Characterizing the appearance of *ex vivo* remineralized white spot lesions with a novel peptide

SHERWIN HABIBI

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COMMITTEE:
GREG HUANG
GEOFF GREENLEE
HANSON FONG

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ABSTRACT
Characterizing the appearance of *ex vivo* remineralized white spot lesions with a novel peptide

Sherwin Habibi

Chair of Supervisory Committee:
Greg J. Huang
Department of Orthodontics

Background: White spot lesions (WSLs) are unfortunate post-orthodontic sequelae that can compromise the final esthetics of treatment, result in caries, and persist indefinitely. Current methods to treat WSLs may promote remineralization, but have not demonstrated consistent improvement with respect to the appearance of the lesion. **Purpose**: This study explores the remineralizing potential of a novel peptide, amelogenin-derived peptide 5 (ADP5), in comparison with MI Paste Plus (MIPP) and topical fluoride treatment. **Methods**: Artificial WSLs were created on *ex vivo* human molars. Teeth were sectioned into samples and randomly assigned to one of four arms: (1) a daily regimen of ADP5 solution, (2) a daily regimen of MIPP, (3) a daily regimen of 20,000 ppm fluoride solution, and (4) incubation in an artificial saliva control. Samples were treated for a period of 2 weeks. Photographs were taken prior to and after treatment. Two panels comprising 5 dental professionals and 5 laypersons assessed before and after pairs of photographs in a blinded fashion. Scanning electron microscopy (SEM) was performed on most samples after treatment to assess the subsurface lesion microstructure and the effects of treatment. **Results**: After creating WSLs, 11 of these were randomly assigned to the ADP5 group, 12 to the MIPP group, 12 to the fluoride group, and 10 to the control group. The mean improvements assessed by the expert panel were 16%, 16%, 15%, and 8% in the ADP5, MIPP, fluoride solution, and control groups, respectively. The mean improvements assessed by the lay panel were 16%, 13%, 16%, and 8%, respectively. Single factor ANOVA revealed no difference in the amount of improvement across the 4 study groups. SEM images were qualitatively assessed. Samples treated with ADP5 showed an appositional layer of approximately 5 μm and a subsurface remineralization depth of 25 μm. Control samples did not appear to display any apposition or any remineralization. MIPP and fluoride samples were more variable in their presentation, with subsurface remineralization depths ranging from 0 to 25 μm in both groups. **Conclusion**: Treatment of WSLs with ADP5 did not produce a significantly greater visual improvement when compared to the other treatment groups or the control over the 2-week period. SEM imaging revealed a more consistently achieved remineralization in ADP5 groups compared to MIPP and fluoride groups, as well as a thin appositional layer that was often present.

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INTRODUCTION

Background:

1. The problem

The first clinically visible stage in the development of dental caries is the incipient, or white spot, lesion. In orthodontics, white spot lesions (WSLs) are common post-treatment findings seen on facial surfaces, typically around the area where brackets were once placed (Figure 1). WSLs may be seen within six months of starting fixed orthodontic appliances (Tufekci et al., 2011), and can remain indefinitely after the completion of treatment (Maxfield et al., 2012). Enaia and colleagues (2011) found that while 32% of their patients had some form of WSL prior to orthodontic treatment, after treatment, the number of patients with WSLs had increased to 74%. Moreover, 10% of these ultimately resulted in cavitated lesions requiring restoration (Enaia et al., 2011). The prevalence of post-orthodontic treatment WSLs has been reported at 5-97% (Gorelick et al., 1982; Boersma et al., 2005). First molars, upper lateral incisors, and lower canines are most commonly affected, and WSLs are predominantly found in the gingival third of the crown (Øgaard, 2008).

Like dental caries, the etiology for WSLs stems from the proliferation of cariogenic bacteria, which can arise around brackets during orthodontic treatment. These bacteria produce acid which decreases the mineral content in tooth material (Naranjo et al., 2006). In many orthodontic patients, the demineralization is arrested before cavitation, but the visible white marks persist indefinitely. WSLs compromise the final esthetics of completed orthodontic care, are difficult to fully reverse, and may even lead to frank cavitation requiring restoration. They can increase costs, time, and trauma to patients. Thus, there is great interest in developing a technique to reverse the demineralization and the accompanying discoloration of the enamel.

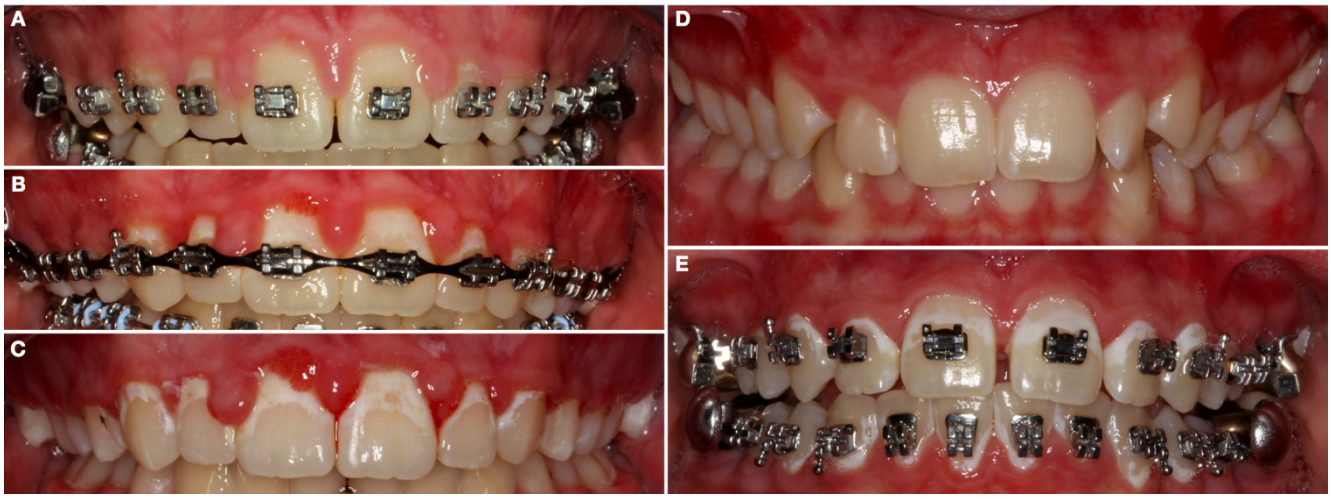

Figure 1. Examples of white spot lesions developing during orthodontic treatment. **A-C**, a patient 6 months into treatment, at 18 months, and at debond. This patient's treatment was terminated earlier due to poor oral hygiene, generalized gingivitis, and extensive WSLs. **D-E**, a different patient prior to the start of treatment, and only 6 months into treatment.

2. Prevention

Prevention of WSLs is ideal, and effective diet control as well as brushing and flossing is of paramount importance. Literature exists on the prevention of WSL and dental caries in children and adolescents with the administration of fluoride (Marinho et al., 2003). However, there is little evidence on the optimal concentration, frequency, and mode of fluoride delivery during orthodontic treatment (Benson et al., 2013). Methods of delivery include topical fluoride (found in toothpastes, rinses, gels, and varnishes), dietary fluoride, and fluoride-impregnated bonding materials. Fluoride hinders the demineralization of enamel, enhances remineralization, and inhibits metabolic enzymes in acid-producing bacteria (Lynch et al., 2006; ten Cate, 2013). Periodic fluoride varnish application every six weeks during orthodontic treatment has been shown to reduce the incidence of WSLs by nearly 70% (Stecksen-Blicks et al., 2007), but similar findings have yet to be substantiated by further research. There is very limited high-quality evidence on the use of fluoride to prevent WSLs (Benson et al., 2013), and unfortunately, whatever degree of prevention that is observed requires repeated application.

More recently, Recaldent (a complex of casein phosphopeptide and amorphous calcium phosphate) has been marketed under the brand of MI Paste to reduce the incidence of WSLs and to remineralize enamel lesions after orthodontic treatment. The active agent is thought to aid remineralization by localizing calcium, phosphate, and fluoride at the tooth surface, releasing it slowly in the hope of remineralizing deeper layers in a WSL (Reynolds, 2008). MI Paste (with or without the addition of sodium fluoride) was shown *ex vivo* to decrease enamel demineralization (Sudjalim et al., 2007).

3. Treatment

Various strategies to deal with the lesions have been studied, including trying to facilitate remineralization of the tooth structure, restoring the lesion, or camouflaging it with shade alteration. An ideal treatment for a WSL would involve a non-invasive complete re-mineralization of the lesion, as well as a return of the tooth surface to its original appearance prior to the WSL.

Application of topical fluoride has shown to increase the rate of remineralization of WSLs after orthodontic treatment, and to help normalize tooth appearance (Øgaard, 2008). Due to its topical nature, the remineralization is characteristically on the superficial layers of enamel, and has difficulty reaching inner demineralized layers, especially in deeper WSLs (Bishara & Ostby, 2008; Castellano & Donly, 2004; Mellberg et al., 1985; ten Cate et al., 2008; Trairatvorakul et al., 2008). For this reason, there is concern that the use of fluoride to treat a WSL will preclude the remineralization of the innermost portions of the WSL, and the visual appearance of the white spot may persist (Garcia-Godoy & Hicks, 2008; Linton, 1996; Øgaard et al., 1988; Willmot, 2008).

In addition to its application in WSL prevention, MI Paste has been used to remineralize WSLs, mostly in *in vitro* studies (Iijima et al., 2004; Kumar et al., 2008; Manton et al., 2008; Reynolds et al., 2003; Shen et al., 2001; Walker et al., 2006; Walker et al., 2010; Willershausen et al., 2009). Reynolds and colleagues (2008) showed that 100 µm WSLs treated *in situ* with MI Paste had microradiographic improvements that returned the demineralized portion to a

radiographic appearance resembling that of sound enamel. Unfortunately, they did not supplement these findings with photos of the sampled teeth to enable assessment of improvement in surface appearance. There is a general lack of studies to show clinical improvements with the use of MI Paste compared with regular home care. MI Paste "Plus" (MIPP), which includes 900 ppm sodium fluoride, has been shown *in vitro* to re-mineralize WSLs to an even higher degree (Reynolds et al., 2008; Chen et al., 2013).

It has been noted that WSLs sometimes diminish or disappear on their own, with only a regular home oral hygiene regimen. Huang et al. (2013) conducted a clinical trial where the appearance of WSLs was evaluated after treatment with fluoride varnish, MIPP, or without any additional treatment beyond a natural home care regimen with regular fluoridated toothpaste. They found that there was no difference over an 8-week period in any of the three groups. However, while some WSLs improve over time, orthodontic patients still have significantly higher incidences of WSLs compared to those with no treatment (Øgaard, 1989).

Other strategies when dealing with WSLs include camouflaging them by relative whitening of the surrounding tooth structure with dental bleaching. While this addresses the relative appearance, it does nothing to restore mineral content. As such, the susceptibility to future caries increases in lesions treated by bleaching (Knösel et al., 2007). Therefore, this treatment modality is best restricted to patients with lower caries risk and great oral hygiene.

Finally, the most invasive treatment of WSLs involves restorative procedures. These can include crowns or veneers in extreme cases. While these do nothing to restore mineral content, and further subtract enamel, esthetics can be improved. More recently, superficial composite resin infiltration techniques have been shown to be minimally invasive and successful in masking WSLs, with success documented *in vitro* exceeding lesion depths of 400 µm (Paris et al., 2007). While infiltration with resin such as the "Icon Infiltrant" system has shown an *in vitro* stability of at least 8 weeks (Senestraro, 2013), long-term stability has yet to be determined.

It is clear that the best approach to deal with WSLs is to prevent them. When they must be treated, an ideal and stable treatment involves increasing the mineral content throughout the entire depth of the lesion, in addition to returning the enamel in a non-invasive manner to its original appearance. None of the above treatments has shown superior remineralization and improvement in appearance without sacrificing layers of enamel.

4. New strategy

Amelogenin is one of the principal proteins involved in the mineralization of enamel during tooth formation. It controls both the dimensions and direction of forming hydroxyapatite crystals in the enamel matrix (Bartlett et al., 2006). A recent study has identified a 22-amino acid long peptide chain within the wild type mouse amelogenin protein (rM180) that can mineralize hydroxyapatite tooth structure at a comparable rate to that of the full-length protein (Gungormus et al., 2012). This peptide, named amelogenin-derived peptide 5 (ADP5), has been shown to absorb on the demineralized root surface of human teeth. It yields the apposition of a mineral layer integrated with underlying tooth structure by attracting soluble calcium and phosphate ions, increasing local saturation, and exerting kinetic control over the catalysis of calcium phosphate (Gungormus et al., 2012). A more recent study has demonstrated this

peptide's effectiveness in remineralizing enamel (Dogan et al., 2018). The catalytic characteristic of ADP5 has implications in clinical applications, such as reducing dentinal hypersensitivity, treating caries, and most pertinent to this study, in treating WSLs. There is a unique challenge in treating WSL, however, in that mineralization must take place within the already porous enamel in order to restore mineral density and appearance. An aim of this study was to find a mineralization strategy, using the ADP5 peptide, that favors subsurface mineralization within the WSL as opposed to surface apposition, as shown in Figure 2.

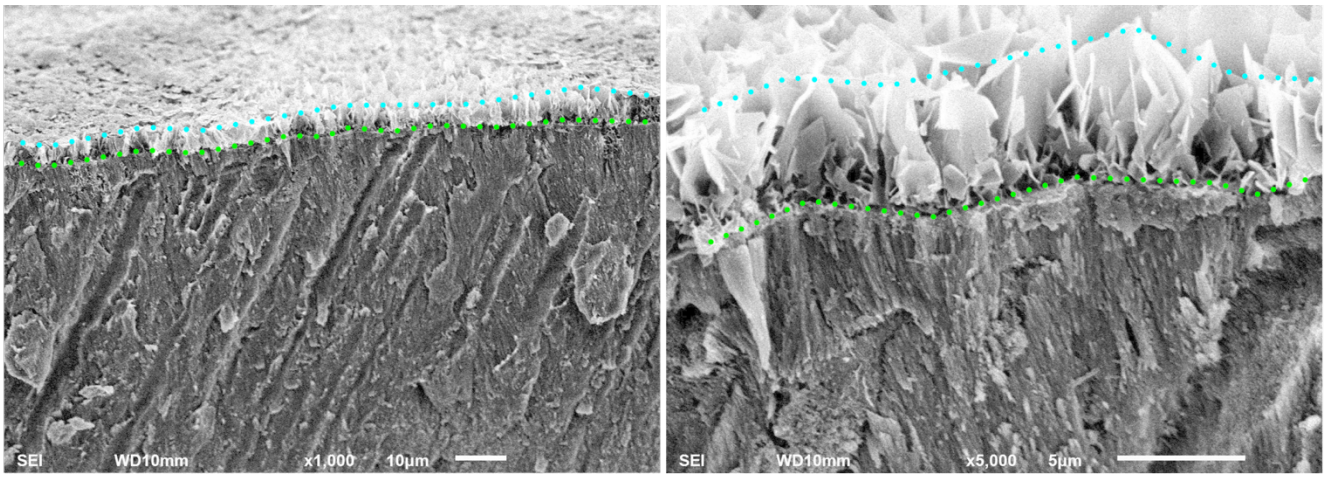

Figure 2. Preliminary SEMs showing an appositional layer on the tooth surface using higher ion concentrations. This resulted in a very crystalline and clear structure of the appositional layer, but limited visible subsurface mineralization**.** The shown apposition was achieved with a KH_2PO_4 concentration of 2.80 mM and a $CaCl_2$ concentration of 4.80 mM. The green line marks the junction between the original enamel surface (extending upwards) and the specimen in cross section (downwards). Blue lines demonstrate an appositional layer. Two different magnifications are shown, with scale bars visible for each SEM.

Significance and objectives:

The potential depth of ADP5 remineralization has not yet been characterized, nor has a comparison been made about the appearance of teeth treated with ADP5 compared to other treatment. Therefore, the purpose of this study is twofold. First, to develop a peptide-guided mineralization strategy that is able to drive mineralization within the WSL (mineral penetration), and second, to determine the change in appearance using ADP5 on *ex vivo* teeth with artificially created white spot lesions, in comparison to MI Paste Plus and fluoride solution. The depth of remineralization using ADP5 will also be assessed in comparison to the other treatments.

METHODS

Sample collection:

De-identified human molars were collected from the University of Washington School of Dentistry and other non-academic dental offices. Teeth were cleaned of debris and soft tissue, placed in a 10% sodium hypochlorite solution for two hours at room temperature for disinfection, washed with distilled water and then stored in a 50% ethanol solution (as per institutional guidelines). Samples were exempt from human participants' approval by the Human Subjects Division at the University of Washington. Through visual inspection, teeth with at least one surface devoid of developmental defects, cracks, caries, WSLs, restorations, or areas of discoloration were selected for inclusion in the study. Teeth with endodontic treatment were excluded.

A priori power calculation determined the need for a total sample size of 32 (8 per treatment arm). This was based on 4 treatment groups, an effect size of 0.83, alpha of 0.05, and a power of 0.95. The effect size was generated by assuming remineralization depths for the four treatment groups as inferred from the literature (Reynolds et al., 2008). Sample sizes of 14 and a standard deviation of 40 were used for all four groups. Mean remineralization values of 80, 80, 25, and 0 μm were used for peptide, MIPP, fluoride, and control, respectively.

Tooth preparation:

21 teeth were selected and polished with medium grain laboratory pumice slurry (Henry Schein, Melville, NY, USA) to restore a glossy surface. The teeth were then painted with two coats of red, acid-resistant nail varnish (Wet n Wild, Los Angeles, CA, USA), leaving an exposed window (approximately 3 x 3 mm) on the defect-free surface for demineralization. Up to 6 windows per tooth were prepared in this manner, as the crown surface could accommodate. Buccal, lingual, and interproximal surfaces were used. The nail varnish was kept on throughout the demineralization process and was removed prior to remineralization. The entire tooth preparation process is summarized in Figure 3.

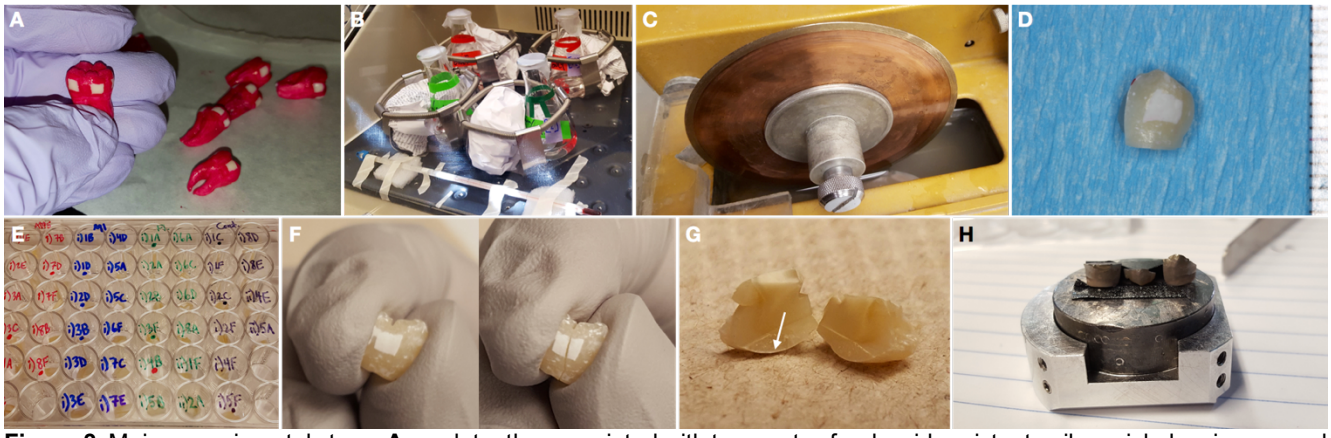

Figure 3. Major experimental steps. **A**, each tooth was painted with two coats of red, acid-resistant nail varnish, leaving several 3x3 mm unvarnished windows per tooth. **B**, teeth were incubated in a temperature-controlled shaker in acidic solution for 14 days, with a 6 hour period of mineralization solution each day. **C**, after varnish removal, teeth were sectioned into individual window samples with a diamond coated rotary disk. **D**, photographs were taken for each sample before and after treatment with standardized conditions and a ruler for scale. **E**, samples were randomly allocated to the 4 arms of the study. **F**, after treatment, samples were fractured through the WSL to produce **G**, segments ready to be examined in cross section (arrow indicates a visible depth of WSL). **H**, fragmented samples coated with Au/Pd and ready for SEM.

Demineralization:

Artificial WSLs were produced by 14 days of daily cycling at 37°C between demineralization and neutral solutions for 18 hours and 6 hours, respectively, as shown in Jones et al. (2006). Demineralization solution was an aqueous solution containing 2.0 mM KH_2PO_4 (USB Corporation, Cleveland, OH, USA), 2.0 mM $CaCl_2$ (Johnson Matthey Inc., Seabrook, NH, USA) and 75 mM acetic acid (EMD Chemicals, Savannah, GA, USA) at pH 4.3. Mineralization solution was an aqueous solution containing 0.9 mM KH_2PO_4, 1.5 mM $CaCl_2$, 150 mM KCl (Johnson Matthey Inc., Seabrook, NH, USA) and 20 mM Tris (Fisher Sci., Hapton, NH, USA) buffered at pH 7.0. WSLs of approximately 100 µm in depth were consistently created by this method, as determined by preliminary SEM imaging (Figure 4). Each exchange of solutions was preceded by a thorough rinse with distilled water. This demineralization-mineralization cycling was found through previous pilot work to allow for fewer cavitations during the creation of WSLs. After samples were created, they were stored in distilled water and refrigerated at 4°C until remineralization could begin.

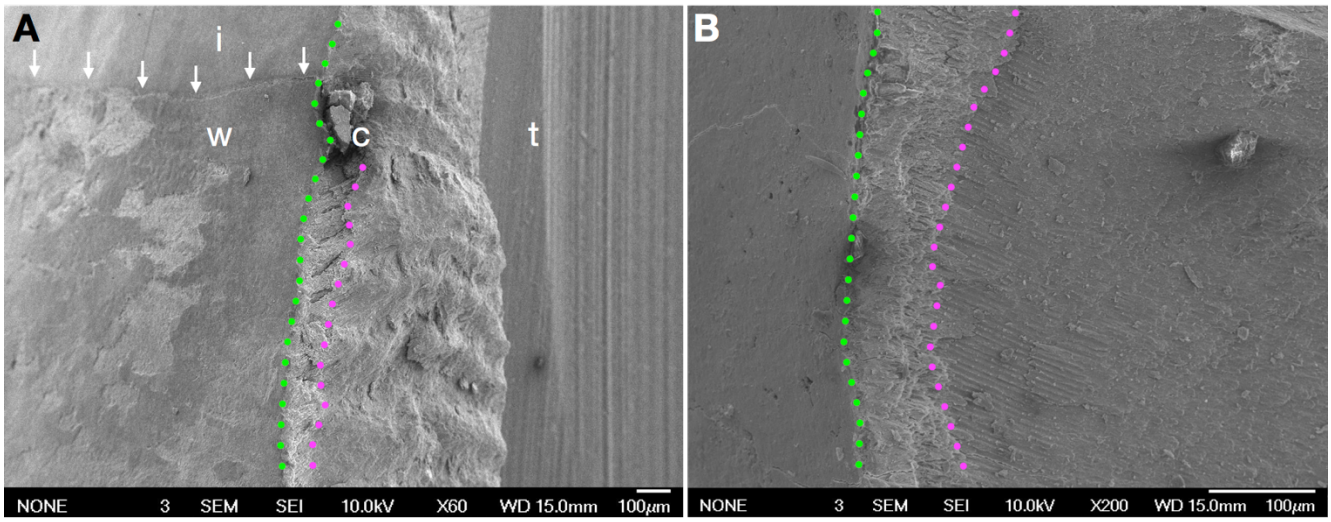

Figure 4. Control samples. **A**, cross section through both the WSL window and the intact enamel. The green line indicates the junction between the enamel surface (extending to the left) and the specimen in cross section (to the right), and the fuchsia line delineates the extent of the WSL, shown here to be approximately 100 µm. Arrows denote the edge of the surface window, with the demineralized zone below (w), and the intact tooth structure above (i). This cross section also shows the striations from the diamond disk used to notch samples (t), as well as a small area of lesion cavitation (c). **B**, higher magnification of a different control sample, showing that white spot lesions of approximately 100 µm were consistently produced using the described method. Scale bars are indicated for each image.

Remineralization:

Varnish was removed by placing the teeth in acetone for 5 minutes, gently swabbing away residual varnish, followed by two more acetone rinses, and a final rinse in distilled water. Roots of all molars were sectioned away at the CEJ by use of a diamond coated rotary disk. Each crown was then carefully sectioned into discrete pieces, each containing 1 demineralized window, and with as much peripheral non-WSL enamel surface as possible. Using this method, 45 samples were generated. With the same rotary disk, notches were cut into each sample from the most interior portion, outward to the DEJ. This was done to facilitate fracturing the samples later on, and because adding these notches post-mineralization could disturb the sample windows. Characteristic data for each sample was recorded, including the tooth from which the sample came, molar type (1^{st}, 2^{nd} or 3^{rd} molar), the specific tooth surface, and the condition of the WSL.

Samples were randomly divided into 4 experimental groups: the mineralizing peptide (ADP5), fluoride solution (F), MI Paste Plus (GC America, Alsip, IL, USA), and a no treatment control. 45 samples were treated. Experimental conditions unique to each group's treatment type were repeated every day for two weeks and are described below.

The control group was stored in artificial saliva at 4°C for two weeks. Artificial saliva consisted of 50 mM KCl, 0.4 mM $MgCl_2$-$6H_2O$ (JT Baker, Phillipsburg, NJ), 1.1 mM $CaCl_2$, 0.6 mM KH_2PO_4, and 20 mM Hepes (Fisher BioReagent, Fair Lawn, NJ), with pH adjusted to 7.

For peptide-directed mineralization, the following procedure was used (after a series of experimentations to achieve mineral penetration into the WSL). Peptide solution (0.8 mM, 300 µL) was pipetted to completely cover each lesion. After ten minutes at 37°C, the peptide solution was replaced with 500 µL of 1.89 mM KH_2PO_4 and 3.20 mM $CaCl_2$ at pH 7.4. Samples

were stored 37°C for 2 hours, and then the ion solution was replaced by artificial saliva for the remaining 22 hours at 37°C.

A similar process was followed for the F solution group except there was no peptide treatment. Samples were submerged in 500 µL of 1.89 mM KH_2PO_4, 3.20 mM $CaCl_2$, and now 20,000 ppm NaF (J.T. Baker, Phillipsburg, NJ, USA) at pH 7.4. Samples were stored 37°C for 1 hour, and then replaced by artificial saliva for the remaining 23 hours at 37°C.

The at-home application process of MIPP was adapted to a laboratory setting from the manufacturer's instructions. A pea-sized amount of MIPP was smeared onto each lesion for 3 minutes in a 100% humidity chamber at 37°C. Thereafter, the excess was wiped off with a cotton swab, leaving a visible film of paste on the tooth surface for an additional 30 minutes in 100% humidity at 37°C. Then, without disturbing the tooth surface, samples were transferred to 1 mL of artificial saliva for the remainder of the 24 hour cycle, at 37°C.

Visual data collection:

Photos were taken of samples after demineralization and after mineralization phases. Photography, lighting, and position was standardized to the extent possible, with samples blotted dry prior to photography with a Canon T5i (manual mode, aperture f/32, shutter speed 1/125, ISO 100) and Canon Macro Ring Lite MR-14EX II (manual mode, flash at ¼ output). Each image was captured with manual focus and an infrared remote shutter to reduce vibrations. Each sample was given an identifier for blinding, and before and after photos were digitally uploaded in pairs to slides on Keynote (Apple, Cupertino, CA, USA) and matched for magnification and rotation. Photos were cropped to remove the background. A slideshow was prepared for a panel of peers to assess, and 3 versions were made with a shuffled sample order.

A visual analogue scale (VAS) was used for assessing changes in WSL as done in Huang et al. (2013), and calibration was performed for each rater: if the WSL had stayed unchanged (0% improvement, or worse), had completely vanished (100% improvement), or anywhere in between. Rater calibration was conducted using a series of standardized photos with the opacity of the WSL digitally reduced on Photoshop (Adobe, San Jose, CA, USA) as seen in Figure 5. The rating panel consisted of 5 dentists and 5 lay people; raters were compensated with gift cards for their time. Each rater used approximately ten minutes to assess 45 samples on a standardized computer monitor. For each sample, a median VAS score was determined in each panel. Improvement scores were then averaged per treatment type and the mean improvement was calculated for each panel.

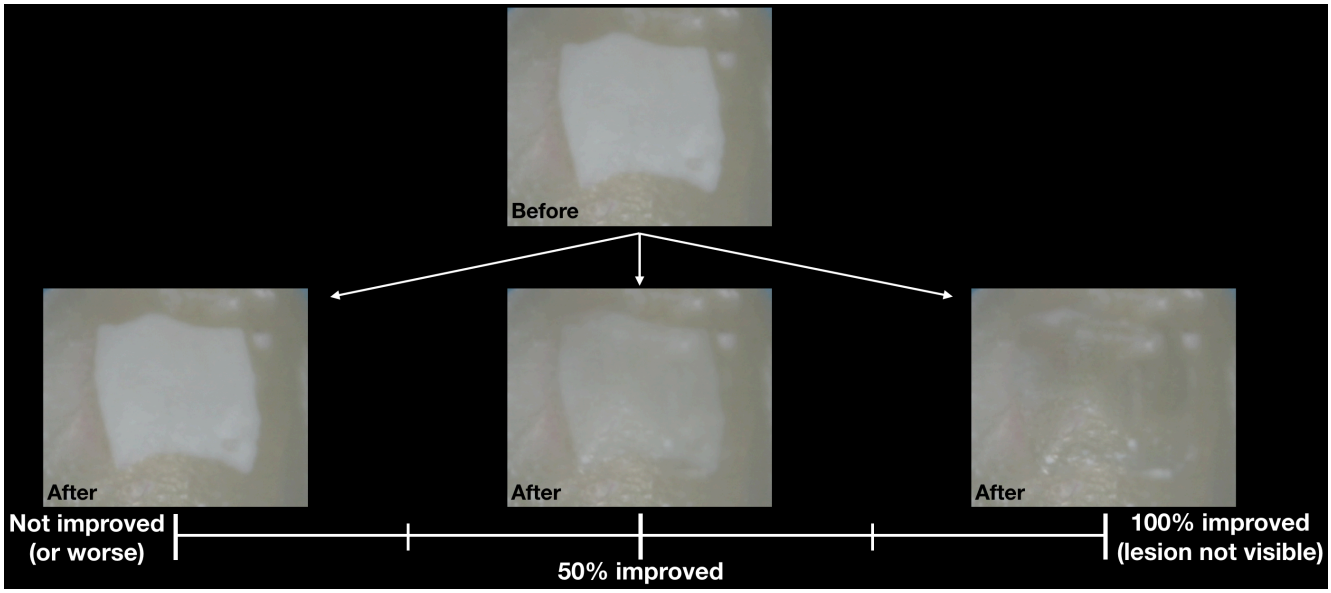

Figure 5. A representation of the calibration shown to each assessor, prior to VAS rating.

Scanning electron microscopy data collection:

After all photographic imaging was completed, teeth were fractured through the WSLs by applying pressure on either side of the previously created notch. Each sample was coated with 5 nm of Au/Pd for electrical conductivity. Scanning electron microscopy (SEM) images were recorded of cross-sectioned lesions using a JEOL JSM 7000F SEM (JEOL, Peabody, MA, USA) at 10 kV, in secondary electron imaging mode.

Under SEM, samples were surveyed for a representative area that could show the depth of WSL. Snapshots were taken at 200x, 1500x, 4000x, and 10,000x magnification, to highlight the surface boundary, any visible appositional layer, and any penetrated remineralization (Figure 6). Layers were differentiated by noting differences in apparent density and porosity, with unaffected enamel appearing the most dense and least porous, while demineralized WSLs appeared the least dense and most porous. Subsurface remineralization had an appearance approaching that of unaffected enamel, but did not necessarily appear as dense. Interfaces between visible layers of apposition, remineralization, and WSL were demarcated. WSLs were identified in a blinded fashion by two different investigators in order to increase reliability.

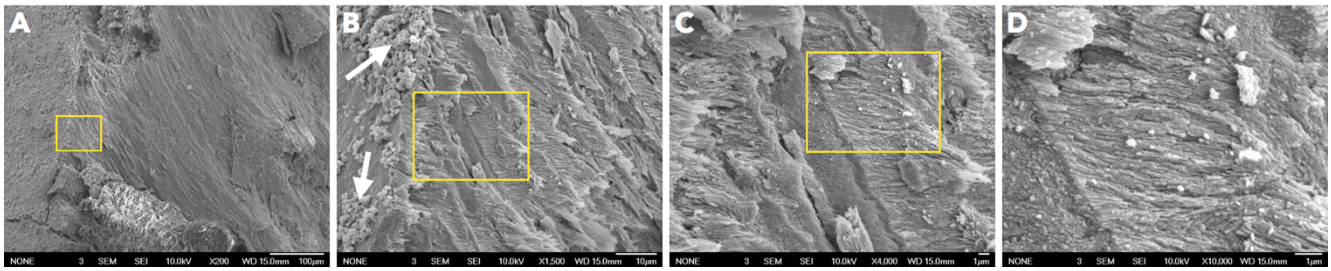

Figure 6. SEM images were captured at various magnifications to the lesion at large in cross-section. **A-D** represent images of the same sample at 200x, 1500x, 4000x, and 10,000x magnification, respectively, and focusing near the tooth surface. Yellow boxes highlight the region magnified to capture the subsequent image. In each image, the tooth surface is oriented on the left, while the inner tooth structure in cross-section is on the right. Clusters of calcium phosphate likely precipitated in solution and inadvertently bound to the tooth surface (as noted by white arrows in **B**). Scale bars are indicated for each image.

RESULTS

Descriptive analysis:

A total of 21 teeth were prepared for this study, with each tooth contributing 4 to 6 surfaces. Of these, 4 teeth were randomly selected and used as pre-treatment controls to confirm WSL depths achievable and optimal treatment parameters. The remaining 17 teeth gave rise to 45 samples, which were randomly assigned to treatment arms. Of the 45 samples, 11 were allocated for treatment with the peptide, 12 with MIPP, 12 with fluoride solution, and 10 with the control solution. The 4 arms of the study were well matched in tooth characteristics (Table I). Characteristics examined included: if the tooth was a 1st or 2nd molar as opposed to a 3rd molar, the type of surface upon which the sample was created, and the surface condition of the sample (i.e., if there was a cavitation anywhere on the surface). Baseline characteristics across the 4 arms were compared with the Fisher-Freeman-Halton exact test statistic. P values of 0.401, 0.989, and 0.852 were calculated when comparing baseline molar type, surface type, and condition, respectively. None of these were significant.

Table I. Comparison of baseline characteristics across the 4 treatment arms

	Peptide (n = 11)		MIPP (n = 12)		Fluoride (n = 12)		Control (n = 10)		Total (n = 45)	
	N	%	N	%	N	%	N	%	N	%
Molar type										
1st / 2nd	8	73	11	92	9	75	6	60	34	76
3rd	3	27	1	8	3	25	4	40	11	24
Surface										
Buccal	4	36	4	33	4	33	2	20	14	31
Lingual	4	36	4	33	4	33	4	40	16	36
Proximal	3	27	4	33	4	33	4	40	15	33
Condition										
Pristine	8	73	7	58	9	75	7	70	31	69
Minor cavitation	3	27	5	42	3	25	3	30	14	31

Visual improvement:

The averages of the median improvements assessed by the expert panel were approximately 16%, 16%, 15%, and 8% in the peptide, MIPP, fluoride solution, and control groups, respectively (Table II). The mean improvements assessed by the lay panel were approximately 16%, 13%, 16%, and 8%, respectively. ANOVA revealed no significant difference in improvement among the 4 arms of the study, with p values of 0.442 and 0.313 for the expert and lay panels, respectively (Table III).

Table II. Improvement scores by treatment group

	Peptide (n = 11)		MIPP (n = 12)		Fluoride (n = 12)		Control (n = 10)	
Assessment	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Expert panel	15.7	12.8	15.5	16.6	14.9	10.1	8.0	7.8
Lay panel	16.0	9.7	12.8	13.3	16.3	14.9	7.5	8.1

Table III. Single factor ANOVA output for expert panels, comparing by: treatment, tooth type, surface type, and sample condition

A. Treatment arms

Summary

Groups	*Count*	*Sum*	*Average*	*Variance*	*St. Dev*
Peptide	11	173	15.727	165.018	12.846
MIPP	12	186	15.500	276.636	16.632
Fluoride	12	179	14.917	101.720	10.086
Control	10	80	8.000	61.556	7.846

ANOVA - single factor

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	426.702	3	142.234	0.916	**0.442**	2.833
Within Groups	6366.098	41	155.271			
Total	6792.8	44				

B. Tooth type

Summary

Groups	*Count*	*Sum*	*Average*	*Variance*	*St. Dev*
1st/2nd molars	34	474	13.941	168.360	12.975
3rd molars	11	144	13.091	123.091	11.095

ANOVA - single factor

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6.009	1	6.009	0.038	**0.846**	4.067
Within Groups	6786.791	43	157.832			
Total	6792.8	44				

C. Surface type

Summary

Groups	*Count*	*Sum*	*Average*	*Variance*	*St. Dev*
Buccal	14	203	14.500	246.577	15.703
Lingual	16	206	12.875	143.050	11.960
Interproximal	14	189	13.500	106.269	10.309

ANOVA - single factor

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	19.886	2	9.943	0.061	**0.941**	3.226
Within Groups	6732.750	41	164.213			
Total	6752.636	43				

D. Sample condition

Summary

Groups	*Count*	*Sum*	*Average*	*Variance*	*St. Dev*
Pristine	31	448	14.452	130.256	11.413
Minor cavitation	14	170	12.143	217.978	14.764

ANOVA - single factor

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	51.408	1	51.408	0.328	**0.570**	4.067
Within Groups	6741.392	43	156.777			
Total	6792.8	44				

Since no difference was found by treatment group, single factor ANOVA was also carried out to assess if any of the sample characteristics shown in Table I had any effect on visual improvement, irrespective of the treatment method used. All samples were assessed in this manner, once for the expert panel, and once for the lay panel. As an example, output tables for the expert panel are shown in Table III. The expert panel had p values of 0.846, 0.941, and 0.570 for comparing tooth type, surface type, and sample condition, respectively. The lay panel had p values of 0.868, 0.964, and 0.984, respectively. In all instances, there were no significant findings, and all characteristic groupings had similar VAS scores.

Overall, expert and lay panels had similar assessments and variations. Scatterplots of the improvement in WSLs over the 2-week treatment period, stratified by assessment panel and treatment arm, showed a wide variation in WSL visual improvement (Figure 7). Scores were generally distributed evenly across the improvement scale.

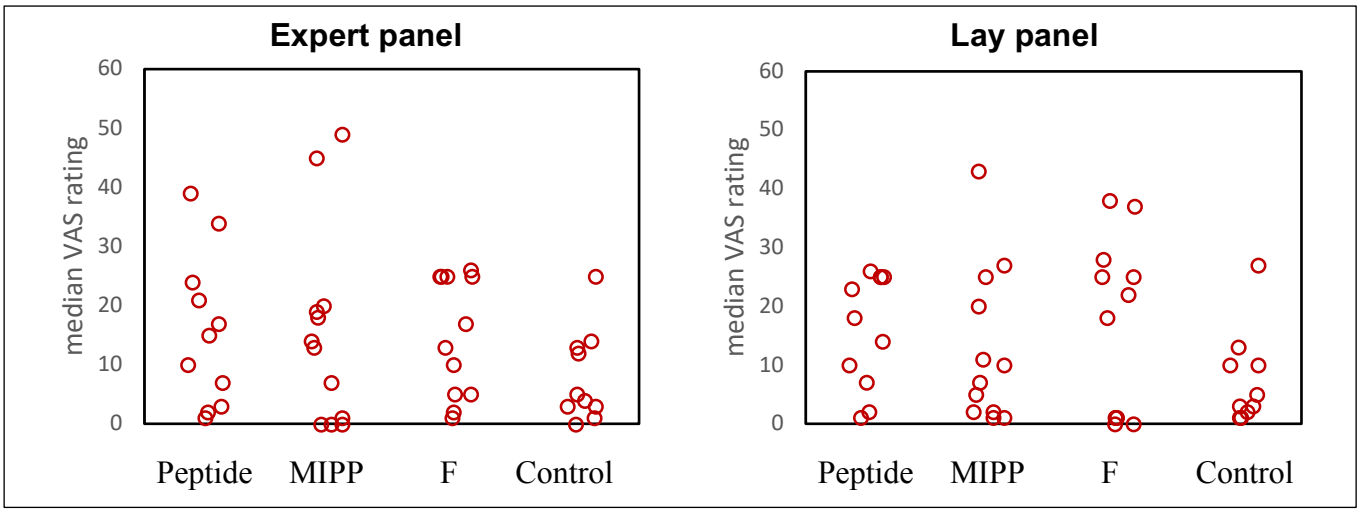

Figure 7. Scatterplot showing improvement in WSLs, displayed by assessor panel and then by treatment type.

Scanning electron microscopy assessment:

SEM images were captured at four different magnification levels for most of the remineralized samples. If present, apposition and penetration were assessed at the 1500x magnification, while penetration (again) and the depth of WSL were assessed at the 200x magnification. In total, 9, 8, 10, and 5 images were captured from peptide, MIPP, fluoride, and control samples, respectively. Due to the limited quantity and some variability in the quality of specimens/images, a quantitative approach was not possible and instead, the interpretation focused on qualitative approach.

In general, image interpretation followed the following guidelines to demarcate the untreated WSL regions, apposition layers, penetrated mineral layers, and unaffected, healthy enamel (Figure 8). Regions that appeared porous, especially with crystals pointing in random orientations were interpreted as untreated WSL regions. In contrast, healthy enamel presented with clear enamel rod demarcations and well-aligned crystals within rods. Regions immediately below the tooth surface showing a mineral layer that was discontinuous from the underlying enamel were interpreted as apposition layers. Directly beneath the apposition layer, and appearing denser than the WSL region, was interpreted as penetrated remineralized layer, which resembled the appearance of health enamel.

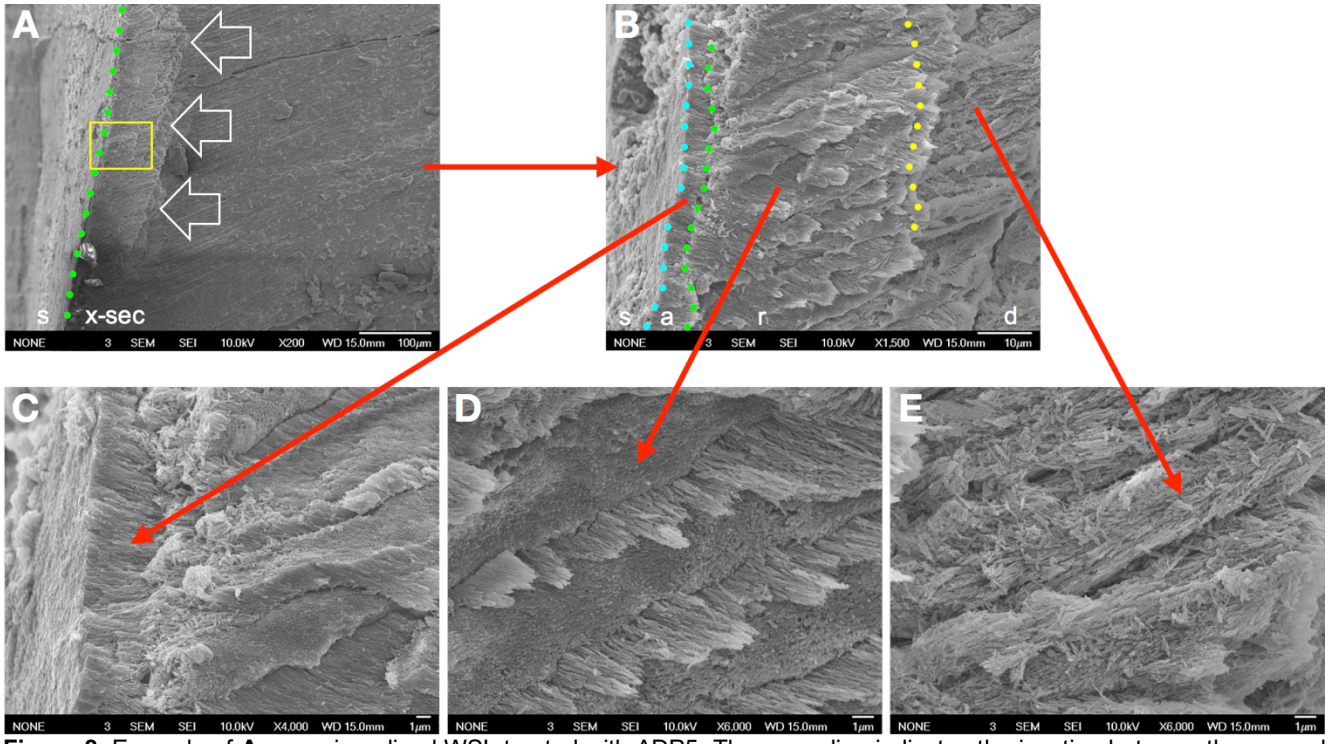

Figure 8. Example of **A**, a remineralized WSL treated with ADP5. The green line indicates the junction between the enamel surface (extending to the left) and the specimen in cross section (to the right). White arrows indicate the WSL depth boundary, shown here to be 100 µm. **B**, a magnified remineralized region displaying a cross section and visible structural layers of tooth surface (s), appositional layer (a), subsurface remineralization (r), and the remainder of the demineralized WSL (d). These four layers are separated, in order, by blue, green, and yellow lines. Yellow lines were placed between regions of decreased porosity on the left and regions of increased porosity on the right. Magnified regions are shown: **C**, the apposition layer; **D**, a penetrated subsurface remineralized WSL; and **E**, unaffected and persistent WSL. Scale bars are indicated for each image.

Mineralization layers were demarcated by two assessors in a blinded fashion. Then, images were grouped based on their treatment group in order to ascertain any visual trends within and between groups. As previously noted, imaged WSL depths were all approximately 100 µm. In general, samples treated with the peptide showed an appositional layer of approximately 5 µm and a penetration depth of approximately 25 µm (interpreted to be remineralization). Control samples did not typically display any apposition or penetration. MIPP and fluoride samples were more variable in their appearance, with penetration depths ranging from 0 to 25 µm in both groups. Two samples in the MIPP, as well as a surprising one sample in the fluoride group, displayed what appeared to be about a 5 µm appositional layer. Figure 9 displays representative SEM images, along with the accompanying visual changes that reflect these observed trends.

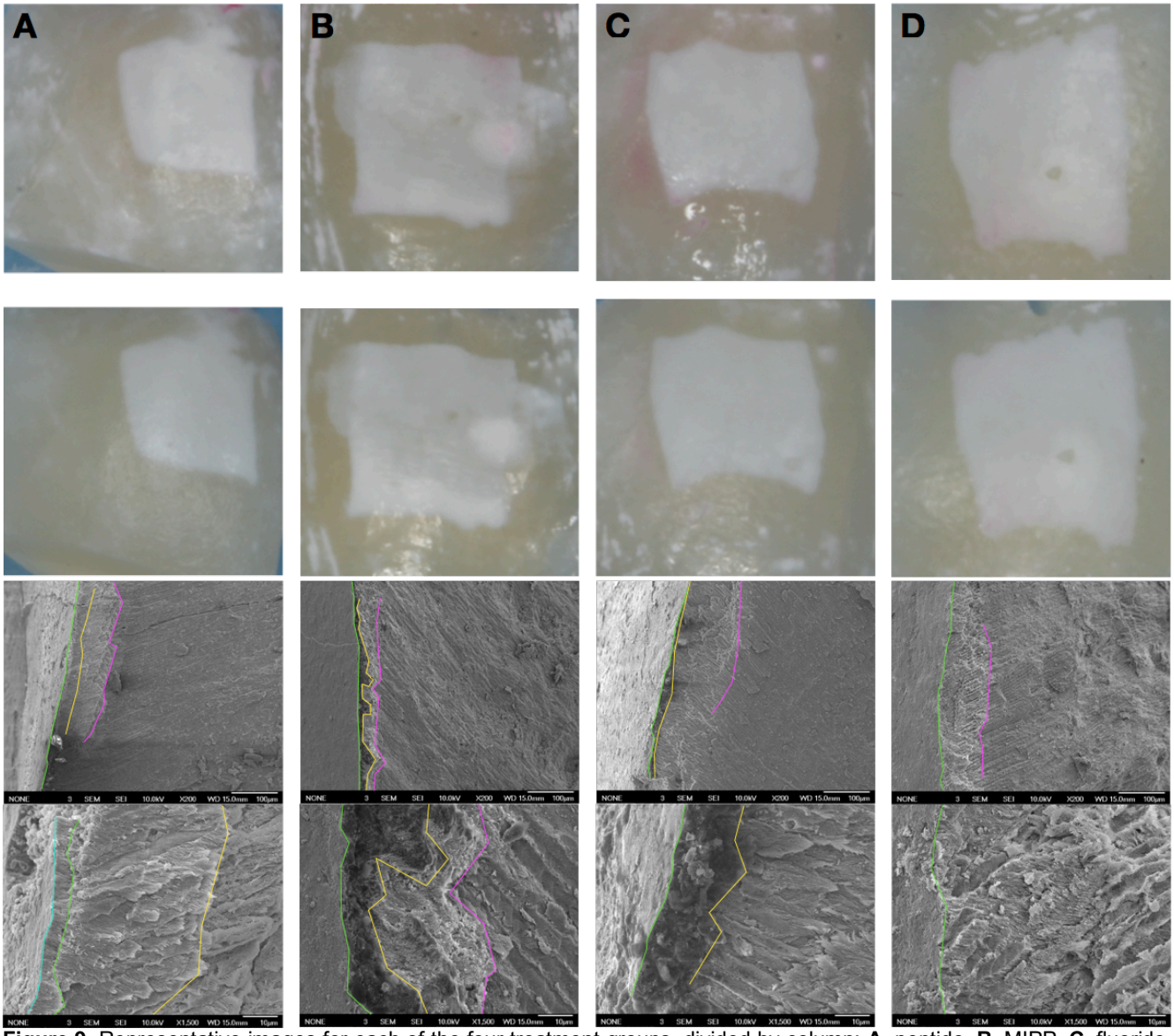

Figure 9. Representative images for each of the four treatment groups, divided by column: **A**, peptide, **B**, MIPP, **C**, fluoride, and **D**, control. In descending order, rows represent pre-treatment (post-demineralization) photos, post-treatment photos, 200x magnification SEMs, and 1500x SEMs. Scale bars are visible for each SEM. The green line marks the junction between the original enamel surface (extending to the left) and the specimen in cross section (to the right). Yellow lines delineate the interface between penetrative remineralization on the left and the remainder of the WSL on the right. Fuchsia line delineates the extent of the WSL. When present, blue lines demonstrate an appositional layer.

DISCUSSION

This study primarily aimed to evaluate the improvement in visual appearance of artificially created *ex vivo* WSLs after treatment with a novel peptide, and to contrast it with the change observed in other established treatment protocols (MI Paste Plus and fluoride solution) during a 2-week period. The secondary objective was to determine the depth of remineralization possible using the peptide and characterize it in relation to the other treatments.

Effects of ADP5:

The opaque, chalky appearance of WSLs arises from the light-scattering effect in porous, partially demineralized enamel. The hypothesis of this study is, therefore, by restoring the enamel mineral density, i.e. eliminating porosity, via peptide directed remineralization, the natural tooth color appearance will be restored, hence, result in improvement in WSL appearance. Based on the results of the VAS study, at least at the levels of remineralization achieved, there is no obvious association between the level of remineralization and improvement in WSL appearance. There were samples with increased apposition and/or penetration, but with modest improvement in VAS score. Likewise, there were samples with minimal or no apposition and/or penetration that showed relatively high VAS score improvements. ADP5 did not produce superior improvement of the appearance of WSL when compared to the other two treatments, and while all three treatment arms were scored at approximately twice that of the control samples, the difference between all groups was statistically insignificant.

There is currently no clinical data on the visual effectiveness of the ADP5 peptide, since it is a relatively novel peptide, and is still in the laboratory testing phase of research. However, plenty of randomized controlled trials exist on the effects of MIPP and fluoride. Most of the previous studies have used a 12-week treatment period, and measure results using clinical observation, photography, and/or some form of fluorescence imaging. It has been reported by some that fluorescence imaging techniques such as Quantitative Light-induced Fluorescence are comparable to visual assessment (Höchli et al., 2017). The results on the effect of MI paste and MIPP have been contradictory. Some authors have shown that both MI Paste and MIPP significantly improved WSLs after a 12-week period in comparison to home regimen controls, with reports of 31-38% better improvement over controls (Bailey et al., 2009; Andersson et al., 2007; Clark, 2011). Conversely, both MI Paste (Shell, 2012) and MIPP (Shell, 2012; Beerens et al., 2010; Huang et al., 2013) have also been shown to produce no significant improvement over regular home care, also over a 8 to 12-week period. The treatment period that was closest to our study was 4 weeks, and according to that study, MI Paste decreased the lesion size, but not to a statistically significant extent (Bröchner et al., 2011).

The inconsistent findings persist when examining the effects of fluoride. Fluoride varnish was reported to improve the appearance of WSL size significantly when compared to controls, when measured by fluorescence and over 24-week periods, with relative improvements of 8-12% over controls (Du et al., 2012; He et al., 2016). Our findings on fluoride solution were similar (7-9% improvement), but were not statistically significant. At the same time, while some

degree of improvement was observed, other studies failed to show a significant difference between fluoride varnish groups and home regimen controls over similar treatment durations (Huang et al., 2013; Miresmaeili et al., 2012). Willmot (2004) reported a similar finding with low-fluoride mouth rinses over a 12 and 26-week treatment period, where they observed a decrease in size of the WSL, but to no statistically significant improvement over the control.

While the current body of literature is by no means consistent, these interventions for WSLs often provide statistically insignificant improvements in either the visual appearance or enamel fluorescence. However, even when there are statistically significant changes, their clinical significance is still questionable when compared to typical home care regimen (Höchli et al., 2017). We treated samples for a 2-week period, and this may have been too short to show maximal effect, in contrast to the aforementioned studies. The implication of this is that continuing treatment for a longer period of time might allow for increased visual improvements, since there would be more time for the effects of treatment to manifest.

This *ex vivo* study found that all three treated groups did show approximately twice the visual improvement seen in the no-treatment control group. However, single factor ANOVA demonstrated that there was generally no statistically significant difference in the mean VAS scores between any of the treatment groups over the course of the 2-week period. Both the expert panel and the lay panel showed similar results. The peptide appeared to offer minimally better visual improvement, when compared to the MIPP or fluoride groups, and was not statistically different from the control. Visual assessment of ADP5 treatment has not been done before, so a *post hoc* power calculation was performed using data in Table II, showing that our experiment had a power of 52%. Had we wished to achieve a power of 80%, we would have needed 20 samples in each group (total sample of 80).

According to our SEM analyses, peptide samples were relatively consistent in showing approximately 1-5 μm of layer apposition, as well as up to 25 μm of penetrative remineralization. Data on durability of the apposition layer and region penetrated by new mineralization was not generated in this study, but will be the subject of investigation in future studies. However, it is noted that in an *ex vivo* study of ADP peptides, Gungormous et al. (2012) found that ADP5 produced a mineral layer of 10 μm on dentin with comparable properties to human cementum, and overall remained intact after mechanical abrasion and ultrasonication. While additional analyses are planned to determine the exact composition of the appositional and penetrative layer, based on the morphology and previous pilot research using Energy Dispersive X-ray Spectroscopy (EDXS), ADP5-guided mineralization likely yielded a penetrative layer that was hydroxyapatite, as well as an appositional layer that was likely a mixture of hydroxyapatite and small amounts of octacalcium phosphate.

Prior to treatment, penetrative remineralization was maximized and apposition minimized by varying ion concentrations and pH. The most optimal combination was concentrations of 0.8 mM, 1.89 mM, and 3.20 mM for the peptide, KH_2PO_4, and $CaCl_2$, respectively. The most optimal pH was found to be 7.4. Further optimization of peptide concentration will be examined in future studies, with the hope of deeper remineralization. Ion concentrations that were higher resulted in more crystalline appositional layers, but limited the extent of subsurface mineralization (Figure 2).

In contrast, our control samples generally did not show any apposition or remineralization. The MIPP and fluoride SEMs were more variable in their appearance, with

remineralization depths ranging from 0 to 25 μm in both groups. Only two samples in the MIPP and one of the samples in the fluoride group displayed what appeared as around 5 μm of appositional layer. Apposition layer with MIPP treatment is believed to be a combination of calcium phosphate, silicon oxide, and a number of organic substances which primarily act as thickening agents. This is based on previous EDXS, knowledge of the chemical content listed on the MIPP label, as well as the darker appearance of the appositional layer compared to the enamel mineral. The darker appearance translated to the content having a lower average atomic number, most likely carbon-rich organic content that was deposited onto the enamel surface. While mechanical tests are needed to confirm the mechanical properties of the MIPP layer, because of the significant amount of organic content present, it is likely that the MIPP-treated layers were softer than the healthy enamel mineral layers or peptide-treated mineral layers.

Topical application of fluoride is believed to enhance remineralization by adsorbing onto demineralized enamel crystals and attracting calcium and phosphate ions, forming stronger, fluoroapatite crystals. Casein phosphopeptide combined with amorphous calcium phosphate (the active components of MI Paste) also attract and stabilize calcium and phosphate, promoting subsurface remineralization conditions (Reynolds et al., 2008). Similarly, ADP5 is thought to act by attracting calcium and phosphate ions to oppositely charged peptide residues, creating a local supersaturation that promotes remineralization. Unlike topical fluoride, ADP5 appears to have insignificant binding affinity to hydroxyapatite (Gungormus et al., 2012). While samples from peptide, MIPP, and fluoride groups did show subsurface remineralization as seen by SEM, it was rare for remineralization to extend much farther than 25 μm. It is of interest to further optimize the remineralization strategy to maximize mineral penetration into WSL and determine if there is a maximum threshold beyond which remineralization cannot progress. This will need to be coupled with mechanical tests to compare the mechanical properties of the remineralized WSL to that of healthy enamel. It is possible that some deeper WSLs may not be fully reversible through remineralization, and would require invasive treatments for full esthetic resolution.

An interesting observation throughout this study was that peptide-treated samples did show some improvement in WSL appearance in the periphery of the 3x3 windows. These smaller WSLs arose unintentionally during the demineralization process due to flaking away of some of the protective varnish and were likely shallower lesions. This study did not investigate these smaller lesions in cross-section with SEM, but it would be interesting to explore the possibility of complete visual resolution of shallower WSLs with ADP5 or other agents.

Limitations:

The study did encounter several limitations, and they were related to variations in specimens within groups, as well as SEM imaging. For example, the WSLs in some of the specimens cavitated during treatment or specimen preparation, rendering them unsuitable for remineralization assessment. Smaller cavitations appeared on the surface of many of the WSLs, despite efforts to demineralize slowly. This could have caused distractions during visual assessments, and spoiled many samples intended for SEM imaging, leading to sample attrition. This concern of cavitation prevented us from demineralizing for longer periods and potentially achieving deeper WSLs that better represent those found *in vivo*, which can extend as far as 400 μm deep (Cochrane et al., 2012). Although our lesions of 100 μm depth were

similar to those reported in other studies (Reynolds et al., 2008), deeper lesions may have been achieved if a smaller window (such as 3 mm x 1 mm) were used, or if fluoride cycling were included in the demineralization process.

Furthermore, in order to visualize the extent of mineral apposition and penetration by SEM, lesion windows had to be fractured in cross-section. This process resulted in some of the specimens fracturing along the boundaries of the lesions and prevented accurate assessment of treatment in their respective groups. For these reasons and constraints with resources, not all samples were imaged, reducing the originally intended image numbers. It should be noted that possible bias may have been exercised in the SEM analysis because it was performed by an investigator not blinded by sample types.

Additionally, due to non-uniform specimen shapes, a non-standardized angulation of samples resulted in differing and unmeasurable degrees of foreshortening, which confound any possible linear measurements. For these reasons, it was established that the SEM data must be taken more so on a qualitative basis, and statistical analysis was of little value. Comparing each SEM image to its VAS scores quickly highlighted that increased penetration did not necessarily correspond to a better visual improvement. The appositional and penetrative layers have not yet been analyzed regarding their composition, and it should be emphasized that we are assuming the penetrative layer indicated remineralization.

Finally, fracturing and preparing samples in cross-section for SEM prevented us from viewing the internal structure of the same sample both before and after treatment. Non-destructive imaging techniques such as micro CT were considered, but the resolution of the imaging available was not enough to capture some of the smaller phenomena, such as the very thin appositional layers.

The teeth which benefit esthetically the most are the maxillary incisors. For tooth collection, ideally, only pristine maxillary incisors extracted for periodontal reasons would have been used, with WSLs created only on facial surfaces. However, since tooth collection proved to be challenging and extracted incisors so scarce, the study had to rely mainly on extracted molars, including third molars, and required the use of as many different surfaces per tooth as possible. The anatomical variation and variable environmental exposure of third molars was also a limitation. Finally, despite clear instructions to the many dental offices supplying the extracted teeth, there was no guarantee that teeth would be rinsed and stored only in the provided 50% ethanol. For example, some offices may have placed the teeth in bleach for prolonged periods of time. This could desiccate and possibly compromise the integrity of the samples. Prolonged exposure to sodium hypochlorite has been shown *in vitro* to decrease the elastic modulus and flexural strength of dentin (Sim et al., 2001).

As seen in Huang et al. (2013), there was a wide range of VAS scores, resulting in a large standard deviation. The subjective nature of WSL assessment is a large limitation, but it is also reflective of the subjective nature with which WSLs are evaluated on a day-to-day basis. Precautions were taken by calibrating assessors and by controlling display conditions on the computer monitor. However, the largest challenge came in standardizing photograph conditions. While camera and room settings were matched, tooth position was very difficult to keep precisely the same between pre and post-treatment samples. This manifested in differential glare, angulations, etc.

Strengths and future research:

Strengths of this study include that each sample was randomly assigned to a group, eliminating systematic error based on tooth type, surface type, or surface integrity of the sample. Descriptive analyses confirmed that the division of teeth per treatment arm was generally random and uniform (Tables I and II). Blinding the investigators during remineralization was not possible, as each treatment had a different protocol. However, the more important instance of blinding was achieved in the subjective evaluations by lay persons and dental professionals.

To reduce proficiency bias, three different versions of the VAS Keynote presentation were prepared, employing a shuffled order of samples. These were consecutively dispersed to each panel member. Five assessors were chosen for each panel, so that a median improvement per sample would be easy to determine. Having an expert panel (comprising 2 orthodontic residents and 3 orthodontic faculty members) as well as a lay person panel (5 individuals chosen at random after ensuring they had no formal education or work experience in the dental field) was important since our primary outcome concerns itself with the subjective opinion that both dental professionals and the public might have on treated WSLs. Radiography and advanced imaging is helpful, but ultimately, the esthetics of WSLs are of primary concern.

Assessors were given standardized instructions to regard only the change in the square lesions, and not peripheral areas. They were also advised to ignore, as best as possible, any cavitations, cracks, or glare. No intra-examiner reliability testing was performed. Assessors were blinded to the sample treatment groups and were only told that the image on the left was “before” and that they were to rate the percentage improvement at the image on the right (“after”). The median % improvement was selected for each sample between all panel assessors to control for outliers. Two different raters independently measured VAS scores for each assessor, and discrepancies larger than 1 mm were re-measured to confirm accuracy. Both raters remained blinded as well.

ADP5 is a newly proposed treatment for WSLs, and as such, laboratory research is required prior to clinical research. The laboratory setting provided many advantages at this stage, such as the ability to guarantee perfect “compliance” with treatment due to tight control and monitoring of sample treatment. There was also a negligible variance of protocol application between samples within the same arm, given that treatment was applied by the same individual for all samples. While WSLs present with a variety of shapes and depths in patients’ mouths, the laboratory setting could standardize the size and depth of lesions to minimize unforeseen confounders. Of course, this strict control is not fully generalizable to a clinical setting, and eventually, clinical experimentation is a necessary step.

Future research can attempt to optimize the mineralization step further to enhance penetration. Furthermore, it can explore the efficacy of involving both peptide and fluoride to determine if there is any synergistic effect, as observed when fluoride is added to MI Paste (Reynolds et al., 2008). Furthermore, a longer treatment period with an increased sample size, clinical trials, and comparison to home-care regimens are important. This study shows that there appears to be subsurface remineralization potential for ADP5, at a level comparable to the other treatment methods, and with similar visual improvement.

CONCLUSION

In this *ex vivo* study, we found that by SEM imaging, a more consistent mineral penetration was achieved in the peptide-directed remineralization group compared to MIPP, fluoride, and no treatment control groups. A thin appositional layer was also present on the enamel surface after treatment in all of the treatment groups, although the apposition in the MIPP group was most likely rich in organic content. While treatment of WSLs with ADP5 did show mineral penetration, it did not produce a significantly greater visual improvement over MIPP, fluoride solution, or the control over the course of a 2-week treatment period. Given our results, further optimization with the ADP5 peptide remineralization strategy may have the potential to restore WSLs functionally and aesthetically.

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