The effect of triclosan toothpaste on enamel demineralization in a bacterial demineralization model

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Triclosan has been incorporated into toothpaste to enhance inhibitory effects on bacterial metabolism in dental plaque. Many studies have confirmed these effects by showing a reduction of accumulation of dental plaque, gingivitis and calculus. However, there is no evidence for triclosan having an inhibitory effect on the dental plaque-induced demineralization of the dental hard tissues. Therefore, the effect of 0.3% triclosan added to non-fluoride and fluoride toothpaste was tested in an in vitro model, in which bovine enamel specimens were to be demineralized by acids produced in overlaying Streptococcus mutans suspensions. In a first set of experiments the toothpastes were added to the S. mutans suspensions at 1:100, 1:1000 and 1:10,000 (w/v) dilutions. After 22 h incubation at 37°C the suspensions were removed and assessed for calcium and lactate content, and pH. In this set of experiments, triclosan had no additive protective effect to the non-fluoride or fluoride toothpaste. In a second set of experiments, the enamel specimens were immersed daily for 3 min in 30% (w/v) slurries of the toothpastes before the 22 h incubation with the S. mutans suspensions. Under these conditions, triclosan showed an additional protective effect compared with non-fluoride toothpaste at a low concentration of S. mutans cells (0.07 mg cells dry weight per 600 µL suspension). It is concluded that the enamel surface may act as a reservoir for triclosan, which may protect the enamel surface against a mild acid attack. In combination with fluoride, however, as in toothpaste, triclosan has no additional protective effect against demineralization.
of triclosan increased the protection of non-fluoride and fluoride toothpaste against demineralization. This was studied under the simulated conditions of enamel or dental plaque being the oral binding site. The experiments were carried out with a Streptococcus mutans strain. S. mutans is an acidogenic and aciduric oral microorganism that is associated with the development of dental caries.\textsuperscript{17,18}

\section*{Materials and methods}

\subsection*{Demineralization model}

A schematic representation of the demineralization model is given in Figure 1.\textsuperscript{19} Enamel specimens were embedded in methylmethacrylate resin (Vertex, Dentimex, Zeist, The Netherlands) and fixed in polypropylene tubes (Greiner, Nürtingen, Germany), which could be closed by a screw-cap. On top of the specimens 600 \( \mu \)L of acidogenic S. mutans suspension was pipetted. The suspensions were prepared by mixing thawed stock cultures of S. mutans C180-2\textsuperscript{20} with YEPC (0.5\% yeast extract, 0.1\% peptone, 0.85\% NaCl, 0.05\% L-cysteine HCl and 10\% w/v glycerine), 0.75\% (w/v) agarose and 50 mmol/L glucose. The final suspensions contained 0.66 or 0.07 mg dry weight S. mutans cells/600 \( \mu \)L (OD\textsubscript{660} = 3 and 0.3, respectively). After application of the suspensions the devices were incubated for 22 h at 37°C.

Then, the suspensions were removed and stored at \(-80°C\) until they were assessed for calcium and \( \ell \)-lactate. Before applying the bacterial suspensions, the enamel specimens were exposed for at least 1 h to a 26 W ultraviolet source with a wavelength of 254 nm (UVSL-58, Ultra-violet Products, San Gabriel, CA, USA) at a distance of 10 cm for surface sterilization.

\subsection*{Preparation of enamel specimens}

Samples were cut perpendicularly to the buccal surface of freshly extracted bovine incisors with a hollow drill (diameter 6 mm). The specimens were carefully embedded in methylmethacrylate resin, leaving the outer surface of the enamel specimens free. These surfaces were ground flat using silicon carbide abrasive paper from grit 200 to grit 600. The surface area of all specimens (approximately 22 mm\textsuperscript{2}) was measured with an image analysis method. Subsurface lesions were then formed as follows; the specimens were placed in a glass tray with the outer surface up and suffused with 150 mL of 8\% methylcellulose gel. After 24 h, filter paper was placed on top of the gel and 150 mL of 0.1 M lactic acid at pH 4.6 was poured over it. The tray was covered and incubated at 37°C for 1 week.\textsuperscript{21} After the lesions were formed, the specimens were incubated twice for 22 h with acidogenic S. mutans suspensions as described above. The calcium content of the suspensions was measured. The specimens were then allocated to experimental groups in such a way that the mean calcium loss per surface area during these two pre-incubations did not differ between the groups. Each group consisted of five specimens.

\subsection*{Bacteriological procedures}

The bacterial strain used was S. mutans C180-2. All S. mutans cells to be used in one set of experiments were grown in a single batch overnight culture in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA). Cells were harvested in the late-exponential phase by centrifugation (30 min, 3000 \( \text{g} \), 4°C), resuspended in YEPC and incubated at 37°C, continuously adjusting the pH to 7 until titration was no longer necessary, indicating that the endogenous carbohydrate reserves were depleted. Then the cells were washed three times, resuspended in YEPC and stored at \(-80°C\) until use in the demineralization model.

\subsection*{Toothpastes}

A paste with 0.3\% triclosan (triclosan paste), a paste with 0.24\% NaF (NaF-paste) and a paste with 0.24\% NaF + 0.3\% triclosan (triclosan–NaF paste) were used in addition to a control paste without any therapeutic addition (non-F paste). All toothpastes had the same composition except for the therapeutic addition. They did not contain propylene glycol, which is known to be incompatible with
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The pastes were kindly donated by Dr V enema of Sara L ee/D E, H & B C R esearch (A mersfoort, The Nether-lands).

Experimental protocols

Two sets of experiments were designed. In the first set of experiments, the specimens were incubated successively in bacterial suspensions, in which the toothpastes were diluted w/v 1:10,000, 1:1000 and 1:100, respectively. Between the incubations with toothpaste the specimens were incubated for 22 h with a toothpaste-free S. mutans suspension to measure any carry-over effect. In the second set of experiments the enamel specimens were treated for 3 min with 5 mL of 30% (w/v) slurries of the experimental toothpastes; followed by rinsing for 30 s in sterile de-ionized water to remove the toothpaste. The excess of water was soaked off with an absorbing tissue and the S. mutans suspensions were immediately applied. This experiment was continued for 5 days, with daily treatments with the toothpaste slurries.

Calcium and lactate measurements

The stored suspensions were thawed and centrifuged (5 min, 16,000g, E ppendorf, H amburg, G ermany) and samples of the supernatant were taken for the determination of calcium and lactate. Calcium was measured by atomic absorption spectroscopy after the samples were diluted in lanthanum reagent [0.5 wt% La(NO$_3$)$_3$.6H$_2$O (Merck, Darmstadt, Germany) in 0.05 M HCl] to suppress phosphate interference. The reproducibility and accuracy of this procedure is very good with an error of $\leq 3\%$. The detection limit is approximately 0.02 mmol/L. L-lactate was measured enzymatically. The reproducibility and accuracy of the procedure is good with a coefficient of variance of $<4 \pm 3\%$.

Statistical analysis

The data from each series of experiments were analysed using A nova with 95% confidence limits. If this analysis revealed differences, then D uncan's multiple range test was used to identify homogeneous subsets of groups with P set at 0.05.

Results

Figures 2 and 3 show the calcium loss from the enamel specimens when the toothpastes were diluted in the bacterial suspensions. It shows that all toothpastes could protect the enamel specimens. The maximum dilution for protection was found to be between $10^{-3}$ and $10^{-4}$. Triclosan had no additional effect when it was added either to the non-fluoride or to the fluoride toothpaste. A II toothpastes were more effective when the suspension contained fewer bacteria. A II toothpastes inhibited lactate production (data not shown). A gain, triclosan added to either the non-fluoride or the fluoride paste did not increase the inhibitory effect on lactate production. The intervening incubations without added toothpastes revealed that there was no carry-over effect.

Figures 4 and 5 show the cumulative calcium loss when the specimens were treated daily with the toothpastes before demineralization. The Table shows the means ± s.d. of the cumulative calcium data after five days. In addition, the levels of significance within and between the homogeneous subsets of the experimental groups are given as found by D uncan's multiple range test. Under the severest demineralization condition (i.e. the highest bacterial density), only the fluoride toothpastes reduced the calcium release. Under the milder demineralization condition, triclosan seemed to have an additive effect to the non-fluoride, but not to the fluoride toothpaste. No significant effect of
Discussion

After application, a therapeutic agent will be cleared from the mouth. The clearance is often biphasic with a rapid initial decrease of the oral concentration when the non-bound or loosely bound fraction of the compound is cleared, followed by a slower decrease when a more firmly bound fraction of the compound is released from the oral retention sites. The clearance of triclosan takes approximately 8 h. The antimicrobial effect during this period may consist of a variety of effects at above and at sub-MIC concentrations. The effects may be different since bacterial species may vary in their sensitivity to an inhibitor.

Table. Means ± s.d. of the cumulative calcium data over five experimental days and the significance levels within and between the homogeneous subsets of the experimental groups found with the Duncan’s multiple range test in the experiments where the enamel specimens were pretreated with toothpaste.

<table>
<thead>
<tr>
<th>Second set of experiments specimens pretreated</th>
<th>Cumulative calcium data over 5 days (mean ± s.d.)</th>
<th>Significance levels within and between homogeneous subsets of groups as found with the Duncan's multiple range test</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. mutans suspensions at OD&lt;sub&gt;660&lt;/sub&gt; = 3</td>
<td>No toothpaste: 21.8 ± 0.7; Non-F-paste: 21.8 ± 0.7; Triclosan: 20.9 ± 0.7</td>
<td>NS, P = 0.66 ( Significant P ≤ 0.05)</td>
</tr>
<tr>
<td>(see Figure 4)</td>
<td>NaF: 16.0 ± 0.3; Triclosan-NaF: 16.6 ± 0.6</td>
<td>NS, P = 0.58</td>
</tr>
<tr>
<td>S. mutans suspensions at OD&lt;sub&gt;660&lt;/sub&gt; = 0.3</td>
<td>No toothpaste: 15.5 ± 0.8; Non-F-paste: 16.5 ± 0.4</td>
<td>NS, P = 0.5</td>
</tr>
<tr>
<td>(see Figure 5)</td>
<td>Triclosan: 12.9 ± 0.8; NaF: 11.3 ± 0.3; Triclosan-NaF: 10.0 ± 0.3</td>
<td>NS, P = 0.08</td>
</tr>
</tbody>
</table>

NS, not significant.
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under the various conditions. The effects at sub-MIC concentrations may be dominant, as these concentrations will last longest during oral clearance. The minimum concentration of triclosan to inhibit growth of mutans streptococci is 0.001%. However, at these and lower concentrations triclosan affected the composition of a mixed continuous growth culture, being particularly inhibitory against S. mutans. In our experiments, both above and sub-MIC concentrations of triclosan were added to the bacterial suspensions, but triclosan did not improve the protective effect of the non-fluoride or fluoride toothpaste at any of the levels used. A reason for this may be that any antimicrobial effect of triclosan was not complementary or additive to that of other antimicrobial compounds of the toothpastes or to the effect of fluoride.

Previously, Gilbert & Watson showed that triclosan binds to saliva-coated enamel in a time- and dose-dependent way. During a 1 min exposure to a 0.2% triclosan toothpaste enough triclosan was absorbed to inhibit subsequently the growth of Escherichia coli in an in vitro zone of inhibition assay. A apparently, also in our second set of experiments, triclosan adsorbed to the enamel specimens to contribute subsequently to the protection of the enamel specimens under the mildest attack. However, when the toothpaste also contained fluoride the additional protective effect of triclosan could not be demonstrated.

In pH-stat experiments, triclosan inhibited the acid production of mutans streptococci. The mechanisms of action are not entirely clear. Triclosan adsorbs to the bacterial cell and increases the cell permeability. High bactericidal concentrations cause membrane lesions that permit leakage of the cellular content. There is a linear correlation between inhibition of acid production and adsorption of triclosan by S. mutans cells. Therefore, the effect of triclosan is in fact not dependent on the concentration of triclosan in solution but depends on the ratio between the amount of triclosan and the number of cells that have to be inhibited. This also explains why there was a triclosan effect in the assays with the lowest numbers of mutans streptococci but not in those with the highest numbers.

Some toothpastes are formulated with triclosan in combination with zinc citrate. The mode of action of both compounds is different and additive inhibitory effects have been demonstrated in pH-stat experiments, mixed culture chemostat studies and in clinical studies to control plaque and gingivitis. In other toothpastes triclosan is formulated with a copolymer of vinylmethylether maleic acid. This combination has proven to be more effective against oral bacteria and to increase the uptake of triclosan by hydroxyapatite. Therefore, it may be expected that in the assays used in this study toothpaste with both zinc citrate and triclosan or with triclosan and the copolymer are more protective than the present triclosan toothpastes.

In conclusion, the present study was designed to measure the effect of triclosan formulated in a non-fluoride or fluoride toothpaste in an in vitro bacterial demineralization model. A n effect was found when enamel was used to simulate the oral binding site and when the demineralization conditions were relatively mild. Under more severe conditions or in combination with fluoride no effect was observed. When the bacterial suspension was used to simulate the oral reservoir triclosan did not increase the efficacy of the toothpastes, probably because a possible antimicrobial effect was not additive to the effect of other antimicrobial compounds of the toothpaste or to fluoride.

References


19. Van Loveren, C., Spitz, L. M., Buijs, J. F., Ten Cate, J. M. & Eisenberg, A. D. (1991). In vitro demineralisation of enamel by F-sensitive and F-resistant mutants streptococci in the presence of 0, 0.05 or 0.5 mmol/L NaF. *Journal of Dental Research* 70, 1491–6.


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