell populations showed identical MIC and MBC against ampicillin. Reduced efficacy of chlorhexidine against detached cells was observed with an MIC of 1.25 ug/ml and MBC 2.5 ug/ml (Table 1). This multi-drug resistance characteristic may contribute to their survival in adverse environment and colonization of new susceptible sites.

Followed we investigated the effect of the three antimicrobials on biofilm detachment extent and detached cells viability by measuring the absorbance of flowthrough at 550nm and counting of the culturable bacteria on BHI agar plate (28). The results were expressed as percentage of detachment and viable cells compared to untreated biofilm. 2,000 ug/ml NaF inhibited biofilm detachment regardless of treatment time (P<0.05) (Fig.1A) and killed 66.80±7.40% of the detached cells after 3hr (Table 2). NaF (1,000 ug/ml) also showed inhibitory effect after 3 hr and 6 hr (65.56±8.87% and 73.01±6.0%, P<0.05) (Fig.1A) but had little effect on cells viability (Table 2). At all concentrations tested, ampicillin inhibited biofilm detachment and reduced detached cells viability. 0.04 ug/ml and 0.32 ug/ml ampicillin reduced the detachment to 51.86±3.7% within 1 hr and to 30.74±2.8% after 6 hr respectively (P<0.05) (Fig.1B). Overall reductions in detached cells viability of greater than 80% were observed after exposure to ampicillin (0.16 ug/ml and 0.32 ug/ml) (Table 2). The effect of chlorhexidine on biofilm detachment varied depending on treatment time and concentrations. Chlorhexidine treatment at sub-MIC levels for 3 hr and 6 hr increased detachment from 125.13±3.40%
to 136.6±2.23% (P<0.05), whereas 1 hr treatment showed no effect (P>0.05). 1 ug/ml and 2 ug/ml chlorhexidine showed detachment inhibitory effect (P<0.05) (Fig.1C) and 2 ug/ml chlorhexidine decreased viable detached cells percentage to 8.00±0.30% and cells were virtually all killed after 6 hr (Table 2). The decreased detachment percentage in our study may result from the self-protection mechanism regulated by biofilm cells (2, 8, 23).

In addition, the effect of the antimicrobials on S. mutans biofilms structures was assessed using confocal laser scanning microscopy (CLSM) (17, 19). Biofilm thickness and live/dead cell ratio were calculated using the COMSTAT software. Since the prerequisite for successful antimicrobial treatments is that bacteria within biofilms are exposed to adequate concentration of antimicrobials (12), we selected the highest concentrations tested for CLSM sample. Untreated biofilms showed an elaborated architecture with a thickness of 16.85 ±0.75 um (Fig. 2D). Upon treatment with NaF, ampicillin and chlorhexidine, S. mutans cells were sporadically scattered on the substrate (Fig. 2A-C), while vertical sectioning revealed no thickness change (P>0.05) (Fig. 2E). Ampicillin and chlorhexidine treatment reduced live/dead cells ratio from 1.47±0.08 to 0.36±0.03 and 0.48±0.05 (P< 0.05), while NaF treatment had no significant effect on live/dead cells ratio (Fig. 2F). The structure alteration may partly explain the reduction in live/dead cells ratio (7).

In summary, antimicrobial treatments affect S. mutans UA159 biofilms detachment and detached cells viability. The results emphasize the importance of considering concentration, treatment time and antimicrobial types when using antimicrobials to control S. mutans UA159 biofilm-associated infections.

ACKNOWLEDGMENTS

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REFERENCES


Figure legends

Figure 1. Effect of NaF, ampicillin, and chlorhexidine treatment on the extent of biofilm detachment. Biofilm developed on PLS blocks was further treated with different concentrations of NaF, ampicillin, and chlorhexidine. OD$_{550nm}$ of flowthrough with biofilm-detached cells at various time points after treatment was measured. Results were expressed as percentages, compared to OD$_{550nm}$ from untreated biofilms formed in parallel, which were considered 100% (0.50 after 1 hr, 0.47 after 3 hr and 0.53 after 3 hr). * Significant difference compared to control group (P<0.05). (A) NaF at 1,000 ug/ml and 2,000 ug/ml inhibited biofilm detachment. (B) Ampicillin at all concentrations tested significantly inhibited biofilm detachment (P<0.05). (C) Chlorhexidine at sub-MIC level promoted biofilm detachment, otherwise inhibited detachment.

Figure 2. Biofilm structures analyzed by CLSM before and after NaF, ampicillin, and chlorhexidine treatment for 1 hr. The biofilms were stained by SYTO 9 and PI and examined with CLSM. * Significant difference compared to control group (P<0.05) (A) Biofilms treated with NaF (2,000 ug/ml); (B) Biofilm treated with ampicillin (0.32 ug/ml); (C) Biofilm treated with chlorhexidine (2 ug/ml); (D) Control biofilm. (E) There was no significant difference in biofilm thickness between treated and control biofilms. (F) After ampicillin and chlorhexidine treatment, live/dead cells ratio of biofilm decreased compared to untreated biofilm (P< 0.05).
TABLE 1. Antimicrobial effect of NaF, ampicillin and chlorhexidine against *S. mutans* UA 159 planktonic and biofilm-detached cells.

<table>
<thead>
<tr>
<th>Agents</th>
<th>Planktonic cells</th>
<th></th>
<th>Biofilm-detached cells</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (ug/ml)</td>
<td>MBC (ug/ml)</td>
<td>MIC (ug/ml)</td>
<td>MBC (ug/ml)</td>
</tr>
<tr>
<td>NaF</td>
<td>600</td>
<td>2,500</td>
<td>1,200</td>
<td>&gt;2,500</td>
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<tr>
<td>ampicillin</td>
<td>0.16</td>
<td>0.32</td>
<td>0.16</td>
<td>0.32</td>
</tr>
<tr>
<td>chlorhexidine</td>
<td>0.63</td>
<td>2.00</td>
<td>1.25</td>
<td>2.50</td>
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</tbody>
</table>
TABLE 2. Viability of *S. mutans* UA 159 biofilm-detached cells during NaF, ampicillin and chlorhexidine treatment.

<table>
<thead>
<tr>
<th>Agents</th>
<th>Con (ug/ml)</th>
<th>1 hr</th>
<th>3 hr</th>
<th>6 hr</th>
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<tbody>
<tr>
<td>NaF</td>
<td>250</td>
<td>95.21±0.32</td>
<td>95.44±1.02</td>
<td>94.71±0.63</td>
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<td>500</td>
<td>94.83±0.17</td>
<td>94.30±0.40</td>
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<td></td>
<td>1,000</td>
<td>68.74±4.21</td>
<td>66.86±7.44</td>
<td>64.23±7.70</td>
</tr>
<tr>
<td></td>
<td>2,000</td>
<td>62.54±2.33</td>
<td>66.80±8.70</td>
<td>65.32±3.94</td>
</tr>
<tr>
<td>ampicillin</td>
<td>0.04</td>
<td>64.86±7.23</td>
<td>68.51±3.80</td>
<td>63.46±5.22</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>52.03±13.50</td>
<td>46.33±7.84</td>
<td>40.84±5.80</td>
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<tr>
<td></td>
<td>0.16</td>
<td>11.74±5.32</td>
<td>17.40±5.42</td>
<td>6.93±2.61</td>
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<tr>
<td></td>
<td>0.32</td>
<td>1.65±2.47</td>
<td>0.23±1.34</td>
<td>0.00±0.00</td>
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<tr>
<td>chlorhexidine</td>
<td>0.3</td>
<td>80.26±6.44</td>
<td>83.45±6.10</td>
<td>74.82±4.90</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>73.53±4.80</td>
<td>68.74±2.63</td>
<td>52.15±2.17</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>34.80±2.93</td>
<td>36.15±0.47</td>
<td>23.54±1.80</td>
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<tr>
<td></td>
<td>2</td>
<td>8.00±0.33</td>
<td>7.41±3.25</td>
<td>7.32±0.10</td>
</tr>
</tbody>
</table>

\( ^a \) Results were percentages of viable cells compared to control (6.93 x 10^6 cfu/ml after 1 hr, 7.22 x 10^6 cfu/ml after 3 hr and 1.37 x 10^7 cfu/ml after 6 hr) and were expressed as means and standard deviations.