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Differences between normal and demineralized dentine pretreated with silver fluoride and potassium iodide after an *in vitro* challenge by *Streptococcus mutans*

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Abstract

Background: The application of diamine silver fluoride ($Ag(NH_3)_2F$) and potassium iodide (KI) to demineralized dentine has been shown to inhibit the growth of *Streptococcus mutans*. The purpose of this study was to observe the differences between demineralized and non-demineralized dentine treated with AgF/KI.

Methods: Thirty-five dentine discs were bonded to the bases of 5mL polycarbonate screw top vials which were filled with nutrient medium, sterilized and placed into the overflow from a continuous culture of *S. mutans*. Samples were divided as follows: 10 samples of demineralized dentine; 10 samples of demineralized dentine treated with AgF/KI; 5 samples of non-demineralized dentine; and 10 samples of non-demineralized dentine treated with AgF/KI. Following two weeks connected to the Chemostat, an electron probe microanalysis (EPMA) of percentage weights and penetration depths of calcium, phosphorous silver and fluoride was conducted. Bacterial growth was monitored by taking optical density readings of the growth medium in each vial and outer surfaces of the specimens were examined by scanning electron microscopy (SEM).

Results: AgF/KI treatment of demineralized and non-demineralized dentine prevented biofilm formation and reduced further demineralization by *S. mutans*. AgF/KI treatment of demineralized dentine was more effective in reducing dentine breakdown and the growth of *S. mutans*. Significantly higher levels of silver and fluoride were deposited within demineralized dentine.

Conclusions: A topical treatment with AgF/KI on dentine reduced *in vitro* caries development and inhibited surface biofilm formation. Reduction of *in vitro* caries development and viability of *S. mutans* was more pronounced on the dentine samples that had been demineralized prior to the application of AgF/KI.

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Key words: Dentine, demineralized dentine, silver fluoride, potassium iodide, EPMA, SEM, optical density, *Streptococcus mutans*.

Abbreviations and acronyms: EPMA = electron probe microanalysis; SEM = scanning electron microscopy.

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INTRODUCTION

The arrest of dental caries relies in part upon the formation of a biological seal at the tooth restoration interface even if some carious dentine is left within the preparation.[1] Mertz-Fairhurst *et al.*[2] demonstrated this in a 10-year study with lesions bounded by enamel. The clinical predictability of a restoration would improve significantly if the remaining bacteria were rendered non-viable prior to restoration placement.

Diamine silver fluoride ($Ag(NH_3)_2F$, referred to as AgF), has been shown to be effective as a caries inhibitor in deciduous teeth,[3-5] to reduce caries formation in primary teeth and first permanent molars[6] and *in vitro* will substantially reduce the growth of *Streptococcus mutans* in AgF and AgF/KI treated demineralized dentine.[7] The mechanism of caries protection by the silver moiety in AgF has yet to be determined although it has been linked to the formation of silver phosphate on the tooth surface,[4] fluoride uptake into the dentine, blocking the dentine tubules with silver precipitates or inactivation of cariogenic bacteria that come into contact with it.[8]

Following the application of silver salts to a treatment site, excess free silver ions have been reduced by the application of either eugenol or stannous fluoride,[3,8-10] resulting in the formation of black precipitates. For silver salts such as AgF to be acceptable as a caries inhibitor the untoward side effects of staining tooth structures and adjacent tooth-coloured restorations needs to be eliminated.[8]

The application of potassium iodide (KI) after AgF application overcomes this problem as the precipitates of silver iodide (AgI) are white, as distinct from a black reaction product. AgI has a history of use in dentistry.[11]

The electron probe microanalysis technique (EPMA) has confirmed the transfer of strontium and fluoride ions into the dentine from glass ionomer cement.[12-14] The ability to measure the percentage weights of chosen elements enables an analysis of the amount of tooth loss, the penetration depth of reaction products and their affects upon tooth demineralization.

The purpose of this *in vitro* study was to observe the differences after two weeks in a growth medium inoculated with *S. mutans*, between non-demineralized and demineralized dentine that had the surfaces treated with an application of AgF followed by KI. EPMA was used to measure the reduction in calcium and phosphorous levels and the ion uptake of silver and fluoride. Optical density readings of the turbidity of the liquids in the vials were taken to indicate growth of *S. mutans* after exposure to the different samples and the surface morphology of the samples were observed using SEM.

MATERIALS AND METHODS

Preparation of the dentine discs

The crowns of 35 recently extracted human third molar teeth that had been stored in 0.5% chloramine were sectioned horizontally to produce enamel dentine sections approximately 1.5mm thick. Only sections with flat, sound dentine on either surface were used. Teeth were collected within the guidelines set by the Committee for the Ethics of Human Experimentation at The University of Adelaide.

After initial preparation, 20 of the 35 discs were demineralized[16] and all discs were set into the bases of 35 plastic vials as described by Knight.[7]

Pretreatment of dentine prior to experimentation

Samples were prepared as follows: (1) 10 non-demineralized samples were coated with a solution of 1.8M AgF, followed by a saturated solution of KI and then washed off with copious amounts of distilled water; (2) 5 non-demineralized samples used as a control; (3) 10 demineralized samples were coated with a solution of 1.8M AgF, followed by a saturated solution of KI and then washed off with copious amounts of distilled water; and (4) 10 demineralized samples used as a control.

Preparation of the culture medium

Medium was prepared by weighing out as (w/v) 3% tryptone soya broth (Oxoid, Basingstoke, UK), yeast extract 0.5% (Oxoid Basingstoke, UK) and 20% sucrose.[7] The medium was sealed inside the vials and sterilized using gamma irradiation.[7]

Experimental method

The sterilized vials were then placed into a sterile glass flask that was connected to the outflow from a Chemostat system (New Brunswick Scientific, Edison, USA). This provided a constant supply of viable *S. mutans* (strain Inbritt) grown by continuous culture. The bacteria were grown in the same medium used to fill the 5mL vials. Growth was maintained under anaerobic conditions at an imposed dilution rate of $0.1h^{-1}$ (mean generation time = 7 hours) and the pH was maintained at pH 7.4 by the automatic addition of KOH (2N). The pH of the flask containing the vials was uncontrolled and remained at pH 4.2 throughout the two-week duration of the experiment.

Optical density

After two weeks the samples were removed from the flask and the optical density of the liquids in the vials was measured using a λ 5 dual beam spectrophotometer (Perkin Elmer, Uberlingen, Germany) set to a wavelength of 560nm to determine the growth of bacterial in AgF/KI non-demineralized and demineralized samples and demineralized control samples. There are no data available to give a correlation between the optical density readings and concentrations of *S. mutans* in the solution.

Culture purity in each container was determined by plating aliquots of cultures onto nutrient medium and *Mitis salavarius* agar (Difco-Becton Dickenson, Sparks MD, USA) followed by gram staining to observe cellular morphology.

Preparation of sections for EPMA and SEM analysis

Following optical density readings the discs were removed from the vials, placed in a fixing solution and then dehydrated as described by Knight.[14] The dehydrated discs were halved and prepared for further analysis.

Scanning electron microscopy (SEM)

The surface of each specimen that was exposed to the Chemostat was attached to a mounting stub and carbon coated for SEM analysis (Philips XL30 Field Emission Scanning Electron Microscope, Netherlands).

Electron probe microanalysis (EPMA)

Each of the 35 dehydrated specimens were embedded in resin and prepared for EPMA following the technique used by Knight.[14] Line scans were carried out on the specimens to measure the relative percentage weights of the following elements: calcium (Ca), silver (Ag), phosphorus (P) and fluoride (F). Scans were conducted from the surface of the cavity over the surface of the specimens every 5μm to a depth of 500μm. Three scans were made at each location and the three readings averaged. Measurements were expressed as a relative percentage weight of the identified element as part of the total weight of the sample where the measurement was taken.

DATA ANALYSIS

Since the data were not normally distributed, the Kruskall-Wallis test was used to determine if there was a difference amongst the groups. *Post hoc* testing was

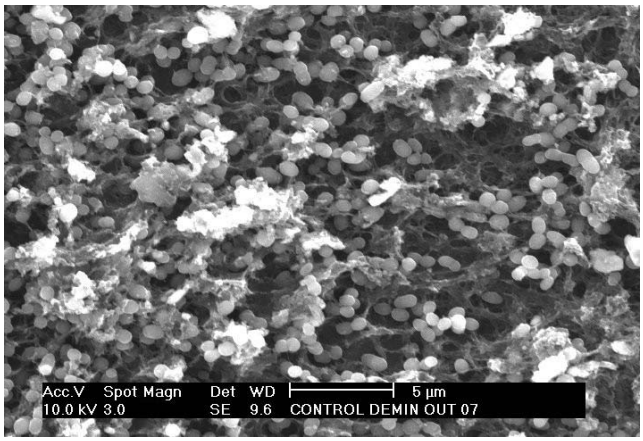

Fig 1. Typical outer surface of a non-treated (control) demineralized dentine disc after two weeks exposure to *Streptococcus mutans*. Heavy biofilm formation is evident.

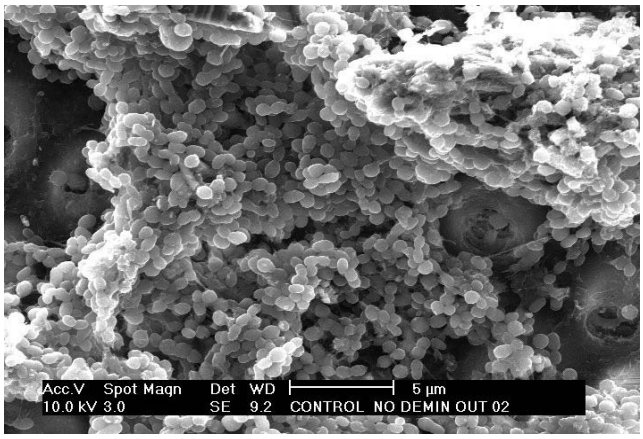

Fig 2. Typical outer surface of a non-treated (control) non-demineralized dentine disc after two weeks exposure to *Streptococcus mutans*. Heavy biofilm formation is evident.

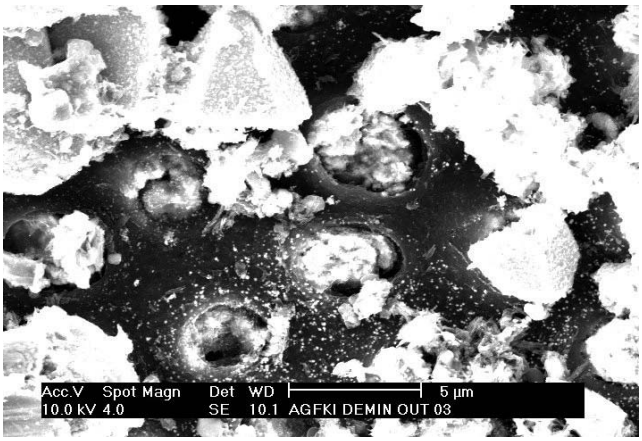

Fig 3. Typical outer surface of a demineralized dentine disc treated with AgF/KI after two weeks exposure to *Streptococcus mutans*. There are deposits of reaction product from AgF and KI on the surface and within the dentinal tubules but no evidence of biofilm formation.

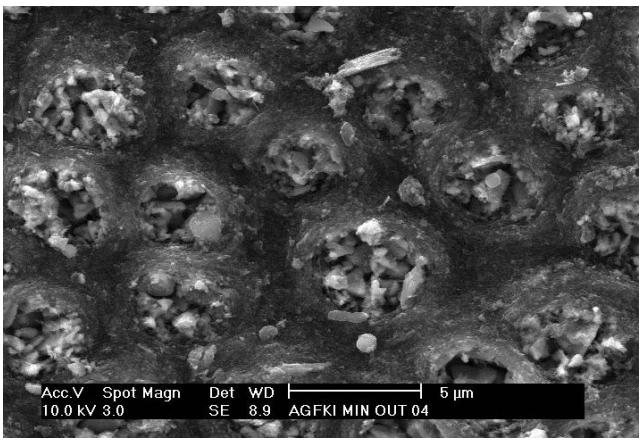

Fig 4. Typical outer surface of a non-demineralized dentine disc treated with AgF/KI after two weeks exposure to *Streptococcus mutans*. There are deposits of reaction product from AgF and KI within the dentinal tubules but no evidence of biofilm formation.

used to make pairwise comparisons with no adjustment made for multiple comparisons. As calcium and phosphorous were being removed from the system, data were compared by calculating the areas above the curve, Delta Z. As fluoride and silver were being added to the system, data were compared by measuring the areas below the curve. Although measurements were carried out to a depth of 500µm, the experimental model was set up to examine the changes occurring at the dentine surface interface and data were not analysed beyond a depth of 300µm, the depth to which secondary demineralization had occurred during the experiment.

RESULTS

Scanning electron microscopy

The SEMs showed extensive deposits of *S. mutans* and mucopolysacharides on all the non-demineralized (Fig 1) and demineralized surfaces of the control sections (Fig 2). No biofilms were observed on the non-demineralized (Fig 3) and demineralized surfaces (Fig 4) of sections treated with AgF/KI. Both non-demineralized and demineralized AgF/KI sections showed crystalline precipitates of reaction products within the tubules and the demineralized sections had reaction product scattered over the dentine surfaces.

Electron probe microanalysis

The graphs in Figs 5–8 show the mean values of each element in dentine for each of the experimental groups up to a depth of 500µm although data analysis was confined to the depth of secondary demineralization (300µm). The left-hand scale represents the percentage weights of each element.

The graphs of the calcium and phosphorus levels (Figs 5 and 6) show the differences between the non-demineralized and demineralized dentine sections and the reductions in further demineralization between the sections treated with AgF/KI and the controls. With both elements there were significant differences between the AgF/KI and the demineralized control samples ($p<0.05$) and between the AgF/KI and non-demineralized control samples ($p<0.05$). There was a significantly improved ability of the AgF/KI demineralized samples to resist further calcium and

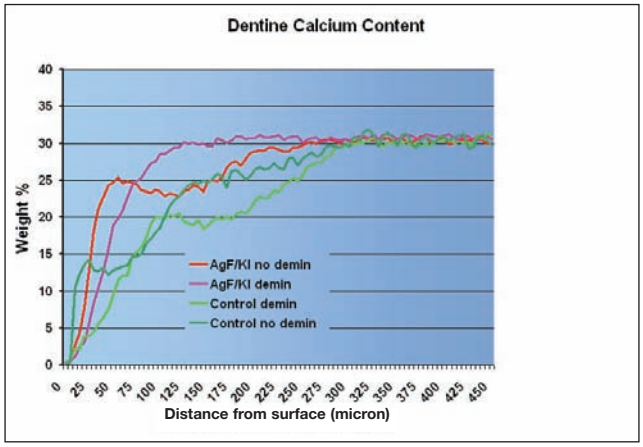

Fig 5. EPMA graph of the average percentage weights of calcium in treated and control specimens. Note that the percentage weights of the non-demineralized AgF/KI samples are significantly less than the demineralized sections ($p<0.05$).
(Statistical analysis: Calcium. Differences at the 0.05 level. NS = not significant. S = significant. AgF/KI demin versus control demin (S); AgF/KI no demin versus control no demin (S); AgF/KI demin versus AgF/KI no demin (S); control demin versus control no demin (NS) AgF/KI demin versus control no demin (S); AgF/KI no demin versus control demin (S).)

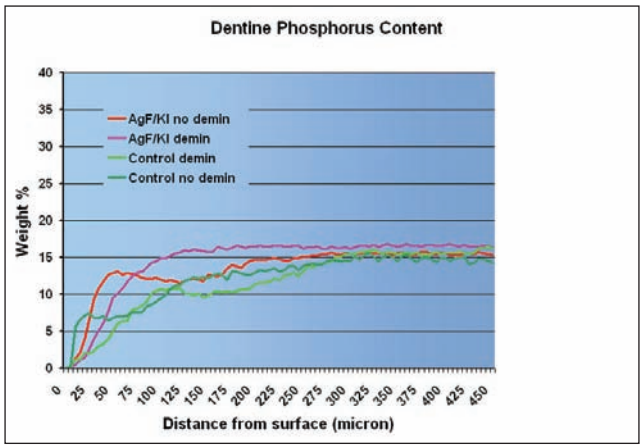

Fig 6. EPMA graph of the average percentage weights of phosphorus in treated and control specimens. Note that the percentage weights of the non-demineralized AgF/KI samples are significantly less than the demineralized sections ($p<0.05$).
(Statistical analysis: Phosphorus. Differences at the 0.05 level. NS = not significant. S = significant. AgF/KI demin versus control demin (S); AgF/KI no demin versus control no demin (S); AgF/KI demin versus AgF/KI no demin (S); Control demin versus control no demin (NS) AgF/KI demin versus control no demin (NS); AgF/KI no demin versus control demin (S).)

phosphorus loss compared to the AgF/KI treated non-demineralized samples ($p<0.05$), but there were no significant differences between the control demineralized and non-demineralized samples in this respect. The graph of silver levels (Fig 8) shows significant differences between AgF/KI samples that were demineralized and AgF/KI samples that were not ($p<0.05$). Silver was not detected in the control samples.

Optical density

Optical density measurements were taken on specimens from both treatment groups and the demineralized control group. *S. mutans* were present

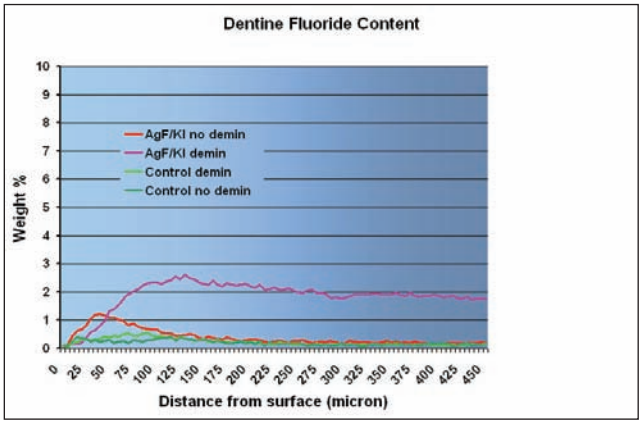

Fig 7. EPMA graph of the average percentage weights of fluoride in treated and control specimens.
(Statistical analysis: Fluoride. Differences at the 0.05 level. NS = not significant. S = significant. AgF/KI demin versus control demin (S); AgF/KI no demin versus control no demin (S); AgF/KI demin versus AgF/KI no demin (S); Control demin versus control no demin (NS) AgF/KI demin versus control no demin (S); AgF/KI no demin versus control demin (S).)

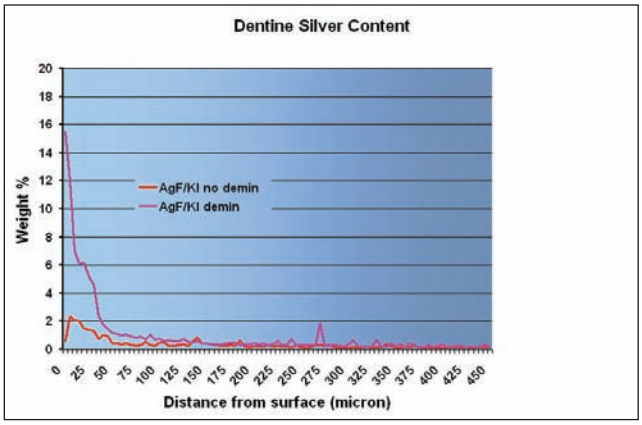

Fig 8. EPMA graph of the average percentage weights of silver in treated and control specimens.
(Statistical analysis: Silver. Differences at the 0.05 level. NS = not significant. S = significant. AgF/KI demin A versus AgF/KI no demin (S). The levels of silver in the control samples were below the detectable limits.)

on every plated culture, confirming the ability of the bacteria to migrate through all the samples irrespective of the surface treatment applied prior to the experiment. The mean and standard deviation of optical density readings are shown in (Fig 9). There were significant differences in readings amongst the three samples ($p< 0.05$).

DISCUSSION

While there was complete inhibition of biofilm on non-demineralized and demineralized dentine discs treated with AgF/KI, the demineralized discs were better able to resist further demineralization than the non-demineralized samples. The demineralized samples also had a greater capacity to reduce the growth of *S. mutans* passing through the discs.

When the outer surfaces of the AgF/KI sections were examined under SEM it was evident that there was more reaction product present on the surface of the

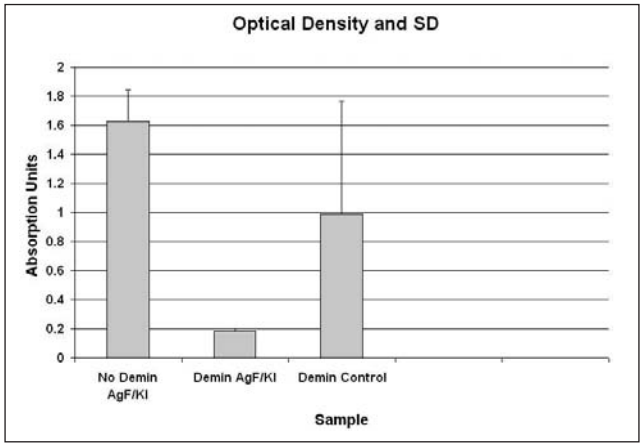

Fig 9. Graph depicting the mean values and standard deviations of the optical density readings in the nutrient broth of the samples. *(Statistical analysis: Optical density. Differences at the 0.05 level. NS = not significant. S = significant. AgF/KI demin versus control demin (S); AgF/KI no demin versus control demin (S); AgF/KI demin versus AgF/KI no demin (S).)*

demineralized samples than the non-demineralized ones. In both sections the dentinal tubules were plugged with reaction product although these precipitates were unable to prevent fully the passage of *S. mutans* through the samples into the nutrient broth chamber beneath. The unencumbered migration of bacteria into a contained nutrient solution should result in a rapid propagation of bacteria over a relatively short period to completely deplete the nutrients available. The low optical density readings associated with the demineralized treated dentine samples suggests mechanisms acting within demineralized dentine that may have interfered with the growth of the migrated bacteria. The narrow range of the readings of the AgF/KI demineralized samples further suggests a predictably reproducible effect on reducing the viability of the invading bacteria. The lower readings of the demineralized control compared to the non-demineralized AgF/KI treated discs suggests the process of demineralization may trigger a natural defence mechanism to bacterial assault that may warrant further investigation.[16]

While both demineralized and non-demineralized AgF/KI treated discs were able to significantly reduce demineralization by *S. mutans* compared to their respective controls, the demineralized samples were more effective. Two factors that stood out in the EPMA data were the higher levels of silver deposited on the surface of the demineralized AgF/KI sections and the increased levels of fluoride present in the demineralized samples.

It has been shown that fluoride can inhibit biofilm formation by *S. mutans* and other bacterial species.[17] In this study, EPMA data showed that while fluoride levels on the surface of the discs were low there were significantly higher levels of fluoride within the demineralized discs that peaked at almost 3 per cent at a depth of 125µm and continued around 2 per cent to a depth beyond 500µm. Fluoride levels within the non-demineralized discs peaked at just over 1 per cent at a depth of 50µm then gradually fell away. While it may be a coincidence, the calcium and phosphorus levels in the non-demineralized discs demonstrated a susceptibility to *in vitro* caries beyond 50µm whereas in the demineralized discs, the higher fluoride levels and penetration depths were associated with an ongoing resistance to *in vitro* caries demineralization. Higher fluoride levels in the AgF/KI treated demineralized discs may have had a minimal effect at the surface on biofilm formation and may certainly have contributed to the improved *in vitro* caries resistance observed in the EMPA data and the low optical density readings.

Silver salts as low as 20ppm have been found to inhibit growth of *S. mutans* and *Staphylococcus aureas*[18] and unspecified higher concentrations were found to be bactericidal. Furthermore, metal cations have been shown to inhibit the activity of several glycosyltranferases[19] that suggests any silver ion release from the tooth surface may have interfered with this enzyme activity. These enzymes are responsible for the synthesis of soluble and insoluble glucans that not only contribute to the bulk of the biofilm but also play essential roles in the sucrose dependent adhesion of the organisms to tooth surfaces.[20]

The AgF/KI treated demineralized dentine had significantly higher levels of silver precipitated (16 per cent) than the AgF/KI treated non-demineralized discs (2 per cent). Both demineralized and non-demineralized discs were able to completely inhibit biofilm formation suggesting that lower concentrations of AgF/KI may be effective in preventing biofilm formation in demineralized dentine. The penetration of silver into both demineralized and non-demineralized AgF/KI treated dentine was around 50µm. This is the depth at which *in vitro* carious demineralization commenced in the AgF/KI treated non-demineralized discs, suggesting the presence of silver may have inhibited *in vitro* caries initiation. The much higher levels of silver on the surface of the AgF/KI treated demineralized discs may have adversely affected the viability of the *S. mutans* to the extent that *in vitro* caries initiation was unable to occur.

The application of AgF/KI onto non-demineralized and demineralized dentine was able to significantly reduce *in vitro* caries progression and prevent biofilm formation. The ability of partly demineralized dentine to take up relatively large amounts of silver and fluoride suggests that this application is more effective as a means of arresting existing dentine caries, where the teeth have already undergone some demineralization, than preventing dentine caries initiation on sound dentine surfaces.

No attempt was made to determine the effects of lower concentrations of AgF/KI on biofilm formation, reducing optical density or dentine demineralization by *S. mutans*, and this warrants further investigation as does an *in vivo* study to clinically confirm the findings of this investigation.

CONCLUSIONS

In this study, the treatment of demineralized and non-demineralized dentine discs treated with AgF/KI followed by two weeks in an *S. mutans* inoculated growth medium suggests that silver-based reaction products on the surface of the treated dentine prevent the formation of a biofilm and that silver and fluoride ions present within the treated dentine inhibit *in vitro* caries progression. The demineralized samples were more effective in reducing the growth of the invading bacteria and inhibiting demineralization of the discs than the non-demineralized samples.

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DISCLOSURE

Authors Knight and Craig are named on a process patent associated with the use of silver fluoride and potassium iodide.

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