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Polyols and Remineralisation of Enamel Subsurface Lesions

Abstract (250 word limit)

Sugar-free chewing gum containing polyols has been demonstrated to reduce caries experience in randomised controlled clinical trials. A range of polyols (mannitol, sorbitol, xylitol and maltitol) can be found in sugar-free gums and it has been claimed that they can facilitate calcium uptake into enamel subsurface lesions promoting remineralisation.

Objectives: The aim of this study was to compare the effect of polyols on remineralisation of enamel subsurface lesions *in vitro* by artificial saliva (AS) and by AS containing the salivary biomimetic casein phosphopeptide amorphous calcium phosphate (CPP-ACP).

Methods: The polyols (12.6% w/v) and CPP-ACP (0.376% w/v) were used at physiologically relevant concentrations approximating those released into saliva during chewing a CPP-ACP/polyol chewing gum. Enamel subsurface lesions were exposed to one of the polyols (xylitol, sorbitol, maltitol, mannitol) in AS or AS containing CPP-ACP for 7 days at 37°C with a change of solution each day. Remineralisation of the enamel subsurface lesions was measured by transverse microradiography.

Results: A statistical test for equivalence showed there was no difference in remineralisation between the AS solutions with or without any of the polyols. The AS + CPP-ACP solution substantially promoted remineralisation over AS alone independently of any polyol added.

Conclusion: This controlled *in vitro* study showed that polyols at physiologically relevant concentrations did not promote remineralisation of enamel subsurface lesions by facilitating calcium uptake into the lesion.

Clinical Significance Statement (no more than 100 words)

The results of this study suggest that polyols do not promote remineralisation of enamel subsurface lesions by forming Ca^{2+}-polyol complexes under physiologically relevant conditions.

1. Introduction

Sugar-free chewing gums containing polyols have been demonstrated to reduce caries experience when compared with no gum chewing in randomised clinical trials [1-4]. The anticariogenic effect of chewing the sugar-free gum has been attributed to the stimulation of saliva [4, 5]. Recently the anticariogenic efficacy of sugar-free gum has been enhanced by the addition of the salivary biomimetic casein phosphopeptide amorphous calcium phosphate (CPP-ACP) which significantly increases the buffering and remineralisation capacity of saliva by providing bioavailable calcium and phosphate ions stabilised by the CPP [6-16]. In a randomised controlled clinical trial a sugar-free chewing containing CPP-ACP with a sorbitol-mannitol (polyol) blend was significantly better than a control chewing gum containing only the polyols in slowing the progression of dental caries and enhancing regression of early lesions in children [10]. Enhanced remineralisation of enamel demineralised lesions and an increase in salivary calcium levels by sugar-free chewing gum containing CPP-ACP have been demonstrated in a number of other clinical studies [6-9, 11, 13-15, 17, 18].

Sugar-free chewing gum with or without CPP-ACP may be formulated using different combinations of sugar alcohols (polyols) such as xylitol, sorbitol, mannitol and maltitol [5]. The polyols act as both binding and sweetening agents in sugar-free gums and are all considered non-cariogenic as they are poorly fermented by oral bacteria [5, 19]. The most common polyols used in sugar-free gums are xylitol and sorbitol although more recently blends of polyols are being used to provide certain taste characteristics [2, 5, 9, 20].

Makinen and Soderling [21] have suggested that sorbitol and xylitol at very high concentrations in a saturated calcium sulphate solution form Ca^{2+}-polyol complexes through the formation of cis-cis-triol coordination complexes. Based on these findings the authors proposed

that these polyols may influence calcium bioavailability in saliva and thereby may directly promote remineralisation of enamel subsurface lesions. Similarly, in an *in vitro* enamel lesion remineralisation study Miake et al. [22] suggested that a remineralisation solution containing 20% w/w xylitol produced less remineralisation in the outer layers of the lesion but greater remineralisation in the deeper layers compared with the solution without xylitol. The authors proposed that xylitol could influence remineralisation of deeper layers of demineralised enamel by facilitating Ca^{2+} movement into the lesion.

These results taken together suggest that the use of different polyols may effect the amount or pattern of remineralisation of enamel subsurface lesions by saliva or saliva/CPP-ACP. However, data from clinical studies suggested that chewing sugar-free gum containing CPP-ACP with either xylitol or sorbitol resulted in comparable remineralisation of enamel subsurface lesions [13], suggesting that, at physiologically relevant salivary concentrations of the polyols, no measurable difference in remineralisation could be detected. These inconsistent findings suggest further research is required to clarify the direct effect of polyols on enamel subsurface remineralisation. To date, no study has directly compared the remineralisation efficacy of saliva or saliva/CPP-ACP in the presence of the four commonly used polyols (xylitol, mannitol, sorbitol or maltitol) at physiologically relevant concentrations released by normal use of commercially available sugar-free chewing gum containing high levels of the polyols. Therefore, the hypothesis to be tested in this current *in vitro* study was that artificial saliva (AS), or AS/CPP-ACP, with and without xylitol, sorbitol, maltitol or mannitol at physiologically relevant concentrations were statistically equivalent with respect to their ability to remineralise enamel subsurface lesions.

2. Materials and methods

2.1 Enamel subsurface lesion preparation

Extracted human third molars were obtained from the Melbourne Dental School, The University of Melbourne after informed patient consent. The study was approved by the University of Melbourne's Human Research Ethics Committee (number 1136929). The teeth were first washed thoroughly in distilled deionised water (DDW) then sterilised with 4.1 kGy of gamma radiation. After sterilisation, any soft tissues were removed from the teeth and sound relatively planar buccal and lingual surfaces free of cracking, staining and fluorosis (as viewed under a dissecting microscope) were selected. The outer enamel surfaces were polished wet to a mirror finish using Soflex™ discs on a slow speed contra-angle dental handpiece. Each polished surface was cut from the tooth as an approximately 8×4 mm block, using a water-cooled diamond blade saw and the whole block was then covered with acid-resistant nail varnish except for two (occlusal and gingival) mesiodistal windows (approximately 1×8 mm each) separated from each other by 1 mm. The blocks were treated to create lesions in the enamel windows by suspending each block in 40 mL of unagitated demineralisation buffer, consisting of 80 mL/L Noverite K-702 polyacrylate solution (Lubrizol Corporation, Wickliffe, OH), 500 mg/L hydroxyapatite (Bio-Gel® HTP, Bio Rad Laboratories, Richmond CL), and 0.1 mol/L lactic acid (Ajax Chemicals, Auburn NSW) pH 4.8, for 4 days at 37°C [23]. A change of solution was made after two days at which time the blocks were removed from the solution, rinsed thrice with DDW, blotted dry and placed into fresh demineralisation buffer. The blocks were similarly rinsed with DDW and dried after four days of demineralisation. This demineralisation procedure produces consistent subsurface lesions of 100 μm depths with intact surface layers, as evaluated by contact microradiography of sections of the lesions. After demineralisation, the enamel blocks were cut

perpendicular to the windows into two 4 x 4 mm half-blocks and the cut surface of each block was covered with nail varnish. One of the half-blocks was retained as the demineralisation control and stored in a labeled 1.5 mL microcentrifuge tube together with a drop of DDW, thereby creating a humidified environment. The other half-block (test half-block) was used for remineralisation (see below).

2.2 Remineralisation protocol

A total of ten solutions were tested. One solution contained only artificial saliva (AS). AS consisted of 50 mM NaCl, 0.5 mM $CaCl_2$ and 0.5 mM Na_2HPO_4/ NaH_2PO_4 pH 7.0. Four AS solutions contained 12.6% (w/v) of one of the following four polyols: xylitol, sorbitol, maltitol, or mannitol. This concentration of polyol was used as it is the maximum concentration attained in saliva while chewing with commercially-available gum (2 pieces of gum) containing these compounds at the upper end of the normal range 0.5 – 1.0 g per piece of gum. The concentration was determined as the maximum concentration achieved in saliva while chewing two pieces of a high dose xylitol gum using stimulated saliva flow rates and release kinetics data [24, 25]. Five AS solutions contained 0.376% (w/v) CPP-ACP and four of these also contained 12.6% (w/v) of one of the following four polyols: xylitol; sorbitol; maltitol; or mannitol.

Each test half enamel block was placed into 5 mL of one of the ten AS solutions and incubated at 37^{o}C for seven days. Each day the 5 mL AS solution was replaced with fresh 5 mL AS solution. Six half-blocks each containing two demineralised subsurface lesions were used for each of the ten AS remineralisation solutions.

2.3 Sectioning and transverse microradiography

Each demineralised control enamel half-block and remineralised test enamel half-block were rinsed in ethanol to remove the nail varnish and washed thoroughly in DDW. Each test half-block was paired with its corresponding control half-block, coded (blinded) and placed into freshly poured transparent cold curing methacrylate resin with the lesion windows parallel. The resin vial was marked at the top corner to identify the test and control half-blocks and the resin was allowed to set at room temperature overnight. Sections approximately 200 μm thick was cut from embedded blocks perpendicular to the lesion surface through the midline of both half-lesions using an internal annulus saw microtome. The sections were lapped down to 95± 5 μm using a RotoPol/RotoForce lapping instrument with 1200 and 2400 grit lapping paper. The lapped sections were removed from the lapping instrument with absolute ethanol and rinsed in deionised water, blotted dry and stored on soft tissue between glass slides. Each section, which contained the remineralised lesions and the demineralised control lesions from the same enamel block, was radiographed along with an aluminium stepwedge of 37.5×7μm thick increments using Microchrome High Resolution glass plates (1 x 3 x 0.06 in., Microchrome, USA) and nickel filtered copper Kα radiation [26] at 20 kV, 30 mA for 8 minutes using a microradiography unit (XMR, Diffraction Technology Pty Ltd) with a PANalytical glass XRD fine focus tube with a copper target (Spectris Australia Pty Ltd) powered by a DF3 generator (Spellman High Voltage Electronics Corporation). Each glass plate was developed in Microchrome Developer D5 for 5 minutes, placed into glacial acetic acid stop bath for 30 seconds and then fixed in Microchrome Fixer F4 for 5 minutes.

2.4 Microdensitometry

Radiographic images of the lesions were viewed via transmitted light through a microscope. The images were acquired by a digital camera and analyzed using imaging software Image Pro Plus Version 7.0. Images of the lesions and the neighboring areas of sound enamel were scanned using the programs line luminance function that gave readings in gray values. An area free of artifacts or cracks was selected for analysis. Each scan comprised 200 readings taken from the tooth surface through the lesion to sound enamel. The aluminum stepwedge image on each slide was scanned and the averaged step gray value readings were plotted against aluminum thickness. The readings of the tooth section image were within the linear portion of the stepwedge curve and linear regression was used to convert the gray value data into values of equivalent thickness of aluminum. The section thickness was measured and the % volume mineral (% vol min) data computed using the equation of Angmar [27] and the linear absorption coefficients of aluminum, organic matter plus water and apatitic mineral (131.5, 11.3 and 260.5 respectively). The image of the median strip of sound enamel between the two lesions was scanned six times and averaged to give a control sound-enamel densitometric profile. The lesion images (remineralisation windows and demineralised control windows) to the gingival and occlusal side of the median strip were similarly scanned, as close as possible to the median strip but avoiding any irregularities commonly found at the lesion edges, and the % vol min profiles computed.

2.5 Remineralisation analysis

The % vol min profile of each enamel block's demineralised and remineralised lesion were compared with the median sound enamel % vol min profile of the same section. The difference between the areas under the densitometric profile of the remineralised lesion and the median sound enamel, calculated by trapezoidal integration, is represented by ΔZd. The difference

between the areas under the densitometric profile of the remineralised lesion and the median sound enamel, calculated by trapezoidal integration, is represented by ΔZr. These parameters were then converted to % change values after remineralisation; % remineralisation (%R) represents the % change in ΔZ values:

$$\%R = \frac{\Delta Zd - \Delta Zr}{\Delta Zd} \times 100$$

2.6 Statistical analysis

2.6.1 Sample size calculation

The number of enamel blocks used for this study was determined using IBM SamplePower package for two one-sided tests for equivalence assuming no difference between treatment means, $\alpha = 0.05$, power $(1 - \beta) \geq 0.85$, equivalence threshold $(\delta) = 12$ and a range of standard deviations (4 - 8). A sample size of n = 6 per treatment provided sufficient power against this range of SD values.

2.6.2 Test for homoscedasticity

Data were analysed with SPSS Version 21. The distributions of residuals for each of the parameters LDd, ΔZd and ΔZd-ΔZr and %R for all AS solutions were determined. Homoscedasticity across all treatments was assessed using Levene's test [28].

2.6.3 Tests for equivalence

Equivalence across all five AS solutions and those five AS solutions containing CPP-ACP with respect to percent remineralisation (%R) was tested using an F-test for equivalence for more than two normal distributions [29] using STATISTICA 12 and Excel 2013 software. Then 10 pair-wise comparisons for the five AS solutions with or without CPP-ACP using two one sided tests (TOST; [30]) of equivalence with a Bonferroni correction for multiple comparisons were performed to test equivalence of each pair of test AS solutions using QI Macros for Excel and Excel 2013 software.

2.6.4 Tests for differences

As the distribution of residuals for the ten AS solutions for LDd, ΔZd and ΔZd-ΔZr approximated a normal distribution and homoscedasticity was confirmed with Levene's test, differences were measured using a one-way ANOVA with *post hoc* Dunnett's tests for comparison with a control group. As the distribution of residuals for percent remineralisation (%R) values for the ten AS solutions was skewed, differences between the %R values were measured with a Kruskal-Wallis test and *post hoc* Wilcoxon Rank Sum tests with a Bonferroni correction for multiple pairwise comparisons.

3. Results

The effect of the four polyols (xylitol, sorbitol, maltitol and mannitol) on remineralisation of enamel subsurface lesions by AS is shown in Table 1. There was no significant difference in size (ΔZd and Ld) of the demineralised lesions before remineralisation and there was no significant difference in the level of mineral gain (ΔZd - ΔZr) between any of the polyol/AS

solutions compared with the AS alone (Table 1). Furthermore, all the polyols were equivalent with respect to the level of remineralisation (%R) observed.

The effect of four polyols on remineralisation by AS/CPP-ACP is shown in Table 2 and Fig 1. With this higher remineralising solution there was also no significant difference in the level of mineral gain (ΔZd - ΔZr) between any of the polyol/AS/CPP-ACP solutions (Table 2), and again all the polyols were equivalent with respect to the level of remineralisation (%R) observed.

The overall mean %R for the AS and AS/polyol solutions was 2.13 ± 0.21% (Table 1) and the overall mean %R for the AS/CPP-ACP and AS/CPP-ACP/polyol solutions was 19.56 ± 0.97 (Table 2), indicating that the addition of 0.376% w/v CPP-ACP to the artificial saliva significantly ($p < 0.001$) increased remineralisation by 9.2 times.

Representative images of the microradiographs of the lesions remineralised by the AS/CPP-ACP alone and the four polyols are presented in Fig 1. These images show the normal pattern of subsurface remineralisation seen with AS/CPP-ACP suggesting no influence of the polyols on the pattern of subsurface remineralisation.

4. Discussion

This controlled *in vitro* study showed that polyols at the maximum physiologically relevant concentration (12.6% w/v) related to that released into saliva by chewing sugar-free gum did not promote remineralisation of enamel subsurface lesions by artificial saliva or by artificial saliva containing the salivary biomimetic CPP-ACP. The polyols studied were xylitol, sorbitol, maltitol and mannitol; the common polyols used in commercial available sugar-free chewing

gums. Hence the results of the current study are not consistent with previous claims that the polyol xylitol can "induce remineralisation of deeper layers of demineralised enamel by facilitating Ca^{2+} movement and accessibility" [22]. These authors used a 20% w/v xylitol remineralisation solution with two weeks *in vitro* exposure. This concentration is above what would be achieved by chewing a normal xylitol-containing gum and this qualitative study did not provide a statistical analysis of the total lesion remineralisation levels, such that the results do not support the promotion of nett enamel subsurface remineralisation by xylitol. It is possible that the very high concentration of xylitol used hindered ion diffusion into the lesion explaining the lower level of remineralisation of the surface layer and perhaps the noticeable difference in pattern of remineralisation. However at the 12.6% w/v concentration used in the current study we could not detect a change in the pattern of subsurface remineralisation in the presence of any of the polyols (Fig. 1), such that it would appear that, at the more physiologically relevant concentration, the polyols have little influence on the diffusion of calcium and phosphate ions into the lesion.

The claim that xylitol and sorbitol can promote enamel subsurface remineralisation by facilitating calcium uptake into the lesion appears to relate back to an earlier study by Makinen and Soderling [21] who investigated the formation of Ca^{2+}-polyol coordination complexes at very high polyol concentrations in a saturated calcium sulphate solution. Under these conditions the authors reported extremely weak stability constants (K) for sorbitol ($K = 0.81\ M^{-1}$) and xylitol ($K = 0.67\ M^{-1}$). They further showed that at 0.5 M xylitol and sorbitol retarded the formation of calcium phosphate solid phases. Based on these findings the authors speculated that these polyols "may favourably govern remineralisation of carious lesions". The belief that xylitol can remineralise enamel subsurface lesions by forming calcium complexes is still

apparent in the more recent literature [31-33] although unequivocal evidence to support the claim is still lacking. The concentrations and conditions used by Makinen and Soderling [21] to define extremely weak calcium-polyol complexes are not physiologically relevant and cannot be extrapolated to the conditions in saliva upon release of the polyols on gum chewing which produce much lower peak salivary concentrations for only a few minutes. Under more physiologically relevant conditions we could not show any direct influence of the four polyols (xylitol, sorbitol, maltitol or mannitol) on remineralisation of enamel subsurface lesions even with constant exposure in artificial saliva for seven days at 37°C which was an exaggerated exposure time relative to the transient exposure time (a few minutes) to the peak polyol concentration achieved in saliva upon chewing sugar-free gum. The findings of the present study are consistent with the *in vitro* study of Amaechi et al. [34] who concluded that xylitol at 20% in artificial saliva did not enhance remineralisation of enamel subsurface lesions over artificial saliva alone.

5. Conclusions

In conclusion, this controlled *in vitro* investigation suggested that polyols (xylitol, sorbitol, maltitol and mannitol) at physiologically relevant concentrations released into saliva upon gum chewing do not promote remineralisation of enamel subsurface lesions by forming Ca^{2+}-polyol complexes and facilitating calcium uptake into the lesion.

Declaration of Interests

The authors declare that they have no conflict of interest.

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Figure Legends

Fig 1. Representative microradiographic images. Representative images of transverse microradiographs of a demineralised lesion before treatment and five remineralised lesions alter treatment with AS/CPP-ACP, AS/CPP-ACP + sortibol, AS/CPP-ACP + maltitol, AS/CPP-ACP + xylitol and AS/CPP-ACP + mannitol. The scale bar on each image represents 100 μm.

Table 1. Effect of polyols on remineralisation of enamel subsurface lesions by artificial saliva.

Solution	LDd (µm)	ΔZd (vol%min.µm)	ΔZd – ΔZr (vol%min.µm)	%R
AS	91.30 ± 17.67*[a]	2240.53 ± 713.35[a]	53.70 ± 62.00[a]	2.41 ± 2.96[a]
Xylitol/AS	91.12 ± 13.15[a]	2545.78 ± 304.25[a]	51.02 ± 85.80[a]	2.00 ± 3.26[a]
Sorbitol/AS	91.04 ± 22.80[a]	2458.66 ± 362.09[a]	51.05 ± 88.42[a]	2.07 ± 3.23[a]
Maltitol/AS	103.03 ± 9.91[a]	3138.58 ± 520.61[a]	66.06 ± 124.01[a]	2.10 ± 3.79[a]
Mannitol/AS	92.06 ± 9.88[a]	2113.05 ± 417.23[a]	47.27 ± 77.11[a]	2.26 ± 3.75[a]
F-test for Equivalence				$p < 0.05$[#]
TOST				$p < 0.05$[§]
Overall Mean				2.13 ± 0.21

LDd = Depth of demineralized lesion before remineralisation (µm);

ΔZd = Difference between the areas under the densitometric profile of the demineralised lesion and the median sound enamel, calculated by trapezoidal integration (vol%min.µm);

ΔZd-ΔZr = Difference between the areas under the densitometric profile of the demineralized lesion and the median sound enamel, and the remineralised lesion and median sound enamel calculated by trapezoidal integration (vol%min.µm);

%R = Percent remineralisation - the % change in ΔZ values relative to ΔZd: %R= (ΔZd-ΔZr)/ΔZd) x 100%.

TOST = Two One Sided Tests (for Equivalence)

*mean ± standard deviation (n = 6)

[#]all five test AS solutions using overall F-test are equivalent ($p < 0.05$)

[§]each pair of test AS solutions using TOSTs are equivalent ($p < 0.05$)

[a]no significant difference between values in the same column ($p > 0.05$)

Table 2. Effect of polyols on remineralisation of enamel subsurface lesions by CPP-ACP in artificial saliva.

Solution	LDd (μm)	ΔZd (vol%min.μm)	ΔZd – ΔZr (vol%min.μm)	%R
AS/CPP-ACP	96.22 ± 11.35*[a]	2282.74 ± 404.48[a]	428.42 ± 131.42[a]	18.62 ± 3.81[a]
Xylitol/AS/CPP-ACP	97.81 ± 14.07[a]	2421.86 ± 666.14[a]	495.72 ± 139.80[a]	20.50 ± 2.26[a]
Sorbitol/AS/CPP-ACP	93.23 ± 17.60[a]	2032.32 ± 423.08[a]	392.22 ± 84.43[a]	19.42 ± 2.63[a]
Maltitol/AS/CPP-ACP	95.50 ± 11.33[a]	2080.75 ± 556.25[a]	422.61 ± 106.40[a]	20.62 ± 3.04[a]
Mannitol/AS/CPP-ACP	94.38 ± 21.33[a]	2443.04 ± 793.10[a]	467.69 ± 211.28[a]	18.64 ± 2.69[a]
F-test for Equivalence of AS/CPP-ACP solutions				$p < 0.05$[#]
TOST for AS/CPP-ACP solutions				$p < 0.05$[§]
Overall Mean				19.56 ± 0.97

LDd = Depth of demineralized lesion before remineralisation (μm);

ΔZd = Difference between the areas under the densitometric profile of the demineralised lesion and the median sound enamel, calculated by trapezoidal integration (vol%min.μm);

ΔZd-ΔZr = Difference between the areas under the densitometric profile of the demineralized lesion and the median sound enamel, and the remineralised lesion and median sound enamel calculated by trapezoidal integration (vol%min.μm);

%R = Percent remineralisation - the % change in ΔZ values relative to ΔZd: %R= (ΔZd-ΔZr)/ΔZd) x 100%.

TOST = Two One Sided Tests (for Equivalence)

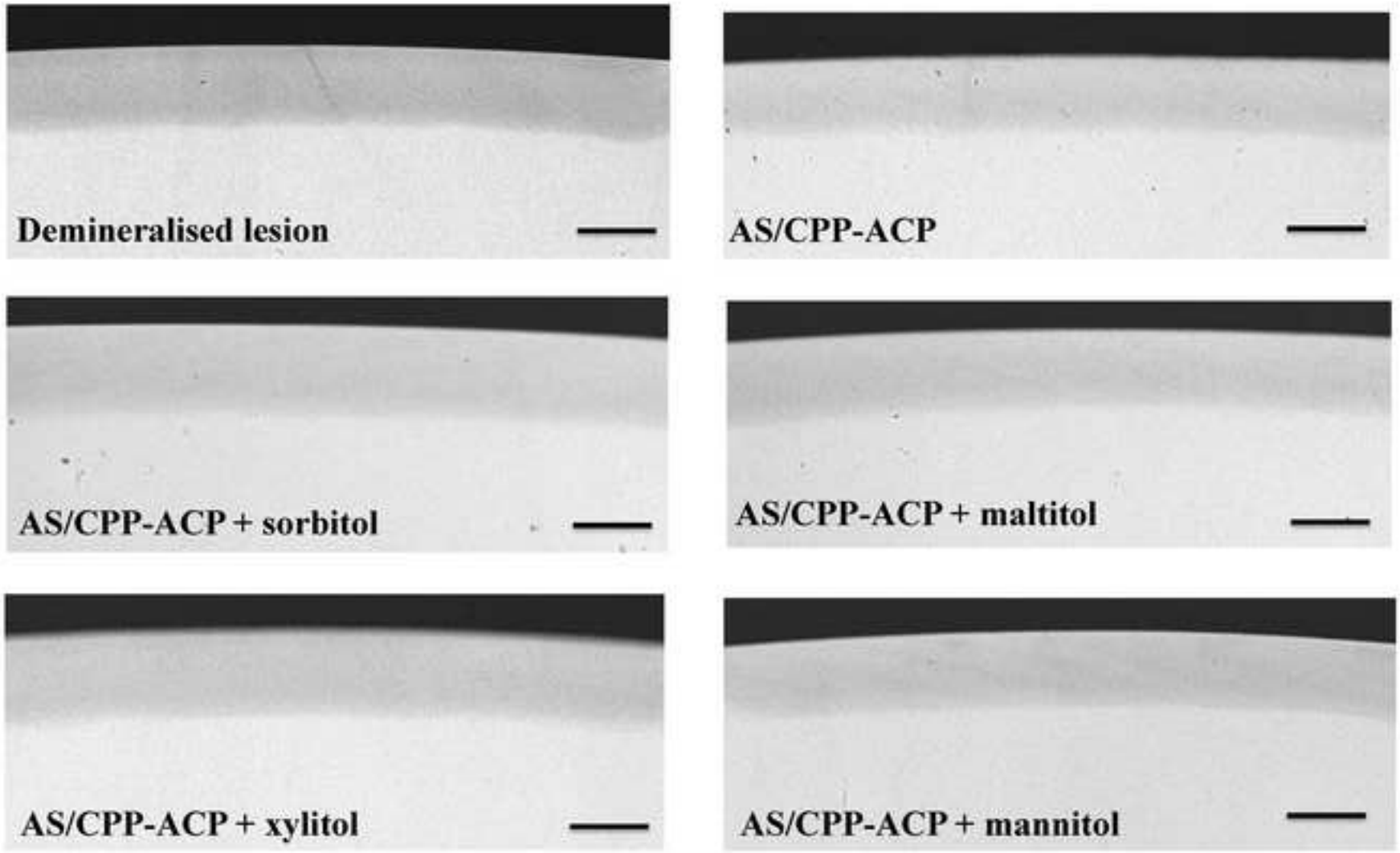
*mean ± standard deviation (n = 6)

[#]all five test AS/CPP-ACP solutions using overall F-test are equivalent ($p < 0.05$).

[§]each pair of test AS/CPP-ACP solutions using TOST are equivalent ($p < 0.05$)

[a]no significant difference between any value in the same column

Figure 1


Demineralised lesion
AS/CPP-ACP
AS/CPP-ACP + sorbitol
AS/CPP-ACP + maltitol
AS/CPP-ACP + xylitol
AS/CPP-ACP + mannitol

Submission to Journal of Dentistry

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