FINAL REPORT TO DR P SHANAHAN RE; THE EFFECTIVENESS OF DENTA-MED IN CONTROLING BACTERIAL GROWTH ON ENAMEL AND ROOT CEMENTUM SURFACES OF HUMAN TEETH

Drs J McIntyre, Mulyani Dalidjan. Dental School, Adelaide University 4.2.08

Summary of Results of tests of Denta-Med

Denta-Med Oral Gel contains three known active therapeutic ingredients, viz. Cetylpyridinium Chloride (0.05%), Zinc Chloride (0.22%) and Sodium Fluoride (0.22%), which have been shown to act as general anti-metabolic agents to bacteria, and/or in the case of sodium fluoride, as a dental caries inhibitor and remineralizing agent. All are safe and effective active ingredients, which have been tested and their therapeutic activity proven individually over a number of years. The patented formulation is chemically stable and all irritants have been removed.

In these tests, Denta-Med was shown to substantially inhibit (x50 times) a *Streptococcus Mutans* bacterial (plaque) growth on the surfaces of both tooth enamel and roots when exposed to a concentrated bacterial population of these organisms over a two week period *in vitro,* when compared to control surfaces. This was demonstrated both by cell counts and SEM analysis. The gel also completely inhibited carious demineralization of both enamel and root cementum/dentine following exposure for two weeks to a pH of 4.5.

Denta-Med Oral Gel also uses a patented proprietary micro-particle, referred to forthwith in this report as the "proprietary ingredient," specifically designed for this application. This proprietary ingredient acts topically, providing multiple receptor sites and a slow release mechanism for the bio-active agents. This property was clearly demonstrated in this study. It is this ingredient that potentiates the other active ingredients and delivers the multiple benefits so clearly demonstrated in these trials through increasing the substantivity of these ingredients.

Overall the study showed that Denta-Med would provide significant protection to both teeth and oral soft tissues against plaque growth, particularly those at high risk of caries and gingivitis. It would be particularly beneficial to the elderly and medically compromised patient as a prophylactic as well as a therapeutic plaque control agent.

Outline of the Tests carried out

This project consisted of two experiments:

A. Analysis of the effectiveness of a regular coating of Denta-Med in inhibiting both bacterial plaque growth and caries effects of plaque bacteria in test enamel and tooth root samples *in vitro*.

B. Determination of the efficacy of the proprietary ingredient liquid as a slow release agent using high concentration proprietary ingredient gels of Denta-Med *in vitro*.

Experiment A. Analysis of the effectiveness of a regular coating of Denta-Med in inhibiting both bacterial plaque growth and caries effects of plaque bacteria in test enamel and tooth root samples 'in vitro.'

General Methodology

The methodology used involved exposure of test slates of enamel and root cementum/dentine of fixed dimensions to a bacterial culture grown within a 'Chemostat,' i.e., apparatus enabling a continuous growth of bacteria under constant growth conditions. Slates of enamel and root surface were exposed to the bacterial culture either unprotected (controls) or protected by a coating of Denta-Med for fixed periods. The effectiveness of the test agent, Denta-Med, in controlling bacterial growth on the test slates, was assessed by SEM analysis of the exposed control and test surfaces, by analysis of bacterial counts of organisms, and by analysis of the caries causing effects of the bacterial growth where it occurred.

Test tooth samples
Ten intact extracted teeth were sectioned to provide two slates of enamel at least 4x4mm square from each tooth, as well as two hemi-sections of root cementum/dentine. These teeth were collected according to the conditions laid down by the Human Ethics Committee of the Adelaide University. These surfaces were painted with nail varnish to leave windows exactly 2x2mm in dimension in the middle of each enamel and root surface. The slates were marked to enable the pairs from each tooth to be identified. Ten samples from different teeth were designated as control slates and the remaining as test slate samples.

Method
A Chemostat was charged with a bacterial growth of *S. Mutans* (Ingbritt), which was obtained from B Krasse, University of Gotëborg, Gotëborg Sweden. It was maintained on Tryptone Soy agar plates (Oxoid, Basingstoke, UK) grown at 37°C in an atmosphere of $N_2/CO_2/H_2$ (90:5:5). Starter cultures (200ml) were grown in 3% Tryptone Soya broth (Oxoid, Basingstoke, UK), Yeast Extract 0.5% (Oxoid, Basingstoke, UK) and 20% sucrose and, after overnight incubation, was used for Chemostat inoculation. Following inoculation cultures were allowed to reach mid-log phase before switching on the medium pump to allow continuous culture.

Two groups of paired 10 slates (i.e., from the same tooth), comprising 5 enamel and 5 root surfaces, were bonded to SEM stubs, and placed in each of 2 Flow Cells, which had been designed to be implanted within the Chemostat. The Flow Cells were connected to a 'Chemostat' machine for 14 days, where the exposed enamel windows were exposed to the culture of *Streptococcus Mutans* (species Inbrit) in growth medium, with sucrose added to result in a pH of around 4.5 during this period. The *Strep Mutans* represented those bacteria considered to be most active in causing dental caries. However as the therapeutic agents within the Denta-Med were of a general bactericidal and bacteriostatic nature, the Strep Mutans bacteria were considered to be representative of other species of oral bacteria in their reaction to Denta-Med.

The Flow Cell containing the test specimens were connected to the Chemostat for two weeks, though was opened to allow two coatings of Denta-Med to be applied daily, one at 9AM and the second at 5PM. The Flow cell containing the control slates was connected to the Chemostat for the same time though had no test agent added.

Assessment methods
Upon removal, all test slates were carefully separated from their stubs.

i. Immediately a sample of bacterial growth covering 0.5x2mm of the 2x2mm of the total exposed surface was collected from each sample and plated on blood agar plates for incubation to permit colony counts.
ii. The slates were then placed in a fixing solution, and upon adequate fixation sectioned using a diamond saw through the midline of each exposed window. This permitted both SEM examination of the window surface on one hemisection, and an Electron Probe MicroAnalysis (EPMA) assessment of Calcium, Phosphate, Zinc and Fluoride ion profiles across the cut surface of the enamel and cementum/dentine adjacent to the exposed surface. The objective in using EPMA was to determine whether any microscopic evidence of demineralization resulted in these surfaces, and if so, the profiles of minerals lost or gained.

The method of preparation for SEM and EPMA analysis was as follows: All the samples to be analysed for Scanning Electron Microscopy (SEM) and Electron Probe MicroAnalysis (EPMA) were placed in a fixative solution overnight, containing 4% Paraformaldehyde /1.25% Gluteraldehyde in PBS + 4% sucrose, pH 7.2. The samples were then washed with washing buffer prior to a dehydration process as follows:

Washing buffer – 3 changes of 30 minutes each.

25% ethanol for 20 minutes
50% ethanol for 20 minutes
75% ethanol for 20 minutes
95% ethanol for 30 minutes
100% ethanol for 1 hour

After dehydration the samples were ready for embedding. The samples were embedded in epoxy resin at a ratio of 100:25 for epoxy resin LC191 and epoxy hardener HY 956 (Adelaide Epoxy Supplies, Adelaide, Australia) under vacuum. For the purpose of EPMA investigation, is it very important to make sure that plano-parallel specimens were obtained. Both sides of the mounting blocks were made parallel to each other using a levelling device. The surfaces to be examined were then polished using polishing discs (Struers, Copanhagen, Denmark) and diamond paste (Kemet, England), starting with 15µm diamond paste at 150rpm and lubricated with DP-Lubricant Green (Struers, Denmark) for 5 minutes at 200N. This was followed with 3µm and finally finished to 1µm at 150rpm and 200N for 3 minutes. All these polishing procedures were achieved using Abramin (Struers, Denmark) polishing machine. Upon completion, all the samples were carbon coated ready to be analysed by Scanning Electron Microscopy (SEM) and/or Electron Probe Micro Analysis (EPMA).

Results from Experiment A

The following results were obtained;

i. Mean nos. of colony forming units of *Strep Mutans* present on each test window compared with those on a similar area of paired control windows
ii. Representative SEM pictures of window surfaces
iii. Mean EPMA profile graphs of Ca, P, Zn and F in cut surfaces adjacent to the exposed windows

i. Mean number of colony forming units

Mean no.colony forming units (i.e.,No. Bacteria) of Strep Mutans /plate after 14 days exposure to Strep Mutans in Chemostat for five samples each of control and test (i.e., twice daily application of Denta-Med; N= 5).

ENAMEL

Control (**without Denta-Med**):	1,430
Test (**with two Denta-Med appns/day**:	26

ROOT CEMENTUM/DENTINE

Control (without Denta-Med):	1,682
Test (with two Denta-Med appns/day	33

Comment

This result indicates greater than a 50x reduction in bacterial numbers growing on both enamel and root cementum/dentine plates after twice daily application of Denta-Med for two weeks. This is **much greater that found with routine antiseptic mouth rinses** (**average approximately a 35%** reduction in bacterial counts after two weeks exposure, though different frequency of application, and not involving a Chemostat).

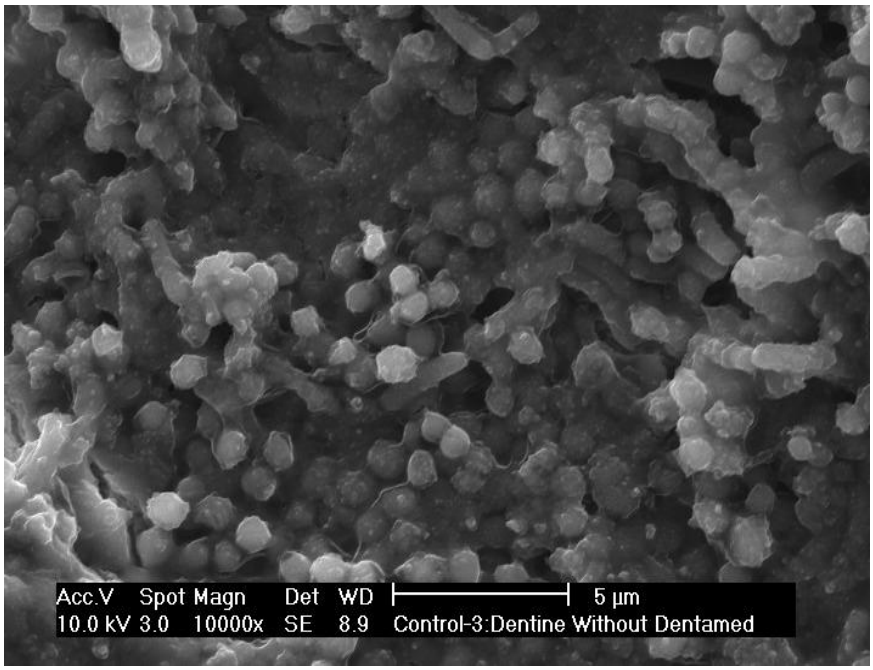
This result has to be considered in relation to the mechanism of action of the Chemostat, where there is a relatively fast flow of bacterial culture over the tooth surfaces, which may remove some of the Denta-Med soon after placement. Hence this method might have not adequately represented what happens *in vivo*. However it must be remembered that it is not ethically easy to measure the effect of this level of contact with Strep Mutans *in vivo,* and as well it is very expensive to run such

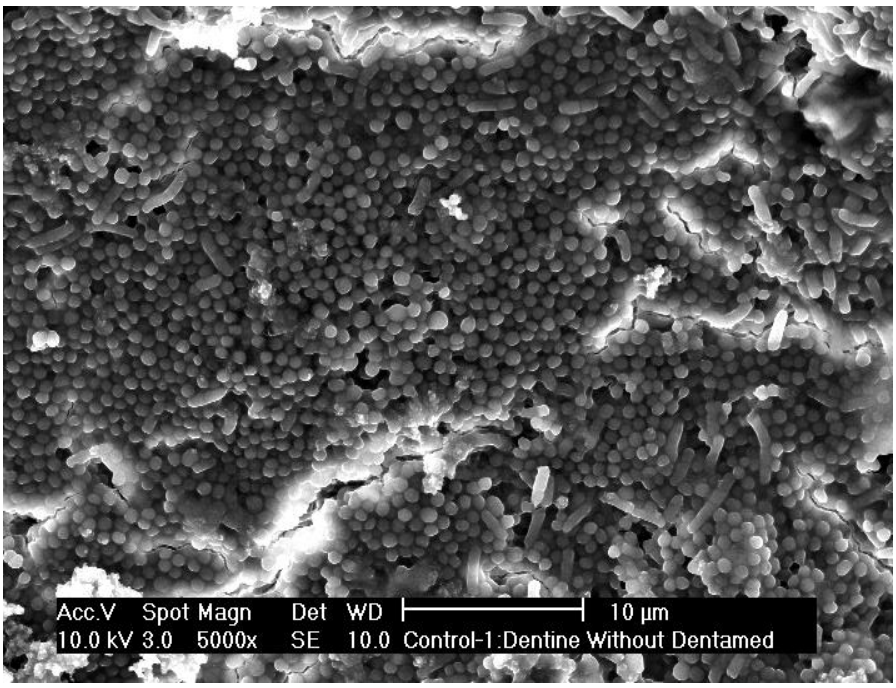
in vivo trials. It would be possible to carry out a comparison between CHX and Denta-Med *in vitro*, though again this would not reflect what happens *in vivo*.

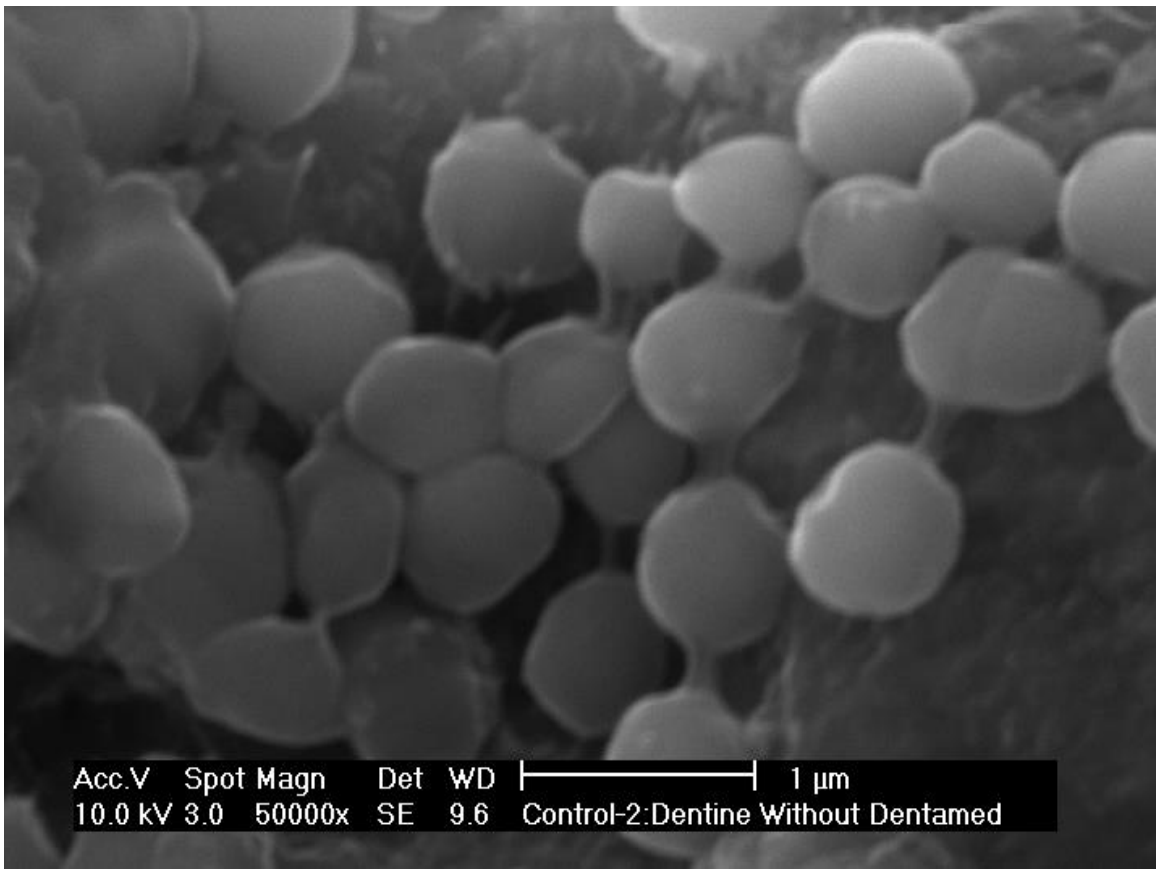
Experiment 2: SEM analysis of surface bacterial plaque deposits

The following are representative SEM images of the surfaces of all categories of test and control samples at different magnifications. These provide supporting evidence to corroborate the above figures, i.e., that very few bacteria could grow on the Denta-Med treated surfaces, while there was prolific growth on the control surfaces. There are numerous SEM views available showing a similar picture to those selected, which can be provided if requested.

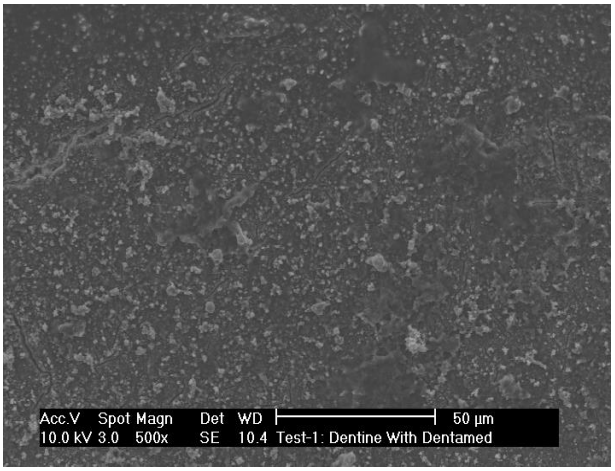
Fig 1; X10000 magnification; Shows root surfaces when no Denta-Med used (i.e., controls). Note the large numbers of Streptococci growing on the surfaces of the roots

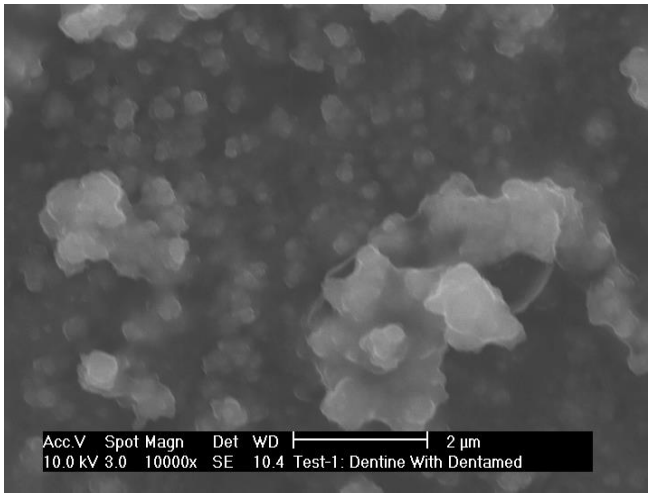






Showing Streptococci in healthy reproductive cycles, with muco-polysaccharide coating still intact
Fig 2; Root surfaces with Denta-Med applied. Top image shows low magnification (x500) to show mainly only proprietary ingredient particles present, which are generally smaller than Streptococci. The x10000 and 20000x magnification shows the proprietary ingredient particles (irregular, conglomerating). It is difficult to see many Strep Mutans, which, as seen in the above images, are mostly small and spherical in shape.

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10.0 kV 3.0 10000x SE 10.4 Test-1: Dentine With Dentamed

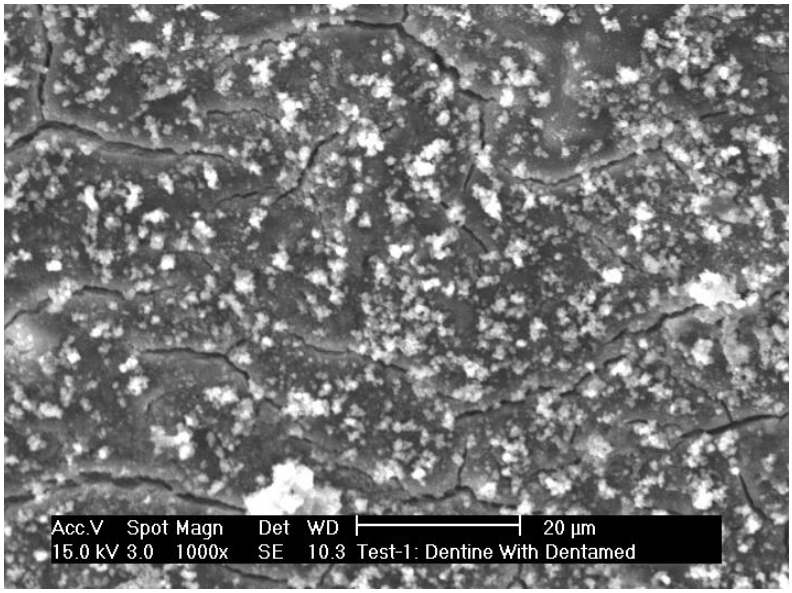
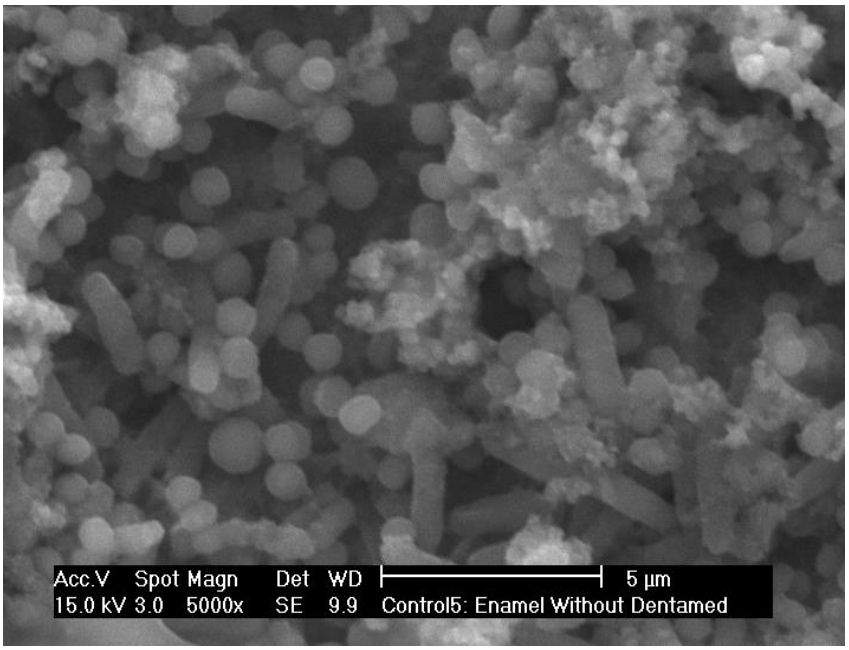

Acc.V Spot Magn Det WD 20 µm
15.0 kV 3.0 1000x SE 10.3 Test-1: Dentine With Dentamed

Fig 3; Enamel windows without any Denta-Med applied (controls). Note the lower image at low magnification (x21) showing sheets of Strep made plaque. The top image at high magnification (x5000) shows masses of Streptococcus organisms, many at the stage of forming chains bound by polysaccharide, indicating rapid growth which enables it to form plaque layers.



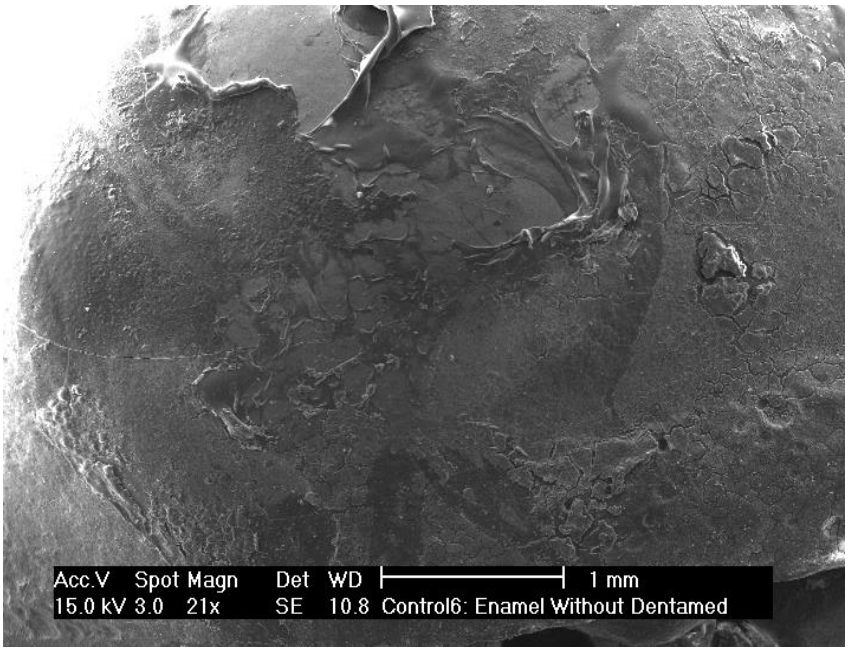
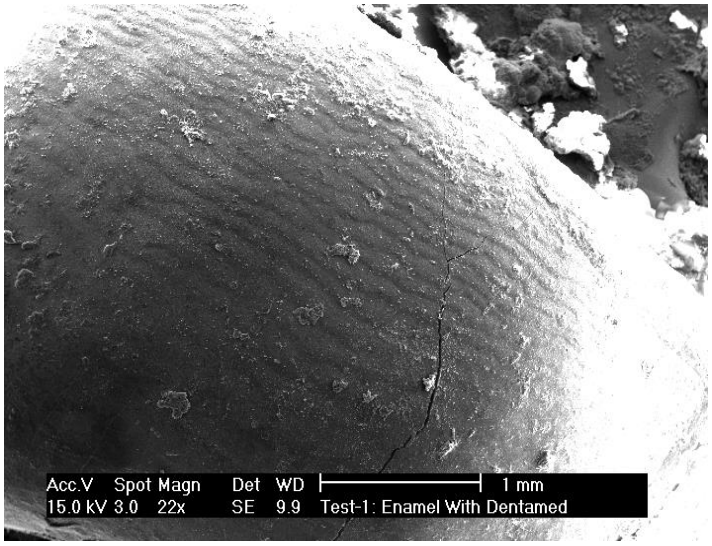
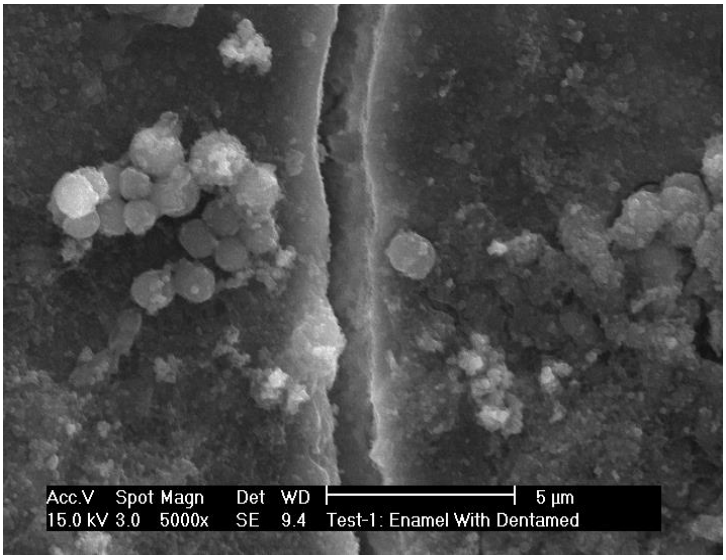
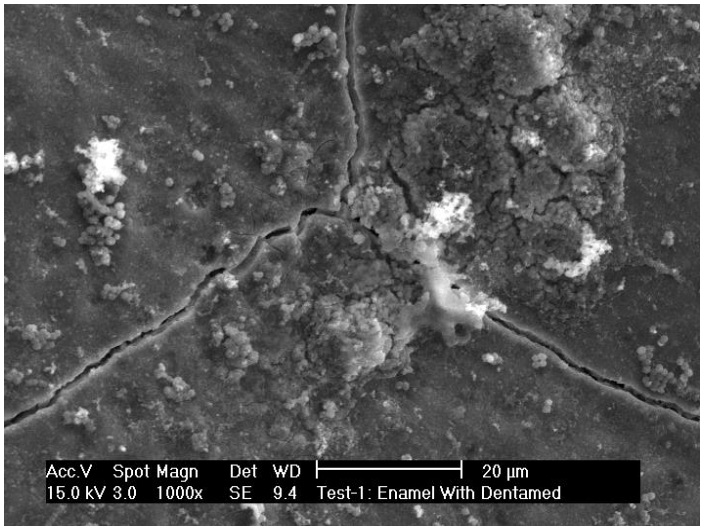


Fig 4; Enamel exposed to Denta-Med. Top image shows the surface perichymata structure of the enamel with mainly the odd patch of bacteria present. The lower images show these at high magnification. The proprietary ingredient did not seem to bind to enamel as much as to root cementum. The crack in the middle is from dehydration caused by preparation for SEM analysis.







Experiment 3; Assessment of outcomes of bacterial plaque contact, viz dental carious demineralization

As any cariogenic demineralization would be microscopic, it was necessary to carry out EPMA examinations in order to determine the presence of mineral changes such as of calcium and phosphorus at the exposed surface. At the same time, this method was used to examine whether any fluoride and zinc ions were taken up above background levels, even though the amounts would be small considering the expected low level of surface demineralization after two weeks exposure.

(Fig 5); The first Figure shows profiles of the elements of interest as means of data from 5 control root surfaces. (The graph represents the concentration profiles of the four elements Ca, P, F and Zn in relative weight % from the surface of the root (Y axis) inwards. This graph shows that without Denta-Med present a small carious lesion (i.e., where the Ca and P weight % goes down proportionally) resulted in the root surface after 14 days exposure to the *Strep Mutans* solution at pH around 4.5. Its depth was about 80 µm. These teeth had a relatively high level of background fluoride and zinc ion across their surfaces, which is not unusual.

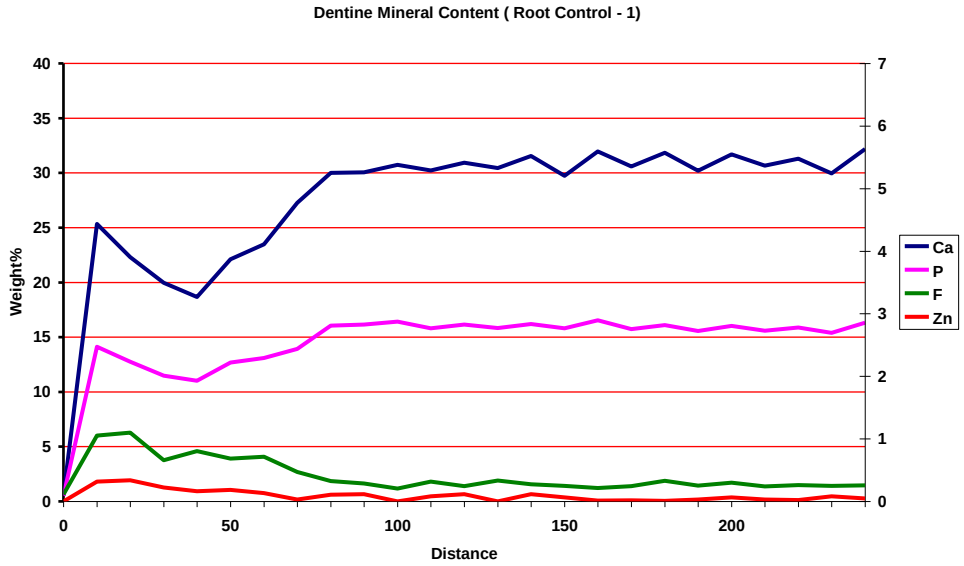
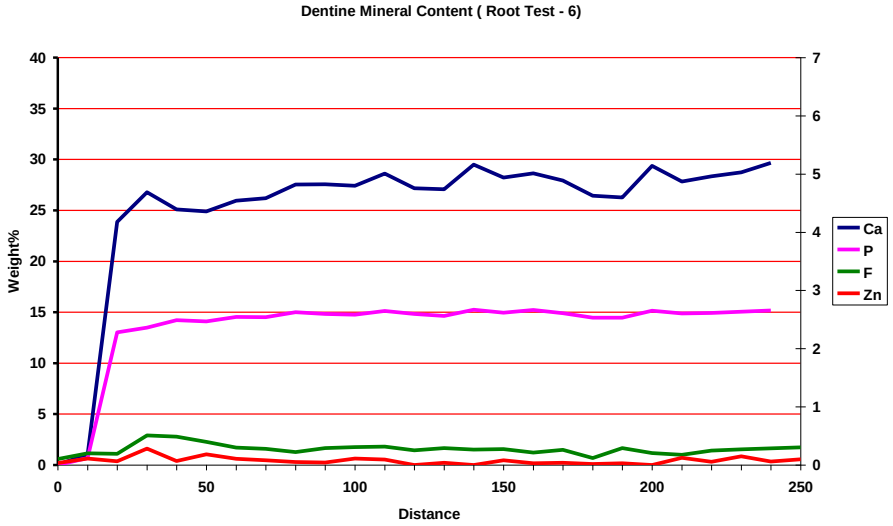


Fig 6; Mean root surface profile of Ca, P, F and Zn adjacent to the exposed root test surface



No loss of calcium or phosphorus, (as a component of phosphate) ion was evident in the test samples of root cementum/dentine. Also this resulted in no uptake of fluoride and zinc beyond background levels.

Fig 7; Mean enamel profile of Ca, P, F and Zn adjacent to the exposed control enamel surface
This graph shows an incipient lesion has developed again to about 150μm deep. Amongst these five lesions in enamel, there was a substantial increase in fluoride and zinc ion uptake into the incipient lesion.

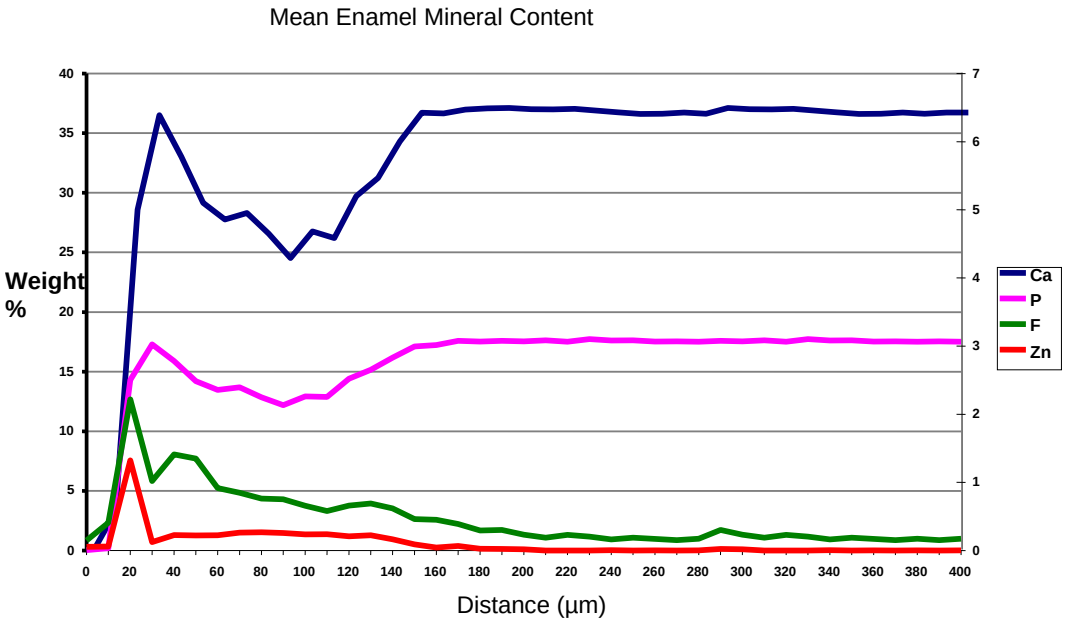
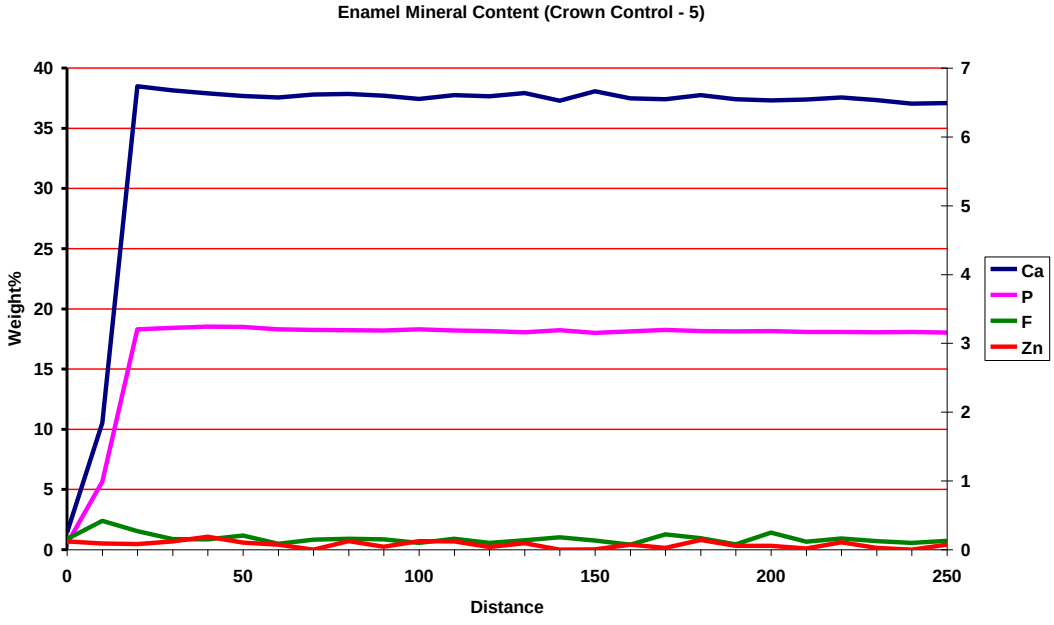


Fig 8; Mean enamel profile of Ca, P, F and Zn adjacent to the exposed surface-test sample.
In this graph, no sign of carious demineralization was evident, and background fluoride and zinc ion concentrations were low.



Overall significance of this data

This data is very supportive of a strongly protective role of Denta-Med against carious demineralization, and also strongly supplements the evidence of bacterial colony growth inhibition by the test agent. This inhibition of bacterial growth would result mainly from the heavy metal Zinc ion, assisted by CPC, and also with a possible contribution from NaF. However the results from the test samples show more-that despite there being a pH of 4.5 in contact with the dentine or root surface for two weeks, demineralization was inhibited, when the pH alone would normally cause it. **This would have been because of the presence of NaF at a relatively high concentration (around 1000ppm, the same as in most commercial fluoridated dentifrices).**

Experiment B. **Determination of the efficacy of the proprietary ingredient as a slow release agent, using high concentration proprietary ingredient gels of Denta-Med *in vitro***

Introduction

There are a number of anti-plaque gels available which aim to reduce the growth of bacterial plaque on teeth. Those agents which are most effective have been those where the therapeutic agents remain in contact with the tooth surface for prolonged periods of time, this property being named 'substantivity'. Denta-Med has been found by a number of clinicians to have a plaque –inhibiting property similar to those known to have a high substantivity, and it is thought that the presence of the proprietary ingredient might contribute this property to this gel. The objective in this experiment was to test *in vitro* for evidence that the proprietary ingredient can retain concentrations of Zinc Chloride, Cetyl Pyridinium Chloride and Sodium Fluoride sufficient to give Denta-Med a therapeutic effect for some hours after placement on teeth. To achieve this, a range of concentrations of proprietary ingredient were used in solutions which contained the same doses of the therapeutic agents as the original gel. The concentrations of proprietary ingredient were increased from 0.01% to 0.1%,1.0%, 5.0%.

The markers used to investigate the substantivity of therapeutic agents in Denta-Med were Zn and F ions. It was not possible to find markers for CPC specifically. Fluoride ions are readily quantitated by Fluoride Specific Electrodes and Zn by Inductively Coupled Plasma Optical Emissions Spectrophotometry (IPC-OES).

Baseline concentrations of Zinc and Fluoride ions

The baseline concentrations of both $ZnCl_2$ and NaF in Denta-Med are stated to be 0.22% concentrations by weight. This results in approximately 1000ppp of both F and Zn ion concentration by weight in the gel (i.e., mg/L), there being almost equivalent weights of Zn and CL and Na and F ion in the solutions. There was a special request to the manufacturers to make solutions which had the same concentrations of these ions and CPC as Denta-Med, though as three different solutions containing 0.1, 1.0 and 5.0% proprietary ingredient nanoparticles, this being to enable a range of readings to be determined at higher concentrations of the proprietary ingredient which would provide readable resulting concentrations of Zn and F ion. As all the supernatants were diluted for measurement purposes, concentrations of F and Zn ions in the original solutions were thus adjusted to reflect these original concentrations.

Materials and Methods;

Outline of Methodology; The method used was to centrifuge 10mL samples of each solution using a high speed centrifuge (10,000 RPM) so as to precipitate the proprietary ingredient nanoparticles, and to re-suspend the pellet of proprietary ingredient in fresh solutions of the base material (i.e., free of the therapeutic agents). After allowing the suspension to sit for defined periods up to three hours, the process was repeated and the pellet again resuspended.

Method.

Initially 10mL of each concentration were centrifuged at 10,000RPM for 20 minutes using a high speed centrifuge, and the supernatant removed and stored at 4oC. Ten mL of the 'Base' solution (ie containing the protein based solution required for the proprietary ingredient to maintain a stable association with the Therapeutic agents) were added to the precipitated pellet of proprietary ingredient particles, and the container vortexed to resuspend the particles of the proprietary ingredient**.** These re-suspended suspensions were allowed to sit at room temperature for one hour, and the same process was repeated. The supernatants were again collected and stored on each occasion. The resuspended suspensions were allowed to sit for a further three hours, and again centrifuged, and the supernatants resuspended this time in DDW.

The supernatants were tested as follows to determine the concentrations of Zn and F ions present in each initial solution, in each subsequent supernatant, and for F ion only in the remaining pellet

(suspended in DDW), the aim being to determine whether there is evidence of a slow release of these elements from the proprietary ingredient**,** and if so, the rate of release.
Zinc concentrations were analysed using an Inductively Coupled Plasma Optical Emissions Spectrophotometer (IPC-OES) at the CSIRO Laboratories, Waite Research Institute. Standards of Zn Cl ranging from 0.1ppp to 1000ppm were used.
Fluoride concentrations were measured using a Fluoride specific electrode, following separation of fluoride ions from the solutions using the Taves (1968) acid hydrolysis method.

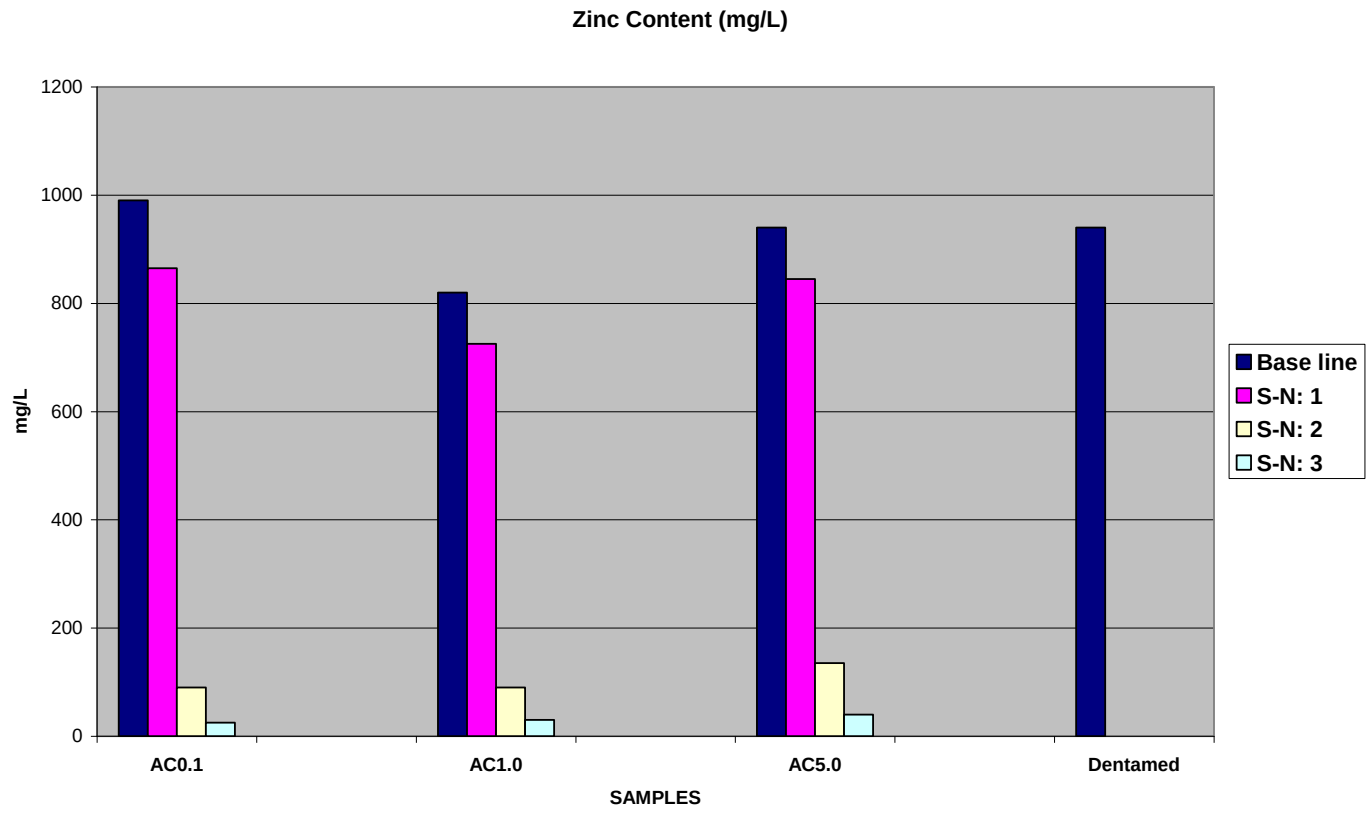
Results

The results show tables and graphs of means of F and Zn ion concentrations initially present in the 0.1, 1.0 and 5.0% proprietary ingredient suspensions, in the 1st Supernatant following centrifugation at 0 hrs, re-suspension of the proprietary ingredient pellet in base solution for 1hr followed by a further centrifugation (2nd Supernatant), and again re-suspension for another 3 hours and final precipitation (3rd Supernatant).

Table 1: Zinc retention (ppm) in initial solution, in initial suspension, after 1hr re-suspension and after a further 3hrs re-suspension (ie cumulative 4hrs suspension)

	Base line	1st Supernatant	2nd Supernatant (after 1hr)	3rd Supernatant (after 4hrs)
AC0.1%	990	865	90	25
AC1.0%	820	725	90	30
AC5.0%	940	845	135	40
Denta-Med	940			

Figure 9; Graph showing retention rates over 4 hrs in ppm (mg/L)

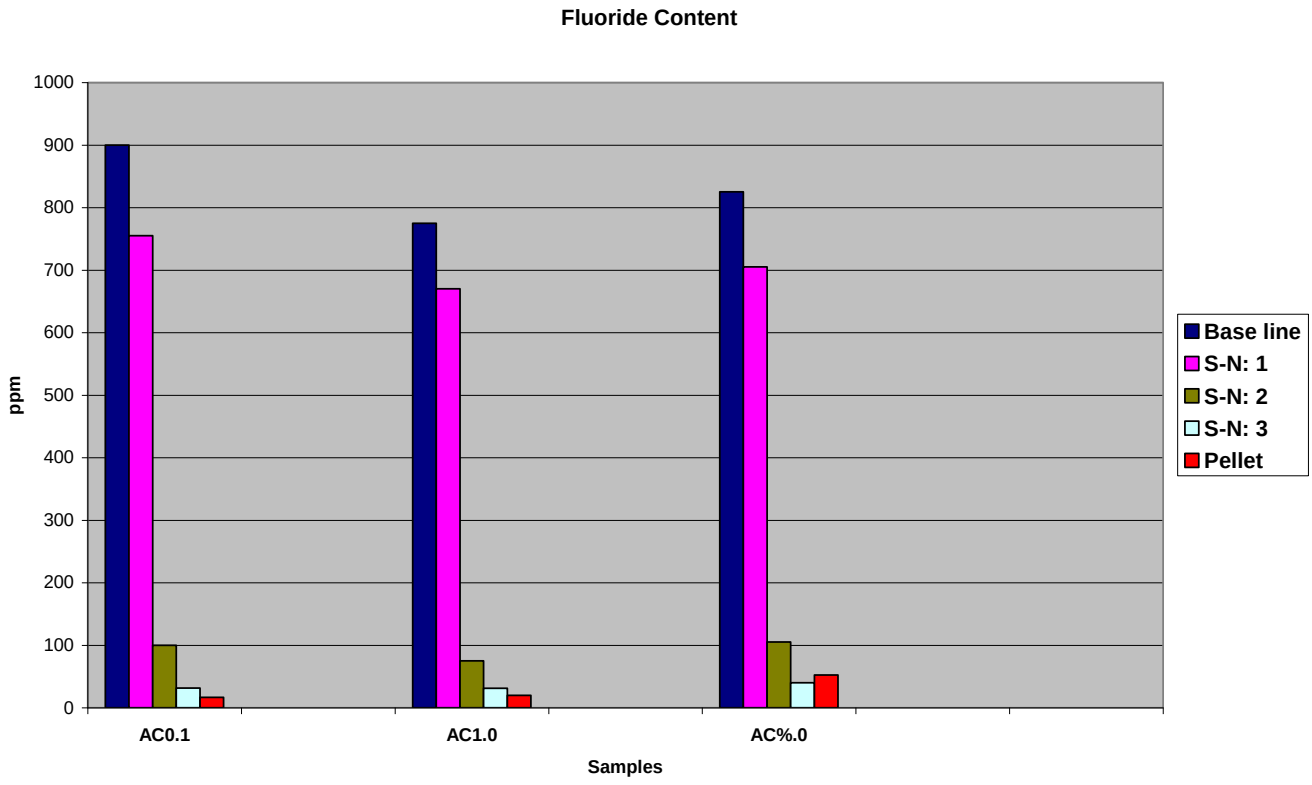


Comment
The Zinc ion concentrations in the original solutions were generally equivalent to that in the Denta-Med Gel, with the exception of the 1.0% solution. The concentrations of Zn ion were similar for all supernatants within the different proprietary ingredient concentrations, indicating that a threshold retention level for this ion had already been reached at lower concentrations than those tested.

Table 2; Fluoride ion retention (ppm) in initial solution, in initial suspension, after 1hr re-suspension and after a further 3hrs re-suspension (ie cumulative 4hrs suspension)

Suspensions	Base line	1st Supernatant	2nd Supernatant (+1hr)	3rd Supernatant (+ 4hrs)	Pellet
AC0.1%	900	755	100	31.5	16.5
AC1.0%	775	670	75	31	20
AC%.0%	825	705	105	40	52.5
Denta-Med	1015				

Figure 10; Fluoride ion retention up to 4hrs



Comment
Again the concentrations of F ion in the original solutions were generally similar to those in Denta-Med gel, with a slight reduction for the 1.0% suspension. There was a small loss within the first supernatant collection for each proprietary ingredient concentration, this allowing for the amount retained by the nanoparticles. Again the concentrations released from the proprietary ingredient particles were around 100ppm after one hour re-suspension and around 30ppm after a further three

hours, as with Zn ion. Again this indicates that the proprietary ingredient concentrations tested, i.e., 0.1, 1.0 and 5.0% were above the threshold level for optimal retention of these therapeutic ions. Also, it is interesting to see that the remnant pellicle of the proprietary ingredient after 4 hours re-suspension in solutes free of these ions still retained small concentrations of F ions (around 20ppm on average).

This evidence supports the use of the 0.01% concentration of the proprietary ingredient in the original Denta-Med gel as providing effective retention of these ions for slow release. While the concentrations of these ions recovered after 1hr and 3hrs was relatively low (around 100ppm after 1 hour and 30 ppm after 3 hrs), these would still be sufficient to help maintain any residual amount of these ions retained at the tooth surface. It needs to be realised that this *in vitro* experiment totally separates the proprietary ingredient particles from the externally dissolved therapeutic agents and re-suspends them in new solute with no $ZnCl_2$ and NaF present. Thus it takes a still picture of what is still retained with the proprietary ingredient particles, which is important to know. In reality, particularly in a drier mouth with low salivary clearance rates, for which cases Denta-Med is particularly prescribed, there would be a slow progressive reduction in the concentration of these ions. It is difficult to say what the effective retention rate might be. However both Zn and F ions are very effective in their respective actions even at 30ppm, and this should indicate that the gel should have a substantive action still at 6 hours at least and possibly longer.

Conclusions

These data overall provide strong evidence of a number of properties of the Denta-Med Gel which would make it very useful clinically. Even though it is *in vitro* evidence, it shows that these properties are intrinsic within the material and should be demonstrable clinically. In particular the plaque inhibition and caries prevention properties were very clearly demonstrated. The evidence of a substantive concentration maintenance property above that of most antiseptic agents should place it within the small list of extremely effective long term plaque control agents such as CHX, and thus as being very effective against those bacteria causing gingivitis. The general antibacterial effect on *Strep Mutans* should be similar for all bacteria growing within dental plaque and within the oral cavity. In addition to this property, the Gel therapeutic agent's totally inhibited carious demineralization in both enamel and root surfaces after two weeks exposure to a pH of 4.5, which CHX would not do. **These properties indicate that the Gel should be most effective for patients with a high oral disease risk, in particular of gingivitis and caries, and to reduce the risk of oral mucosal infections generally.**

As with all such agents, this benefit can only be most effectively demonstrable when tested in clinical trials of patients with a current high risk of these disease problems, and not across the general population. **The demonstrated properties indicate that it should be particularly beneficial for older and other medically compromised people both as a curative and a preventive agent.** There is substantial evidence now available to demonstrate that oral health has a significant impact on general health, in particular on cardiovascular disease, type II diabetes and general susceptibility to infectious disease**. The demonstrated properties of Denta-Med would make it an ideal, safe, daily use preventive agent for all patients at risk of such systemic diseases, with no side effect risks from long term use which are often evident from long term use of chlorhexidine**.

Dr John McIntyre AM,
BDSc, PhD, FADI, FICD
Visiting Research Fellow,
Adelaide University
(Acknowledgement to Dr Mulyani Dalidjan, Technical Officer; Dr P Zilm, Microbiologist)