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Otten, Marieke P. T.; Busscher, Hendrik; van der Mei, Henderina; van Hoogmoed, Christianus; Abbas, Frank; van Hoogmoed, Christianus

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Acute and substantive action of antimicrobial toothpastes and mouthrinses on oral biofilm in vitro


The aim of this study was to compare acute action by killing or disrupting oral biofilms through the use of antimicrobial toothpastes and mouthrinses in vitro and to investigate substantive action by absorption of antimicrobials in a biofilm. Biofilms from freshly collected human saliva were grown in 96-well microtitre plates. After removal of saliva, the wells of the microtitre plates were washed with sterile water (control), or exposed to a dilution series of mouthrinses (Corsodyl, Listerine, Meridol, Crest Pro Health) or to toothpaste slurries (Prodent Coolmint, Colgate Total, Zendium Classic, Crest Pro Health, Oral B Pro Expert, Crest Cavity Protection). Acute action was concluded from reduced growth on top of the treated biofilms. All formulations showed acute action at the highest concentrations studied. Further dilution yielded loss of efficacy, or even stimulation of biofilm growth. Antimicrobial absorption in and the release of effective concentrations of antimicrobials from dead biofilms, was demonstrated for three selected antimicrobial products, indicating that antimicrobials remain bio-available for substantive action on new biofilms.

All surfaces exposed to the human oral cavity are covered within seconds with a layer of adsorbed salivary proteins (‘pellicle’) to which a large variety of different oral microbial strains and species can adhere and grow to form a biofilm (‘plaque’). Under appropriate conditions, the number of pathogenic bacteria in an oral biofilm will increase and, as a consequence, diseases such as caries and periodontal disease (1) can develop. Removal of oral biofilms in vivo by toothbrushing is the most effective way to prevent caries and periodontal diseases, but it is impossible for most people to completely remove the oral biofilm by brushing (2). Interdental areas, fissures and gingival pockets, in particular, are almost completely inaccessible to the bristle ends of a toothbrush and constitute places where plaque is easily left behind and may also be difficult to remove by other mechanical means. Antimicrobials are added to toothpastes and mouthrinses in order to assist in achieving oral health through the killing of oral biofilm organisms (3, 4). A wide range of different antimicrobials, including enzymes, metal ions, stannous fluoride, chlorhexidine, and triclosan, can be added to toothpastes and mouthrinses (3–5). Chlorhexidine is generally considered to be the most effective oral antimicrobial (3, 5), and it is often used as a positive control in studies (6).

Chemical control of oral biofilms by antimicrobials can occur by ‘acute’ or ‘substantive’ actions by killing or disrupting the biofilm (4). Acute action takes place immediately after the use of an oral antimicrobial, leaving behind a dead or partly dead, or disrupted biofilm. Substantivity is defined by the Oxford Dictionary of Dentistry (7) as a characteristic of an antimicrobial product whereby it remains active in the oral cavity for a prolonged period. Recently, we suggested, on the basis of a clinical study, that plaque left behind after brushing, which is inevitably the case in most people, can absorb antimicrobials to yield substantive killing of new plaque (8). In this study we hypothesize that oral antimicrobials can absorb in biofilms to inhibit further growth of and kill new organisms, a process we refer to as substantive action.

Consequently, the aim of this study was not only to measure the acute action by killing or disrupting oral biofilms achieved by a single exposure of antimicrobial mouthrinses and toothpastes, but also to investigate whether antimicrobials, absorbed in dead biofilms, have the ability to exert prolonged action on organisms growing from fresh saliva on top of the dead biofilm.

Material and methods

Saliva, toothpastes, and mouthrinses

In order to study acute and substantive action, stimulated human saliva of five volunteers was collected by chewing...
Parafilm and pooled in accordance with the guidelines set out by the Medical Ethical Committee at University Medical Center Groningen (UMCG), Groningen, the Netherlands. The saliva was sonicated twice for 10 s at 30 W (Vibra Cell model 375; Sonics and Materials, Danbury, CT, USA) in order to break bacterial chains and clumps.

To study acute action, four different mouthrinses and six different toothpastes were commercially obtained; these are listed in Table 1, together with their main active ingredients. Toothpastes were diluted in demineralized water to a 25% (w/w) slurry, which was centrifuged at 10,000 g for 5 min at 10°C (Beckman J2-MC Centrifuge, Fullerton, CA, USA) in order to remove particulate matter. Subsequently, the supernatant of the toothpaste was further diluted to 12.5%, 2.5%, and 0.25% (w/w) with demineralized water. Mouthrinses were used full-strength or diluted to 50%, 10%, and 1% (v/v) in demineralized water. To demonstrate substantive action through antimicrobial absorption in biofilms, Corsodyl and Crest Pro Health mouthrinse [100% (v/v)] and Crest Pro Health toothpaste slurry [25% (w/w)] were used.

**Acute and substantive action of antimicrobial toothpastes and mouthrinses**

Acute action of mouthrinses and toothpaste slurries on microorganisms in 4-h-old, initial oral biofilms was evaluated in sterile 96-well microtitre plates (Greiner Bio-one, Alphen a/d Rijn, The Netherlands). First, a 4-h-old biofilm of initially adhering organisms was grown. To this end, wells were filled with 175 µl of freshly collected human saliva and incubated for 4 h at 37°C, with shaking at 150 rpm (Incubator Shaker, Innova 4000; New Brunswick Scientific, Edison, NJ, USA). After 4 h, the liquid was aspirated from each well and the wells were rinsed three times with 200 µl of sterile water. Control biofilms were exposed to sterile water. Then, 175 µl of mouthrinse or toothpaste (Crystal Violet Solution; Sigma-Aldrich, St Louis, MO, USA) was added to each well. After 30 min at room temperature, excess CV solution was removed by washing the plates four times with 200 µl of ultrapure water. Finally, bound CV was released by adding 225 µl of ethanol/acetone [80:20% (v/v)] to each well (9) and the OD 575 was measured.

**Evaluation of biofilm growth**

In order to quantitatively evaluate the amount of biofilm growth, wells with biofilm were rinsed three times with 200 µl of PBS, and 175 µl of a 2.3% (w/v) CV solution (Crystal Violet Solution; Sigma-Aldrich, St Louis, MO, USA) was added to each well. After 30 min at room temperature, excess CV solution was removed by washing the plates four times with 200 µl of ultrapure water. Finally, bound CV was released by adding 225 µl of ethanol/acetone [80:20% (v/v)] to each well (9) and the OD 575 was measured.

<table>
<thead>
<tr>
<th>Products</th>
<th>Main active components</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouthrinse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corsodyl</td>
<td>Chlorhexidine digluconate 0.2%</td>
<td>GlaxoSmithKìne, Middlesex, UK</td>
</tr>
<tr>
<td>Listerine</td>
<td>Alcohol, phenols, and essential oils</td>
<td>Pfizer Consumer Healthcare, Morris Plains, NJ, USA</td>
</tr>
<tr>
<td>Meridol</td>
<td>Amine fluoride, stannous fluoride</td>
<td>GABA Group, Basel, Switzerland</td>
</tr>
<tr>
<td>Crest pro health mouthrinse</td>
<td>Cetylpyridinium chloride</td>
<td>Procter &amp; Gamble, Cincinnati, OH, USA</td>
</tr>
<tr>
<td>Toothpaste</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prodent coolmint</td>
<td>Sodium fluoride, sodium lauryl sulphate (SLS)</td>
<td>Sara Lee Household &amp; Bodycare, Exton, PA, USA</td>
</tr>
<tr>
<td>Crest cavity protection</td>
<td>Sodium fluoride, SLS</td>
<td>Procter &amp; Gamble, Cincinnati, OH, USA</td>
</tr>
<tr>
<td>Colgate total</td>
<td>Triclosan, polyvinyl methylether maleic acid, sodium fluoride, SLS</td>
<td>Colgate-Palmolive Company, Piscataway, NJ, USA</td>
</tr>
<tr>
<td>Zendium classic</td>
<td>Sodium fluoride, colostrum, lactoperoxidase, lysozyme glucose oxidase, amylglucosidase</td>
<td>Sara Lee Household &amp; Bodycare, Exton, PA, USA</td>
</tr>
<tr>
<td>Crest pro health toothpaste</td>
<td>Stannous fluoride, sodium hexametaphosphate, SLS</td>
<td>Procter &amp; Gamble, Cincinnati, OH, USA</td>
</tr>
<tr>
<td>Oral B pro expert</td>
<td>Sodium fluoride, stannous fluoride, Sodium hexametaphosphate, SLS</td>
<td>Procter &amp; Gamble UK, Weybridge, UK</td>
</tr>
</tbody>
</table>
using a microtitre plate reader (FLUOstar OPTIMA; BMG LABTECH, Offenburg, Germany).

Acute action was concluded from a reduction in the amount of biofilm after growth of the exposed biofilms (OD\text{exposed}) with respect to the control (water treatment; OD\text{control}), while substantive action was concluded from a reduction in the amount of biofilm grown on a dead biofilm (OD\text{exposed}) with respect to a water control. Accordingly, for acute and substantive action, the percentage reduction in biofilm growth was concluded using the following equation

\[
\% \text{Reduction} = \left( \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{exposed}}}{\text{OD}_{\text{control}}} \right) \times 100\%
\]

All OD values were corrected for possible adsorption of toothpaste slurries, mouthrinses, and CV at the walls of the wells, while for substantive action, OD values were also corrected for CV absorption in the dead biofilm.

**Statistical analysis**

All reductions in biofilm growth after exposure to (diluted) mouthrinses or to toothpaste slurries were compared with the control (i.e. water exposure) using the unpaired Student’s t-test, ANOVA, and least-significant difference (LSD) post-hoc test. For statistical analysis, SPSS 16.0 software for Windows (SPSS, Chicago, IL, USA) was used. P-values of < 0.05 were considered to be statistically significant.

**Results**

**Acute action of antimicrobial toothpastes and mouthrinses**

All undiluted mouthrinses and 25\% (w/w) toothpaste slurries reduced the continued growth of an exposed biofilm significantly (P < 0.05), compared with the control, which is indicative of acute action by killing or disruption (Figs 1 and 2). Corsodyl was effective, even at a 1\% dilution (Fig. 1), whereas Listerine and Crest Pro Health rinse lost efficacy at a concentration of 1\% (v/v). Meridol showed loss of efficacy at a dilution of 50\% and even stimulated biofilm growth when used at 10\% and 1\% (v/v) dilutions (P < 0.05).

Toothpaste slurries of Prodent Coolmint were the only slurries that yielded significant reductions in continued growth of exposed biofilms over the entire dilution range (Fig. 2), as most toothpaste slurries lost efficacy at 0.25\% (w/w) dilution. Note that Crest Pro Health paste and Crest Cavity Protection stimulated growth at their highest dilutions (only statistically significant for Crest Cavity Protection at P < 0.05). Zendium Classic lost efficacy and stimulated growth at a 12.5\% (w/w) dilution, although the stimulation of growth was not statistically significant.

**Substantive action through absorption in, and release from, biofilms**

Biofilm growth on dead biofilms after absorption of mouthrinse or toothpaste components was significantly (P < 0.05) reduced for all three antimicrobial products, demonstrating absorption and release of bio-available antimicrobials at effective concentrations (Fig. 3). Growth reduction after absorption of Corsodyl was less than achieved by Crest Pro Health rinse or Crest Pro Health toothpaste slurry, although this effect was only significant for the rinse. The growth reductions observed resulting from the absorption and release of components in 24-h-old dead biofilms were very similar to those observed in the 4-h-old initial biofilms.

**Discussion**

Conceivably, antimicrobial agents can influence oral biofilm formation in different ways, for example, by preventing bacterial adhesion to surfaces, affecting bacterial viability or by disrupting an existing biofilm (4).
Here, we studied acute action on organisms in oral biofilms and substantive action through the absorption and release of antimicrobials in and from dead biofilms. All antimicrobial products included showed acute action when applied at full-strength (mouthrinses) or in 25% (w/w) toothpaste slurries. Moreover, antimicrobial absorption by, and release of bioavailable antimicrobials from, dead biofilm in effective concentrations was demonstrated to contribute to substantive action for three selected antimicrobial products, viz. Corsodyl, Crest Pro Health mouthrinse, and Crest Pro Health toothpaste.

The use of dilution series of mouthrinses and toothpaste slurries to assess the acute action efficacy of these oral antimicrobial products is new. Mouthrinses are always used full-strength and clinically only undergo minor dilution in saliva. For toothpastes, 25% (w/w) slurries are the standard for in vitro evaluations (10), as based on the average amount of toothpaste used during brushing, and the total amount of fluid in the oral cavity. Yet, there are large individual variations, and during brushing further dilution will occur as a result of salivation and swallowing (11). Moreover, most people add water to the toothbrush. Therewith, the decrease in growth reduction of exposed biofilms upon dilution is indicative of the antimicrobial efficacy of the product in clinical use. Most antimicrobial products evaluated for their acute action remain effective up to substantial dilutions, which is in line with clinical observations on their plaque-control efficacy.

In Corsodyl, chlorhexidine is the component responsible for clinical antimicrobial efficacy, while for Listerine, Meridol, and Crest Pro Health rinse the responsible components are essential oils, the combination of stannous- and amine-fluoride, and bioavailable cetylpyridinium chloride, respectively (3, 5, 12). In most toothpaste formulations, fluoride and sodium lauryl sulphate contribute to a certain degree of acute action, as found in this study for Prodent Coolmint and Crest Cavity Protection. Toothpastes with antimicrobial claims include Colgate Total, Crest Pro Health, and Oral B Pro Expert (12, 13) and indeed these toothpaste slurries cause acute action up to substantial dilutions. Note that Oral B Pro Expert differs only from Crest Pro Health with respect to an increased fluoride level (1,450 p.p.m. fluoride in Oral B Pro Expert vs. 1,100 p.p.m. fluoride in Crest Pro Health). The higher fluoride concentration, also present in Prodent Coolmint, is probably the reason why these products still show growth reduction at the highest dilution. Zendium Classic, based on colostrums and enzymes enhancing the host defense system, loses antimicrobial efficacy at a dilution of only 12.5%. Clinical studies (14) have shown that the antimicrobial effect of enzyme-containing toothpastes was minor.

Fig. 2. Percentage reduction in continued growth of an initially adhering, 4-h-old biofilm, after exposure for 2 min to different dilutions of toothpaste slurries, compared with a control (i.e. exposure to sterile water). Note that negative reductions denote increased growth with respect to the control. *A significant (P < 0.05) reduction compared with the control. The SE values were calculated from the results obtained from 18 experiments.

Fig. 3. Percentage reduction in biofilm growth on dead, 4-h- and 24-h-old biofilms, after absorption of antimicrobial components from mouthrinses (30 s absorption) or a toothpaste slurry (2 min absorption) compared with a control (i.e. exposure to sterile water). The asterisk (*) indicates a significant difference with respect to the control, while the hash (#) indicates a significant difference compared with Corsodyl. The SE values were calculated from the results obtained from 18 experiments.
as has also been shown by Aranha et al. (17). However, the stannous-containing formulation, complemented with a high concentration of sodium fluoride (Oral B Pro Expert), does not show growth stimulation, which confirms that fluoride too can exert antimicrobial effects (4).

In addition to their acute action efficacy, components from the antimicrobial mouthrinses Crest Pro Health and Corsodyl and the toothpaste Crest Pro Health are absorbed by dead biofilms and remain bioavailable for subsequent release and substantive action on biofilm organisms growing on the exposed biofilm. Hitherto, the substantiveity of oral antimicrobial products has been evaluated predominantly in vivo (6, 10) and suggested to be caused by the adsorption of antimicrobials to the abundantly available soft tissue surfaces in the oral cavity from which they are slowly released to yield substantiveity (3, 5). This in vitro study confirms our clinical findings that plaque can act as a reservoir for oral antimicrobials (8) and identifies the absorptive capacity of oral biofilm as a factor, contributing to the prolonged activity of antimicrobial healthcare products. Substantivity through absorption in oral biofilms can only be effective if the absorbed antimicrobials remain bioavailable (i.e. can be released). In this respect it is interesting that substantiveity of the chlorhexidine-containing rinse, through absorption in and release from the oral biofilm, is smaller than of the other products, possibly because this large cation had become irreversibly trapped in the biofilm, which consists of negatively charged bacteria (18). We could not establish here with adequate statistical significance that thicker, 24-h-old biofilms had a greater absorption capacity than 4-h-old biofilms.

In conclusion, antimicrobial toothpastes and mouthrinses are able to act acutely on biofilm organisms in vitro, even when they are considerably diluted, as in clinical situations. Furthermore, antimicrobials from mouthrinses and toothpaste slurries may remain bioavailable in a dead biofilm, resulting in prolonged killing of new biofilm, therewith providing evidence in support of a new mechanism of substantive action. This is of clinical importance because patients are usually not able to completely remove all oral biofilm by tooth cleaning. The new mechanism outlined enables them to benefit from active toothpaste and mouthrinse that is absorbed in biofilm left after cleaning.

Conflicts of interest – The authors declare that there are no conflicts of interest in this study.

References