The mechanism of action of chlorhexidine
A study of plaque growth on enamel inserts in vivo


Abstract. Controversy exists concerning the mode of action of chlorhexidine in plaque inhibition. This study attempted to determine whether an oral reservoir of chlorhexidine was necessary for plaque inhibition. Plaque growth on enamel under the influence of topically applied or rinsed chlorhexidine was closely monitored by clinical scoring, bacterial culturing and scanning electron microscopy. Thus, 3 subjects wore removable acrylic appliances containing enamel inserts. In the first regimen, inserts on one side of the appliances were exposed to 0.2% chlorhexidine and on the other, water for 1 min twice a day for 14 days. In the second regimen, subjects rinsed with 0.2% chlorhexidine for 1 min twice a day for 14 days with the appliances in situ. Results demonstrated that plaque growth assessed by the 3 study methods was very small on chlorhexidine-treated inserts by comparison with water-treated specimens. Importantly, inserts treated with chlorhexidine topically or by rinsing could not be distinguished by any method of evaluation. It is concluded that chlorhexidine achieves plaque inhibition as a result of an immediate bactericidal action during the time of application and a prolonged bacteriostatic action as a result of adsorption to the pellicle coated enamel surface. Consistent with other clinical studies, it is apparent that a progressively desorbing oral reservoir of antiseptic is not the mechanism by which chlorhexidine achieves plaque inhibition on teeth.

Key words: chlorhexidine; plaque.

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The use of chlorhexidine in the oral cavity has been the subject of numerous investigations. Reviews on plaque control have concluded that to date chlorhexidine, used as a mouth rinse, is the most effective chemical antiplaque agent (Hull 1980, Kornman 1985, Addy 1986). Despite the extensive information on this bisbiguanide antiseptic, controversy still exists concerning the mode of action of chlorhexidine in plaque inhibition. It appears generally accepted that prolonged retention, by adsorption, of the dicationic antiseptic onto oral surfaces, is a major factor determining the plaque inhibitory action of chlorhexidine (Rolla & Melsen 1975). However, whether it is progressive desorption of chlorhexidine from oral surfaces which maintains a bacteriostatic milieu (Gjermo et al. 1975) or simply activity of the chlorhexidine adsorbed to the tooth surface, has not been elucidated. Certainly, chlorhexidine does show considerable affinity for oral structures (Rolla et al. 1970, 1971, Hjel-
very subjective nature of assessing plaque formation is an additional problem. The aim of this study was to follow closely the colonisation of bacteria on enamel in vivo under the influence of topically applied and mouth rinsed 0.2% chlorhexidine gluconate in the hope of explaining the mode of plaque inhibition by chlorhexidine.

**Material and Methods**

3 male, dentate subjects aged 26, 25 and 39 years, all members of the Department of Periodontology, volunteered for the study. All 3 had a high standard of oral hygiene and on examination had four or less mesial papillae bleeding on gentle probing. For each subject, a lower lingual acrylic plate was constructed to extend from the left to the right second molar and was retained by Adams cibrobs on the lower first molars. Square enamel pieces of approximate dimensions 3 mm × 3 mm by 1 mm were cut from freshly extracted human molar teeth. The enamel specimens were cleaned in a 1% hypochlorite solution, ultrasonicated in 20% alcohol and then washed in sterile water before being fixed by the undersurface in the recesses on the mucosal surfaces of the appliances using sticky wax. When the appliances were in place, the enamel inserts lay approximately 1 mm away from the mucosal surface. By the nature of the investigation the clinical aspects could not be performed ‘blind’ without recourse to a totally independent examiner and therefore the two regimens were not randomised to the subject. During both treatment periods, the subjects practiced normal oral hygiene using the same fluoride containing toothpaste with no mechanical cleaning of the appliance. For the first regimen four enamel inserts were placed into each of the left and right-hand recesses. The subjects were asked to wear the appliances continuously for 14 days only removing the appliance for tooth brushing and twice each day to place one side of the appliance, identified by a cross in indelible ink, into a 0.2% chlorhexidine gluconate solution* for 1 min with the other side of the appliance placed into distilled water for 1 min. After both treatments the appliance was to be washed in running cold water for 20 s to remove any

residual antiseptic solution from the chlorhexidine treated side. The morning soaking regimen was supervised and each day the volunteers were questioned by the supervisor to ensure the evening procedure had been performed. After 7 days, 2 enamel inserts were removed from each appliance, 1 from the chlorhexidine treated side and 1 from the water treated side. These enamel inserts were placed separately into 5 ml volumes of 3% glutaraldehyde in Sorensen’s phosphate buffer for one hour. Following this each specimen was washed in a series of alcohols, 70%, 90% and 100%, then critically point dried** using the volatilizing agent 1,1,2-trichloro 1,2,2-trifluorothane (Arklane). Finally, specimens were sputter coated*** with gold for viewing in a scanning electron microscope.

Representative scanning electron photomicrographs were taken of the surface detail of all specimens. Additionally 10 fields at ×700 magnification per specimen were randomly selected and the bacterial density subjectively scored from a ×2100 magnification inset (Figs. 3–7).

Scoring criteria were as follows:

0 = no bacteria or only occasional single bacteria present within the inset;
1 = bacterial colonies covering up to half the inset;
2 = half to complete bacterial coverage of the inset;
3 = dense multi layers of bacterial forms filling the inset.

The 2nd pair of chlorhexidine and water treated enamel inserts were stained with an erythrocyan disclosing solution and the excess dye removed by washing under running cold water. Any plaque present was then scored according to the following criteria:

0 = no plaque;
1 = isolated specks of disclosed plaque;
2 = partial coverage of the insert with disclosed plaque;
3 = total coverage of the insert with disclosed plaque.

Each insert was then scraped with a small, sharp sterile spoon excavator. The scrapings were made using firstly

overlapping parallel horizontal strokes followed by overlapping vertical strokes until it was judged the whole surface had been instrumented. In the case of specimens revealing disclosed plaque removal was continued until all visible material had been removed. Periodically the material recovered on the instrument was suspended in a 2 ml volume of sterile phosphate buffer. After thorough mixing of the suspension by drawing and expelling the fluid through a 25 gauge needle on a 2 ml syringe, 10 fold dilutions ranging from 0 to 1/10,000 were made in phosphate buffer. Three 0.1 ml aliquots of each dilution were then spread on individual fastidious anaerobic blood agar plates and incubated anaerobically at 37°C in an anaerobe cabinet for 7 days. Bacterial counts were made from the appropriate plates using an illuminated colony counter. The mean count for each specimen was obtained by taking the average of the counts from the three respective plates for the appropriate dilution. Actual counts were then calculated using the dilution factors and expressed as colony forming units per ml. The scraped inserts were removed from the appliances and processed for scanning electron microscopy as described previously. The appliances were then worn for the second 7 days and the same methods employed for study of the plaque on the surface by scanning electron microscopy, plaque scoring and bacterial counting. The second regimen was commenced at least 9 days after the first topical regimen. The appliances were cleaned by brushing with a tooth brush and water, followed by ultrasonication in sterile saline and stored in sterile water during the washout period. Immediately prior to the 2nd regimen enamel inserts were fixed into the appliance as before and these worn for 14 days. The appliances were only removed for tooth brushing of the natural teeth. The volunteers were requested twice each day to rinse with 10 ml of the same 0.2% chlorhexidine gluconate solution for one minute. During rinsing, volunteers were instructed to raise the appliance off the lower teeth to ensure that the chlorhexidine solution contacted the lingual mucosal surfaces of the appliance. Supervision was as with the soaking regimen. 7 and 14 days inserts were removed from the left and right hand recesses and processed as before for scanning electron microscopy. Additionally, the inserts were scored for

* Corsodyl mouthwash. ICI Pharmaceuticals, Alderley Edge, Macclesfield, UK.

** Polaron E. 3000 critical point dryer – Polaron Equipment Ltd., Watford, Herts, UK.


**** I.S.I. Super III A. scanning electron microscope. ISI Technical Services, Kenyon House, Stockport, UK.
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plaque and scraped for bacterial culturing and counting. All techniques used were as described for the topical regimen. Thus, in summary, plaque scores and subsequent bacterial counts were taken from 3 inserts, one from each subject, at each time period, for each of the three regimens, a total of 18 specimens. Scanning electron microscopy was of a second group of 18 inserts obtained from each subject, at the respective time periods for the 3 regimens.

Results

At both the 7- and 14-day appointments of the topical regimen, all 3 volunteers stated there was a distinct difference in the feel to the tongue of the surface of the appliances. Thus, the chlorhexidine side remained smooth and the control side was reported as being rough. Visibly the surface detail differences reported could be seen and a very distinct line between the chlorhexidine and control sides was apparent. During the rinsing regimens at the 7- and 14-day appointments, volunteers reported the appliance surface to be smooth to the tongue and this was confirmed visually.

The plaque score for each individual recorded from the inserts at day 7 and 14 of the topical and rinsing periods are given in Table 1. At 7 days, 2 out of 3 volunteers had complete plaque coverage of inserts exposed to water only. By 14 days, all volunteers had complete plaque coverage of these control specimens. For the enamel inserts exposed to topical or rinsed chlorhexidine, there was either no plaque or only isolated specks of plaque disclosed on the surface at both the 7- and 14-day appointments. No differences in plaque inhibition as scored by the method, could be detected for topical or rinsed chlorhexidine. Individual subject logarithmic bacterial counts from plaque scraped off the enamel surface following the topical and rinsing regimens are shown in Fig. 1. The log mean counts and standard errors of the three subjects at days 7 and 14 for each regimen is given in Fig. 2. Since the inserts were mechanically and chemically sterilised prior to insertion into the appliances the day 0 count was considered as zero. For each individual there was a very large difference in counts from enamel exposed to either topical or rinsed chlorhexidine and those exposed to water. Counts from water treated specimens were essentially similar for each subject ranging from $10^4$ to $10^5$ bacteria. Interestingly, there was also little difference in counts taken at 7 and 14 days. Topical and rinsed chlorhexidine reduced bacterial counts on the surfaces by between 1000 and 100,000 fold. Although there was individual variation in counts recovered from chlorhexidine, topical and rinsed specimens the pattern was not consistently in favour of either. Mean counts for the volunteer group (Fig. 2) revealed almost exactly the same bacterial counts for topical and rinsed chlorhexidine which were almost 10,000 fold lower than the mean count for water treated specimens. As for the control specimens, there was little change in mean counts for chlorhexidine treated speci-

<p>| Table 1. Topical versus mouthrinising with chlorhexidine; plaque scores |
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W = water. R = rinsed chlorhexidine. TC = topical chlorhexidine.

Fig. 2. Mean bacterial counts from enamel inserts exposed to water and to topical and rinsed 0.2% chlorhexidine.
Table 2. Subjective scores of bacterial colonisation of enamel inserts at 7 and 14 days during chlorhexidine or water treatments.

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Thus on all specimens, areas of apparently bacteria free enamel were seen (Fig. 5). The extent of these zones varied between specimens and between subjects and in some cases extended over nearly the whole insert. Bacterial deposits were nevertheless always seen particularly in what appeared to be grooves or surfaces depressions on the enamel surfaces (Fig. 6). The bacterial deposit forming under the influence of chlorhexidine, either topical or rinsed, appeared primarily made up of coccoid forms and in many cases the bacteria
forming this plaque differed from the control plaque having indistinct cell boundaries (Fig. 7). Scanning electron photomicrographs revealed that most, but not all, of the deposit on the chlorhexidine treated specimens was removed by scraping. For control specimens bacterial deposits were still clearly apparent with numerous furrows running horizontally and vertically through the plaque layer (Fig. 8).

The subjective scores of bacterial density from 10 randomly chosen zones per specimen are shown in Table 2. For water treated specimens most of the zones studied were scored as dense multilayers of bacterial forms filling the scanning electron microscopic insert (score 3). Bacterial free zones (score 0) were occasionally seen at Day 7 but none were apparent amongst selected fields at day 14. Topical or rinsed chlorhexidine treated specimens received quite different scorings from water controls. Most randomly chosen sites from specimens at both day 7 day 7 and 14 received a score 0. Nevertheless, some zones received scores 1, 2 or 3, indicating occasional plaques of varying bacterial densities.

Discussion

This study has shown, using clinical indices, bacterial cultures and scanning electron microscopy that the plaque inhibition achieved by the topical application of chlorhexidine was equal to that by rinsing with 0.2% chlorhexidine. This finding is in agreement with a related clinical study using topically applied and rinsed chlorhexidine (Addy & Moran 1983) and contradicts a similar investigation (Waaler & Rolka 1985). However, the latter study employed a 0.1% chlorhexidine preparation and, perhaps more importantly, investigated effects on plaque growth, enhanced unnaturally by sucrose rinses. Moreover, sucrose is known to inhibit the antibacterial activity of chlorhexidine (Davies 1973).

The adsorption isotherms for chlorhexidine uptake onto acrylic and hydroxyapatite suggest the formation of a stable molecular monolayer (Rolla et al. 1970, Emilson et al. 1973, Addy & Moran 1985). The quantity of chlorhexidine adsorbed from the 0.2% solution onto the one side of the lower acrylic appliance would have been very small (Bonesvoll & Olsen 1974, Addy & Roberts 1981, Moran & Addy 1985) particularly as the appliance was rinsed under water before replacement. Thus, we assume that the prolonged effect against plaque growth on the enamel inserts came from the chlorhexidine adsorbed to the specimens. Certainly, there appeared no evidence of a carry-over of chlorhexidine to the control side treated with water. Thus, plaque accumulation on the control specimens was rapid and as expected. Furthermore, on the appliances, a very clear demarcation between plaque free and plaque coated surfaces was noted by the assessor and the subjects, and this demarcation coincided with the immersion level of the chlorhexidine solution.

Salivary levels of chlorhexidine after rinsing have been demonstrated (Bonesvoll et al. 1974); however, as stated, the measurement methods employed did
not necessarily permit the conclusion that the levels resulted from desorption of active chlorhexidine from oral surfaces. Nevertheless, until there is further information it is still possible that the effects observed following topical chlorhexidine treatment of the appliances, were partly due to desorbed antiseptic influencing micro-organisms in close proximity to the inserts including those on the oral mucosa. Against this must be that the total dose of chlorhexidine on one side of the acrylic appliance would have been minute (Addy & Roberts 1981, Moran & Addy 1985). Even were the total dose to desorb rapidly the concentration produced locally in saliva would be below the minimum inhibitory concentration of many oral bacteria (Addy & Wright 1978).

The results of this study also help to explain why some antiseptics with similar activity in vitro to chlorhexidine exert little antiplaque activity. Thus, some antiseptics are either not adsorbed for prolonged periods to oral surfaces including the teeth, or exert minimal antimicrobial action once adsorbed (Addy & Roberts 1981, Moran & Addy 1984, 1985). Moreover the activity of some compounds is vitiated in the oral environment (Addy & Wright 1978, Roberts & Addy 1981).

The oral retention of chlorhexidine after rinsing has been determined as approximately one third of the 20 mg dose contained in 10 ml of a 0.2% mouth rinse (Rolla et al. 1971, Gjermo et al. 1974). Most of this appears to be retained on mucosal surfaces, however, from this study, such a deposit of chlorhexidine appears to play little if any rôle in plaque inhibition on the teeth. This is not to suggest that alternative delivery systems are necessarily indicated, since mouth rinses are still perhaps the most easy and acceptable vehicles for many topical preventive and therapeutic agents. Additional benefits from the use of antiseptic mouth rinses may be obtained and include degerming of the oral mucosa and reductions in salivary bacterial numbers (Addy & Wright 1978, Schiott et al. 1979).

In retrospect, despite the useful information gained from the oral retention studies for chlorhexidine (Jensen & Christensen 1971, Rolla et al. 1971, Bonesvoll et al. 1974) it is surprising that the slowly desorbing reservoir theory for plaque inhibition (Gjermo et al. 1974) was proposed. Much of the available data contradicts directly or indirectly such a theory, demonstrating that plaque inhibition with chlorhexidine still occurs when either the antiseptic was only applied to the teeth and/or when the dosage used was considerably below that necessary to produce levels of retention reported after rinsing with a 0.2% solution (Davies et al. 1970, Davies & Hull 1973, Hamp et al. 1973, Flotra 1973, Usher 1975, Cutress et al. 1977, Reed et al. 1977, Dever 1979, Dever et al. 1982, Addy & Moran 1983, Francis et al. 1987, Kalaga et al. 1987).

As with studies in vitro (Tanzer et al. 1979, Reed et al. 1981), the scanning electronmicroscopy findings indicate that chlorhexidine does not appear to inhibit bacterial attachment but delays bacterial growth by a prolonged surface bacteriostatic action. Specimens exposed to topically applied or rinsed
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Fig. 6. Appearance of enamel after 14 days topical chlorhexidine showing isolated areas of bacterial growth confined to surface depressions. Magnification x700, insert x2100, small bar 5 μ.

colorhexidine revealed bacterial plaques. However, and consistent with the low bacterial counts recovered from cultures, many appeared to contain non-viable cells. It was interesting to note also that despite the visual presence of, in some cases, considerable numbers of organisms after topical or rinsed chlorhexidine, specimens did not stain appreciably with the disclosing dye.

This study closely examined in vivo bacterial colonisation of enamel surfaces under the influence of chlorhexidine. In conclusion, consistent with clinical studies, plaque inhibition appears not dependent on the slow desorption of chlorhexidine from oral sites to produce a prolonged bacteriostatic environment. It is proposed that when mouthrinsed or applied topically to the teeth, chlorhexidine produces an immediate, probably short lived bactericidal effect. Following this there is a prolonged bacteriostatic action solely dependent on antiseptic adsorbed to the pellicle coated tooth surface. Despite these observations, mouth rinsing would appear, for most individuals, the best method of delivery for agents that rely on substantivity to surfaces for effect from which to exert a prolonged antibacterial or antiplaque action.

Zusammenfassung

Der Mechanismus der Chlorhexidineinwirkung. Eine Studie über die Ansammlung von Plaque auf Schmelzeinlagen in vivo


References


Résumé

Mécanisme de l'action de la chlorhexidine. Étude de la croissance de la plaque sur des fragments d'émail incrustés dans des appareils amovibles, in vivo

Le but de la présente étude était d'établir s'il est nécessaire qu'il existe une réserve buccale de chlorhexidine pour obtenir l'inhibition de la plaque. Les scores cliniques, la mise en culture microbienne et le microscope électronique à balayage ont été utilisés pour surveiller la croissance de la plaque sur l'émail en présence de chlorhexidine administrée soit en applications locales soit en rinçages. Chez 3 sujets, on a posé des appareils amovibles de résine acrylique dans lesquels étaient incrustés des fragments d'émail. Dans le 1er type de traitement, les fragments incrustés d'un côté de l'appareil ont été traités à la chlorhexidine à 0.2% et ceux de l'autre côté ont été traités à l'eau, pendant 1 minute 2 fois par jour, pendant 2 semaines. Dans le 2ème type de traitement, les sujets se rinçaient la bouche pendant 1 minute 2 fois par jour pendant 15 jours, avec de la chlorhexidine à 0.2% avec l'appareil dans la bouche. Les résultats ont montré que la croissance de la plaque a été inhibée.

Fig. 8. Topical water treated control specimen showing enamel surface after scraping. Vertical and horizontal lines are apparent through the plaque layer. Magnification x 70.

plaque, telle qu'elle ressortait des 3 méthodes de mise en évidence, était très faible sur les fragments traités à la chlorhexidine par rapport à la croissance sur les spécimens traités à l'eau. On a particulièrement noté que les fragments traités par applications locales ou par rinçages ne pouvaient être distingués par aucune méthode de mesure. En conclusion, l'inhibition de la plaque obtenue par la chlorhexidine résulte d'une action bactéricide immédiate pendant la durée de l'application, et d'une action bactériostatique prolongée résultant de l'adsorption à la surface d'email recouverte par une pellicule. Conformément aux résultats d'autres études cliniques, il apparaît que le mécanisme grâce auquel la chlorhexidine obtient l'inhibition de la plaque sur les dents n'est pas une désorption progressive de la réserve buccale d'antiseptique.


Address:
M. Addy
Department of Periodontology
Dental School
University of Wales College of Medicine
Heath Park
Cardiff
UK