Title
Effect of a new carbon dioxide laser and flouride on caries progression in demineralized enamel

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Effect of a New Carbon Dioxide Laser and Fluoride on Caries Progression in Demineralized Enamel

by

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THESIS

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Dedication

This paper is dedicated to my family.

To my mom and dad, Christine and Stanley Chmiel, who encouraged me to follow my dreams and were there each step of the way. To my brother and sister, Brett and Renee, who provided words of encouragement and inspired me through their own achievements. To my Aunt Jane for her constant love and support.

ACKNOWLEDGEMENTS

The author would like to specially acknowledge my research committee of Drs. John Featherstone, Dan Fried, Arthur Miller and Peter Rechmann who have always been there to guide me through this process and serve as invaluable resources of information. I would like to give special thanks to Marcia Rapozo-Hilo for her indispensable laboratory assistance and patience. I would also like to recognize Beate Rechmann for her considerable knowledge and help with sample preparation. Finally, I wish to thank Charles Le for his help with laser irradiation.

ABSTRACT

Effect of a New Carbon Dioxide Laser and Fluoride on Caries Progression in Demineralized Enamel
Monica Leah Chmiel, DDS

The overall objective of the present study was to further explore the specific set of optimal laser conditions that may be used clinically for the prevention and potential reversal of carious lesions. The specific aim of this study, was to provide experimental evidence that the use of a new prototype carbon dioxide laser (9.6μm, 20 pulses per spot, 20 μs pulse duration, and a 20 Hz repetition rate) combined with fluoride produces a significant protective effect against lesion progression using incident fluences of 2.0 and 4.0 J/cm^2. The hypothesis to be tested is that treatment with a new carbon dioxide laser and fluoride significantly inhibits the progression of artificial caries-like lesions in smooth surface dental enamel to a greater extent than the laser or fluoride treatments alone. This study also explored the effect of fluoride therapy alone, as well as, laser treatment alone and possible sequence effects of combination treatment under these specific laser conditions. Samples (100) of sound enamel were divided into 10 groups. Ninety samples were partially demineralized in a 50% HAP/0.1M lactic acid/carbopol solution (pH 5.0). Experimental groups were exposed to various combinations of laser and fluoride treatments using the above parameters and then submitted to 9 days of pH cycling. New group numbers were randomly assigned by an independent operator to eliminate experimenter bias during sample analysis. Microhardness analysis was performed to determine the relative mineral loss as $\triangle Z$ (volume % x μm). Mean (SD) $\triangle Z$ values for groups I-X were, respectively: 936.17 (770.88); 515.47 (420.84); 703.23 (534.59); 553.57 (260.72); 581.36 (281.76); 488.08 (292.64); 4310.98 (672.52); 4406.15 (1099.18); 5378.44 (644.38) and 2141.70 (1290.43). This new carbon dioxide laser did not have a significant protective effect against lesion progression. Fluoride treatment only with a 5-minute topical gel not only inhibited further lesion progression in a pH-cycling model but also was effective in remineralization of artificial caries-like lesions.

TABLE OF CONTENTS

List of Tables

List of Figures

Introduction

Purpose

The purpose of the present study was to further explore the specific set of optimal laser conditions that may be used clinically for the prevention and potential reversal of carious lesions. Using demineralized smooth surface enamel samples that were exposed to various fluoride and laser treatments, we were able to assess the capacity of these treatments to influence the prevention of lesion progression. This study examined a specific combination of parameters required for the CO_2 laser to produce its positive effects on demineralized smooth surface enamel in the presence of fluoride.

Specific Aims

The specific aims are to explore the effects of:

- Fluoride therapy
- Carbon dioxide laser therapy
- Combination therapy
- Sequence of treatment of combination therapy

Caries Prevention

Caries prevention continues to be a major interest of ongoing research in the dental field. Efforts to find new and more effective clinically relevant ways to combat dental disease have included the use of various strategies. The effectiveness of fluoride as both a preventative and remineralizing agent has been well documented in the literature.[1-4] Professionally applied topical fluoride gel treatments of 4 minutes or more

have been repeatedly proven to be effective in caries reduction.[1] Sodium fluoride and acidulated phosphate fluoride gels are commonly used as topical fluoride treatments in the dental office. There is inadequate support in the literature to substantiate if there is a difference in the effectiveness of sodium fluoride compared with acidulated phosphate gels. The ADA Council on Scientific Affairs approves the clinical recommendations that include fluoride varnish and gel applications at 3 and 6 month intervals for high or moderate-risk individuals, respectively.[4] A reduction in application time without a subsequent decrease in clinical effectiveness would prove beneficial to both the patient and dentist. Patient discomfort would be minimized with shorter treatment times and a decreased risk of accidental swallowing while dentists would be able to provide preventative care with greater efficiency.

There is a growing body of literature regarding the effectiveness of lasers as a preventative agent against enamel demineralization[5-21]. Carbon dioxide lasers have been utilized primarily because they operate at the wavelength range where dental hard tissues have a high absorption coefficient.[16] Carbon dioxide lasers have been widely studied and have demonstrated their caries protective effect when operating at wavelengths between 9.3- to 10.6-μm[22]. Pulsed carbon dioxide lasers have achieved near complete inhibition of demineralization in sound enamel when exposed to an acid challenge.[17] Lakshmi et al. (2001) found similar beneficial effects of laser pre-treatment of enamel with a 10.6 μm pulsed carbon dioxide laser reducing caries-like lesions by 82.7%.[8] Other studies have demonstrated a 50-70% reduction in artificial caries-like lesions after laser treatment in the range of 9-11 μm.[23,24]

The beneficial effects of fluoride are undisputed; however, much interest has been generated regarding its synergistic potential when used in combination with laser treatment. Laser irradiation together with fluoride therapy has shown much promise in its ability to produce beneficial results in caries prevention and, in some cases, reversal of caries progression as shown in laboratory studies.[25-27] The advantageous effects of combined laser and fluoride therapy have been well documented.[18,24,28-30] The key appears to lie in uncovering the specific laser parameters that permit the synergism with fluoride to occur. This blend of treatment modalities may help to avoid some of the observed negative side affects of laser treatment alone. This includes the generation of heat following laser irradiation that may lead to possible melting and cracking of the tooth structure, and pulpal damage when high-energy treatment and lengthy exposure times are used to achieve the desired protective effect.

Wavelength and Fluence

A working knowledge of dental hard tissues and their interactions with lasers is required to determine those parameters needed to produce successful results. Dental tissues preferentially absorb CO_2 laser irradiation leading to increased temperatures that permit carbonate loss leaving a hydroxyapatite-like material that is less susceptible to acid attack than the original acid-soluble carbonated hydroxyapatite mineral.[31,32] Laser conditions using wavelengths that are strongly absorbed by dental enamel produce the greatest effect at the lowest fluence (energy/surface area per pulse) with a minimum amount of heat deposition in the tooth. The preferred wavelengths that have been shown to permit efficient and short heating of dental enamel for caries prevention are with carbon dioxide lasers at wavelengths of 9.3 and 9.6 μm[22,24,31] along with fluences that

reduce acid reactivity reported to be between 3-5 J/cm^2.[19] Consistent with these findings, Featherstone and Fried (2001) demonstrated that using low fluences (2-5 J/cm^2), a repetition rate that permits adequate heat dissipation (10Hz) and a short pulse duration (100μs) consistent with the thermal relaxation time of enamel, result in a 60%-70% reduction in caries progression in a laboratory pH cycling model.[16] A similar beneficial effect was found by Hsu et al., (2000) who demonstrated that a low-energy CO_2 laser treatment resulted in an almost complete inhibition of enamel demineralization in their laboratory artificial caries model.[10] In order for lasers to play an effective role in dental caries prevention, the solubility of the dental tissues must be changed, and the laser energy must be efficiently absorbed without inducing undesirable damage of the underlying or surrounding tissues.[22] This is possible if the correct wavelength is selected to match the absorption properties of enamel so that lower fluences can be used to produce these protective effects without producing excess heat that will heat and destroy the pulp.

Pulse Duration and Repetition Rate

Pulse duration and repetition rate are significant factors that influence the caries preventive effect of laser treatment. The pulse duration should correspond with the thermal relaxation time of enamel.[16] One study that evaluated this factor looked at pulse durations of 50, 100, 200 and 500 μs at 9.3 μm with a fluence of 5 J/cm^2. Pulse durations at 500 μs resulted in minor surface melting while 50 μs pulse duration resulted in complete surface melting.[33] It appears that the shorter the pulse duration used, the lower the fluence required to achieve the same effect. Other studies found that a 5 μs pulse duration was adequate in producing beneficial results.[18,34] Featherstone (2003) found

pulse durations as low as 5 and 20 μs useful for caries prevention.[14] Subsequent measures of the absorption coefficients of enamel allowed a more accurate estimate of the thermal relaxation times. At 9.3 and 9.6 μm wavelengths, the relaxation times are 1 and 2 μs, respectively.[5] Repetition rates have varied from 1-20 Hz in studies reported in the literature.[13,25,34,14,15,18,19,24,35,36] Carbon dioxide laser irradiation at these repetition rates have been shown to produce a protective effect on enamel surfaces while taking into account adequate heat dissipation time and clinical manageability.[16]

Evaluation of Multiple Laser Parameters

Many studies have explored the various combinations of laser parameters used for pretreatment of dental enamel that result in a reduction of solubility of enamel.[13,19,32] Carbon dioxide laser treatment followed by demineralization and remineralization using a pH-cycling model has shown up to an 85% decrease in caries-like progression as a result of specific laser treatments.[21,24] It has been shown that lasers significantly increase the acid resistance of enamel by inducing changes in enamel crystal structure and acid solubility if a specific range of irradiation conditions are used.[19] One study examined the changes in dissolution profiles observed using a carbon dioxide laser at a wavelength of 9.6 μm under a range of fluences (0.5-3 J/cm^2) and pulse durations (5 and 20 μs). Their results indicated that fluences of 1.5 J/cm^2 or less for both pulse durations resulted in a surface layer that had decreased solubility. Higher fluences of 2.0-3.0 J/cm^2 resulted in a more soluble phase formed at the surface layer that covered a more acid resistant mineral below.[14] Featherstone et al., (2005) examined the dissolution profiles of bovine enamel blocks that were irradiated with a carbon dioxide laser at a wavelength of 9.6 μm using

either 20 or 60 pulses per spot with a 20 μs pulse duration, and a fluence of 1.0 J/cm^2.[15] According to this study, increasing the number of pulses per spot resulted in a greater depth of effect in enamel with a decreased dissolution rate. However, the amount of solubility reduction was not as marked.[15] According to Featherstone et al., (1998) laser treatment at a wavelength of 9.3 and 9.6 μm, 100 μs pulse duration, 25 pulses, with fluences in the range of 1 to 3 J/cm^2 produced inhibition of caries-like progression that paralleled that obtained with daily fluoride dentifrice treatments. The use of specific laser conditions inhibited surface solubility and also prevented progression of caries-like lesions up to 80%.[13,16,24] These experimental conditions permitted prevention without causing excessive temperature rises that may risk pulpal damage.

Laser and Fluoride Combination Therapy

Several studies have investigated the combined use of fluoride and lasers in an attempt to find safe and clinically effective parameters for caries prevention. Combination therapy has been show to result in an even greater increase in caries resistance than laser or fluoride treatment alone.[9,26,29,37-39] Studies have shown the percentage of inhibition achieved with both laser and fluoride treatments ranged from 76% - 87% versus 50% - 70% with laser irradiation only.[24,25,36,40] Featherstone et al. (1991) demonstrated that laser treatment with a 9.32 μm carbon dioxide laser with 200 pulses at 15 mJ per pulse combined with a 5-minute fluoride gel treatment entirely inhibited subsequent lesion progression.[9] The benefits of combination therapy have also been shown using an intra-oral model. Laser irradiation (TEA Co_2 laser at 9.6 μm wavelength, 5 μs duration, 10 Hz repetition rate, and 1.5 J/cm^2 per pulse) along with

fluoride treatment resulted in significantly decreased mineral loss in the presence of a severe caries challenge model.[35]

Treatment Sequence of Laser and Fluoride Combination Therapy

Studies have explored the influence of treatment order in the successful inhibition of caries progression. These indicate that not only are the proper laser parameters necessary but, the appropriate sequence of treatment must be present in order for fluoride uptake to be facilitated. [41] There does not appear to be a consensus in the literature regarding what specific sequence is most effective. In one study, there was nearly complete elimination of lesion progression using a pH-cycling model system, when treatment with a carbon dioxide laser (9.32 μm) was followed by fluoride treatment.[9] A study with similar findings observed that fluoride uptake was greater when laser treatment was done before the fluoride treatment. There was less acid resistance of enamel when fluoride treatment was done prior to laser treatment where results were similar to either fluoride only or laser only treatment.[42] Other studies have found the opposite sequence effect exists where the acid resistance of enamel was increased when fluoride treatment preceded laser treatment.[30,37] Similar findings were achieved in another study where treatment of demineralized enamel with fluoride followed by carbon dioxide laser treatment lead to a 62% inhibition of lesion progression versus a 49% inhibition when this sequence was reversed.[25] One study found that prior treatment of tooth surfaces with a fluoride solution followed by laser irradiation resulted in high fluoride uptake and acid resistance leading to decreased enamel dissolution.[26] These findings are consistent with studies using carious enamel treated with fluoride followed

by CO_2 treatment where the modified mineral had a much lower solubility than the original enamel.[36]

Rationale for the Present Study

The overall objective of the present study was to further explore the specific set of optimal laser conditions that may be used clinically for the prevention and potential reversal of carious lesions. It has already been shown that by selecting appropriate laser parameters, it is possible to markedly inhibit further demineralization of enamel tissue. Little work has been done using demineralized enamel to study the effects on early carious lesions. Most of the studies reviewed above started with sound enamel and studies the demineralization inhibition effects on sound tissue. In the present study, we therefore started with preformed artificial caries-like lesions and followed this with laser and/or fluoride treatments. Therefore, this study explored the effect of fluoride therapy, as well as, laser treatment and possible sequence effects of treatment under these specific laser conditions.

Previous studies in our laboratories have used various research carbon dioxide lasers and the results summarized above led to the development of a prototype clinical laser that is also being used to study caries inhibitory effects in an in vivo study. Therefore we examined the effect of a new 9.6 μm CO_2 laser, (Pulse Systems, Inc (PSI), Model # LPS-500) that was designed for clinical use, for both ablation and caries prevention (Pulse Systems, NM). Using demineralized smooth surface enamel samples that were exposed to various fluoride and laser treatments, we were able to assess the capacity of these treatments to influence the degree of enamel remineralization and prevention of lesion progression. No single laser parameter alone dictates the reaction

dental tissue will display under experimental conditions. Various parameters in a myriad of combinations determine the influence lasers will have on dental tissues such as enamel. This study examined a specific combination of parameters required for the CO_2 laser to produce its positive effects on demineralized smooth surface enamel in the presence of fluoride. Ultimately, research of this nature is expected to provide dentists with clinically effective and safe laser parameters, for use in combination with fluoride therapy, necessary to combat caries progression and prevent future decay.

Methods

Experimental Design

One hundred enamel crowns were assigned to nine carious enamel groups (1-9) and one sound enamel group (10). Groups were divided into fluoride and laser treatment groups (Table 1). Group 1 received no further treatment. Groups 2 – 10 were subjected to further lesion progression by a pH-cycling model.

Table 1: Groups used in this study including three control groups. In all groups except Group 1, the treatment was followed by pH cycling demineralization/remineralization, designated by P in the group designation abbreviation (last column).

Group #		Treatment 1	Treatment 2	Group designation abbreviation
1	Demineralized enamel only			D
2	Demineralized enamel	Laser (2 J/cm^2)	APF	DL2FP
3	Demineralized enamel	APF	Laser (2 J/cm^2)	DFL2P
4	Demineralized enamel	Laser (4 J/cm^2)	APF	DL4FP
5	Demineralized enamel	APF	Laser (4 J/cm^2)	DFL4P
6	Demineralized enamel	APF only		DFP
7	Demineralized enamel	Laser only (2J/cm^2)		DL2P
8	Demineralized enamel	Laser only (4 J/cm^2)		DL4P
9	Demineralized enamel	No APF or laser		DP
10	Sound enamel			SP

Tooth Preparation

One hundred human molar crowns were used. The teeth used in this study were extracted previously, for reasons not related to the study, during normal patient care. Confidentiality of the patient was protected as teeth were not labeled with the patient's name, nor linked to the patient in any way. Permanent teeth, with sound enamel, and the absence of any lesions on the buccal and/or lingual surfaces, were selected. Teeth were sterilized by gamma irradiation, their crowns removed from their roots, brushed with warm ivory detergent solution, polished with a 5 μm alumina slurry using a felt wheel for one minute in the incisal-gingival direction, rinsed in double deionized water (DDW), dried with air, and painted with acid resistant varnish leaving one exposed window (approximately 4.0 x 2.0 mm) on one enamel surface. At all times during treatments, the teeth were suspended so that the windows were exposed to the de- and remineralizing solutions.

Caries-like Lesion Production and Fluoride (APF) Treatment

To produce artificial caries-like lesions, ninety crowns were partially demineralized in the windows of the buccal and/or lingual surfaces by immersion individually in 25 ml of lactate buffer for 48 hours. The buffer consisted of 0.05 mol/l lactate, pH 5.0, 50% saturated with hydroxyapatite, and containing carbopol at 2% as described by White and Featherstone.[43] Partially demineralized enamel samples, in fluoride-treated groups, were exposed to a 5-minute topical fluoride gel (NuPro APF by Johnson & Johnson) composed of 1.23% fluoride ion. The specimens were rinsed in DDW and wiped with tissue paper. Enamel blocks were irradiated by laser as described below.

Laser Conditions and Sample Irradiation

The laser used in this study was a Pulse Systems, Inc (PSI), Model # LPS-500, Serial # 030106, manufactured: January 2003 in Los Alamos, New Mexico. This clinical carbon dioxide (CO_2) laser with a 9.6 μm wavelength was used with the following parameters: 20 pulses per spot, 20 μs pulse duration, and a 20 Hz repetition rate. Incident fluences of 2.0 and 4.0 J/cm^2 per pulse were used. The laser energy was measured and calibrated using a calorimeter (Gentec, Model ED 200, Quebec, Canada). A beam diameter of approximately 0.6 mm was used, and the irradiation spots overlapped by 1/3 the beam diameter, as the laser advanced across the sample window, providing uniform coverage over the entire sample window.

PH Cycling

With the exception of Group 1, that had no further treatment, the following pH-cycling scheme was employed after demineralization solution and subsequent fluoride and/or laser treatment. A nine-day pH-cycling scheme, with 6 hrs in demineralizing solution, at pH 4.4, and 16 hrs in remineralizing solution at pH 7.0 each day, was used. This procedure was designed to model a daily demineralization challenge and repair. Each test group consisted of ten enamel crowns with one exposed window on the buccal or lingual surface.

The remineralizing solution consisted of calcium (1.5 mmol/L), phosphate (0.9 mmol/L), potassium chloride (150 mmol/L), and cacodylate (20 mmol/L) buffer to pH 7.0. This solution approximates the degree of saturation with respect to hydroxyapatite in saliva and is similar to that utilized by ten Cate and Duijsters.[2]

The detailed procedure used was as follows:

1. 6 hr demineralization at 37 °C in a demineralizing solution containing 2.0 mmol/l Ca, 2.0 mmol/l phosphate, 0.075 mol/l acetate at **pH 4.4**. Each tooth was immersed individually in 40 ml of solution. Fresh solutions were used weekly.
2. The crowns were removed from solution, rinsed in deionized water (DDW).
3. The teeth were then immersed individually in 20 ml of mineralizing solution (as above) at 37 °C overnight (16 hr) to simulate the remineralizing stage of the caries process. Fresh solutions were used weekly.
4. Prior to the demineralization stage, the crowns were again rinsed in deionized water (DDW).

This cycling scheme was repeated for 9 days as described above and as seen below in Figure 1.

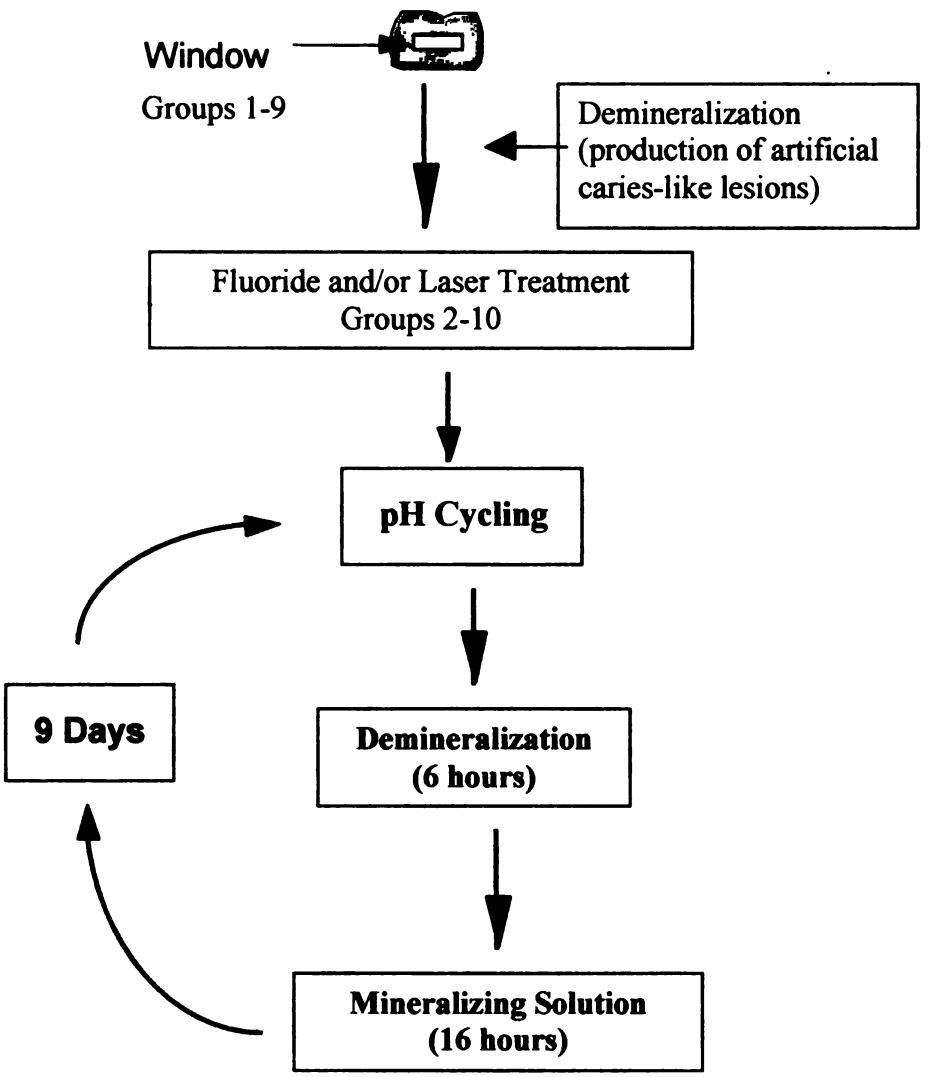

Figure 1: Flow chart describing steps in the pH cycling protocol for demineralization/remineralization caries progression model for human teeth designated to groups 2-10, in the present study.

Methods of Data Analysis
Assessment of De- and Remineralization

At the conclusion of pH cycling, the teeth were thoroughly rinsed in double deionized water, cut into a hemisection through the lesions, and embedded in epoxy resin with the cut face exposed utilizing the methods reported previously.[3,4] New group numbers were

randomly reassigned by an independent operator so that data analysis was completed without the experimenter knowing which group was being evaluated.

After serially polishing the embedded teeth, each lesion was assessed by microhardness analysis. A scatter pattern was used starting from 15 μm from the outer surface, progressing in 5 μm steps up to 50 μm (Figure 2). Indents were then placed at 25 μm intervals from 75 μm to 300 μm from the surface of the tooth across the sectioned lesion along a line perpendicular to the surface and into the underlying enamel (single row per lesion).

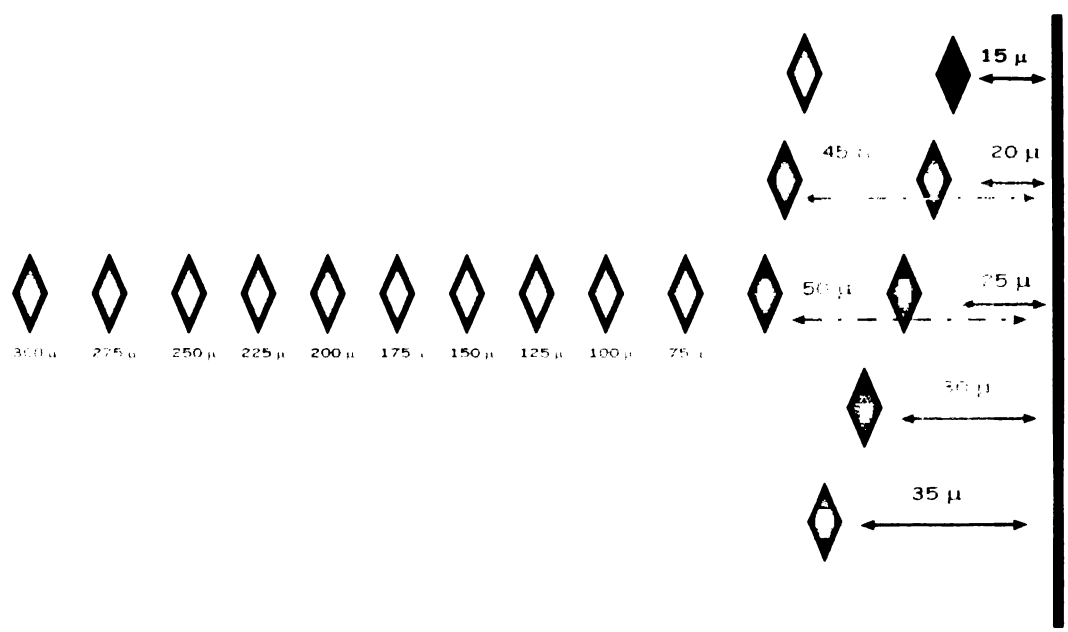

Figure 2: Schematic diagram depicting the sites for evaluation using microhardness indentation.

The indentation lengths were converted via Knoop hardness number to volume percent mineral (vol. %) according to the formula in previous publications.[3,4] Normal enamel has approximately 85% mineral by volume.

The overall relative mineral loss from each lesion was calculated from the data using a numerical integration to provide values as ΔZ (relative mineral loss) for each test group in units of volume % x μm.[1] Mineral loss values were compared statistically among treatment groups to assess their relative ability to inhibit caries progression.

Statistical Treatment of the Data

The ΔZ values were compared to the three control groups (demineralization solution only, fluoride only, laser only and no fluoride or laser treatment) giving a measure of the efficacy of laser and fluoride treatments in inhibition of caries progression in demineralized smooth surface enamel. Comparison of ΔZ values for each of the groups indicated the amount of demineralization/remineralization that occurred under the various experimental conditions. Relevant pair wise comparisons were made by student t-test with the level of significance adjusted by the Bonferroni correction (p-value of .00294). The means of the volume % mineral at depths of 15 μm, 20 μm, and 25 μm were examined by an ANOVA with a post ANOVA Tukey's multiple comparison test ($p<0.05$) to determine the statistically significant differences between groups. The mean volume percentage mineral at each depth from the outer surface was plotted versus depth in μm for each of the groups.

Results

The volume percent mineral profiles for each group are illustrated in Figure 3. The group with demineralized enamel that underwent pH cycling had the highest volume percent mineral loss while the lowest volume percent mineral loss was obtained for the group with demineralized enamel that was treated with fluoride and underwent pH-cycling, as shown in Figure 3.

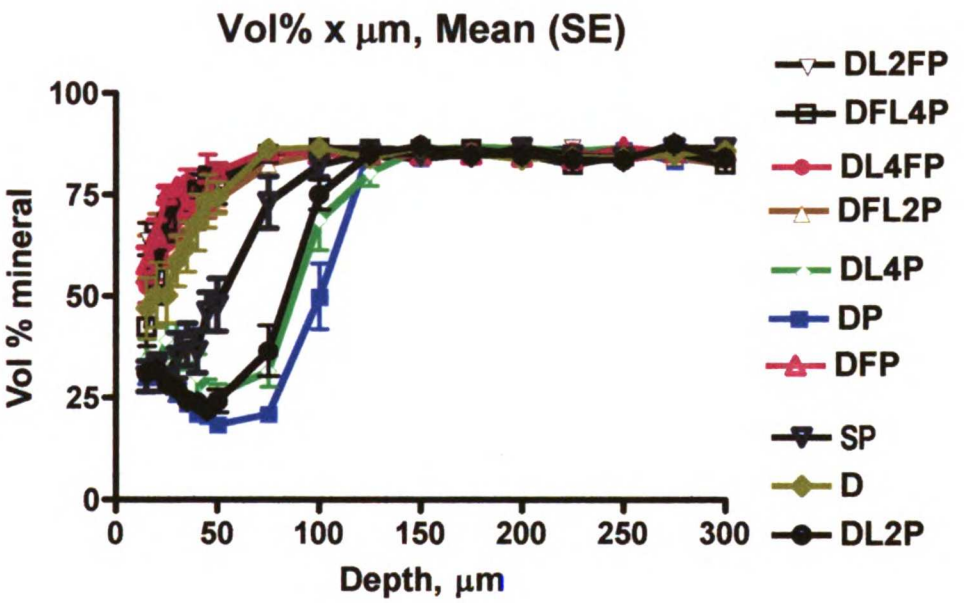

Figure 3: Volume percent mineral profiles for all ten groups from 15 µm to 300 µm.

This study had three control groups including sound and demineralized groups that underwent pH cycling and a demineralized group that did not undergo pH cycling. As shown in Figure 4, adequate caries-like lesions were formed, as outlined in the methods section above. The volume percent mineral profiles for the demineralized group that underwent pH cycling indicates marked demineralization occurring during pH cycling

when the enamel surface was compromised by prior surface demineralization (Figure 4). Lesion progression was less pronounced after pH cycling when an intact and sound lesion was present, as shown in Figure 4.

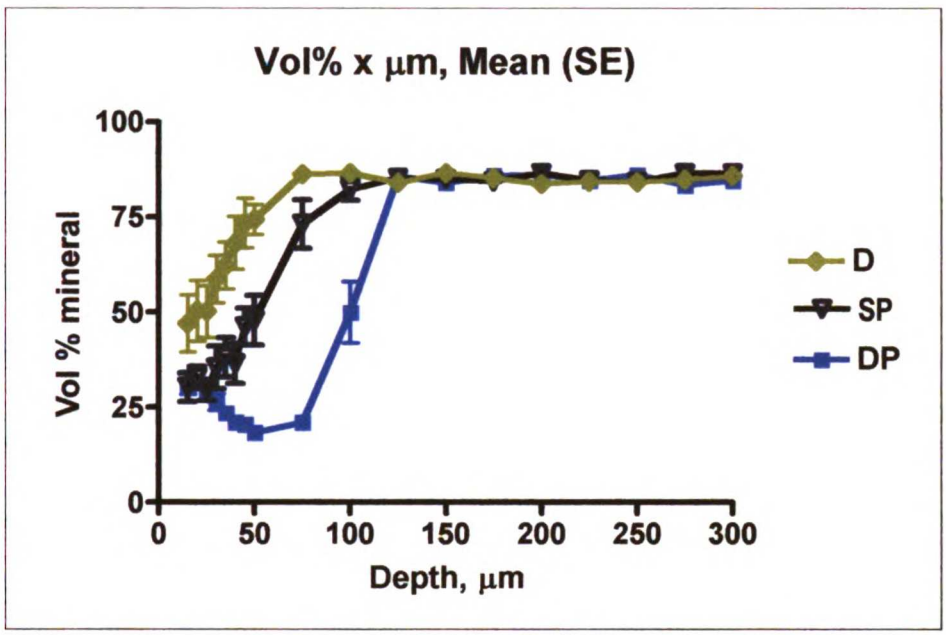

Figure 4: Volume percent mineral profiles for each of the control groups.

There was no sequence effect observed with reversal of combination therapy with laser and fluoride treatment, as shown in Figure 5. The absence of any sequence effect was observed for both laser treatments at 2 J/cm^2 and 4 J/cm^2. Figures 5 and 6 illustrate these findings and display the volume percent mineral profiles of the combination therapy groups compared with the demineralized enamel groups that underwent pH cycling.

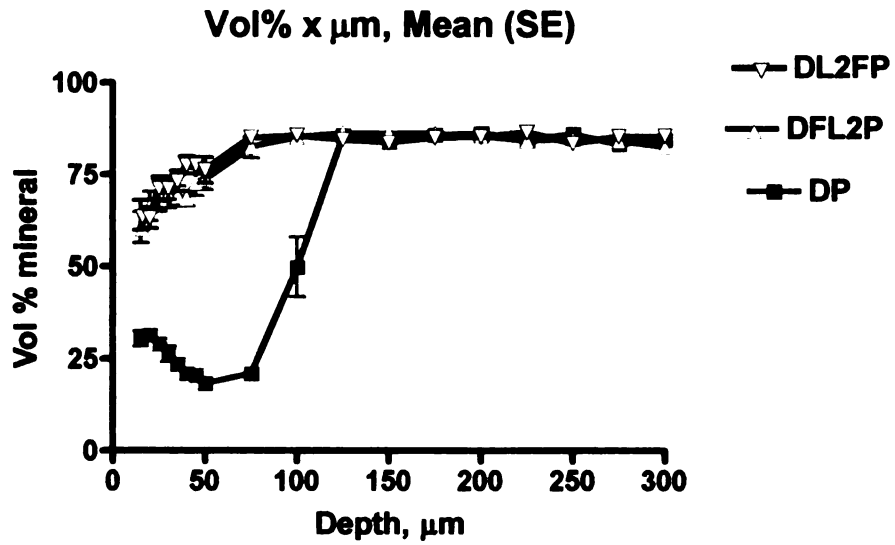

Figure 5: Volume percent mineral profiles for combination therapy with laser (2 J/cm^2) and fluoride treatment compared with the demineralized enamel group that underwent pH cycling.

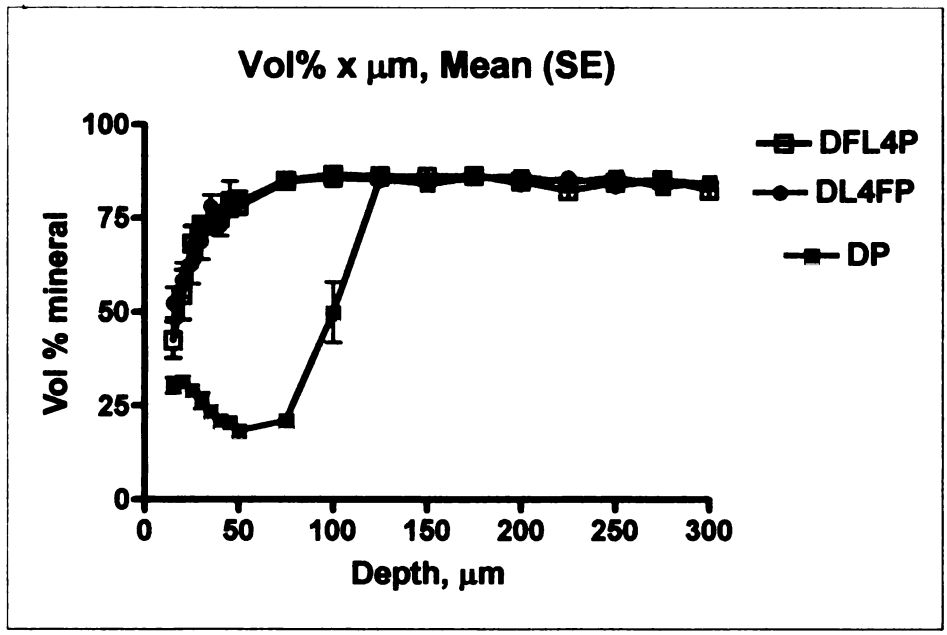

Figure 6: Volume percent mineral profiles for combination therapy with laser (4 J/cm^2) and fluoride treatment compared with demineralized enamel group that underwent pH cycling.

There is no significant difference in the volume percent mineral profiles for groups that underwent laser and fluoride treatment compared with the group of demineralized enamel group that had fluoride only treatment (Figure 7 and 8). This finding was

observed for both 2 and 4 J/cm^2 laser treatments despite the order of treatment rendered, as indicated in Figures 7 and 8.

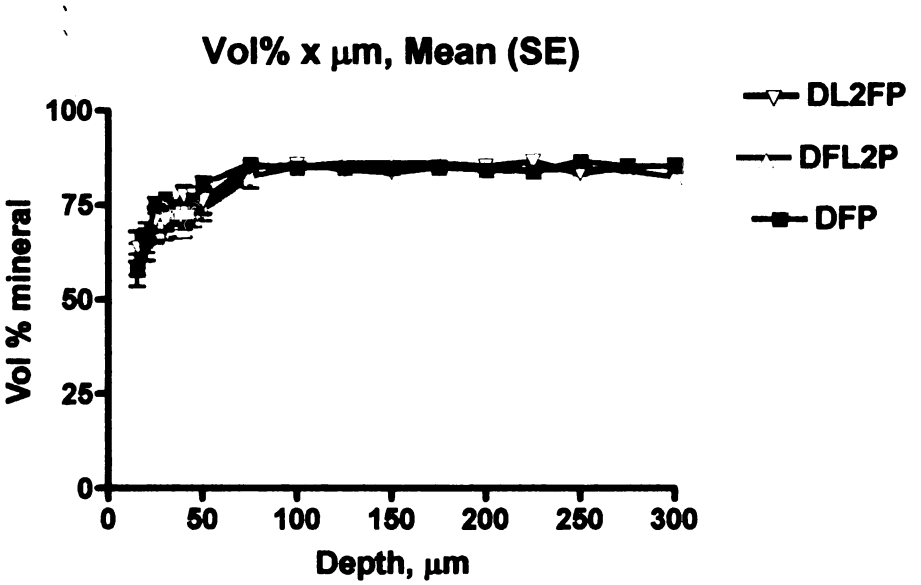

Figure 7: Volume percent mineral profiles for combination therapy with laser (2 J/cm^2) and fluoride treatment compared with demineralized enamel group treated with fluoride treatment followed by pH cycling.

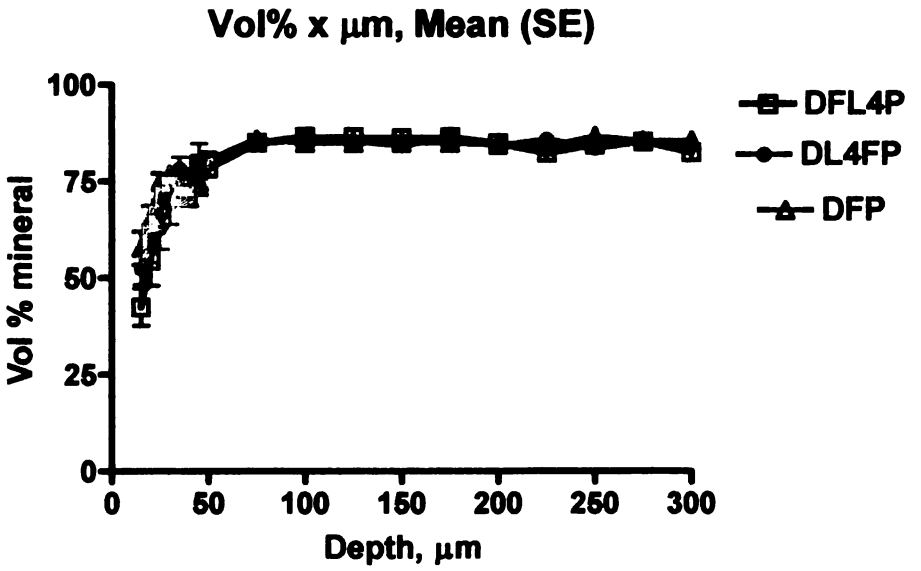

Figure 8: Volume percent mineral profiles for combination therapy with laser (4 J/cm^2) and fluoride treatment compared with demineralized enamel group treated with fluoride treatment followed by pH cycling.

A slight increase in volume percent mineral was observed in those groups that were laser treated with 2 and 4 J/cm^2 (Figure 9).

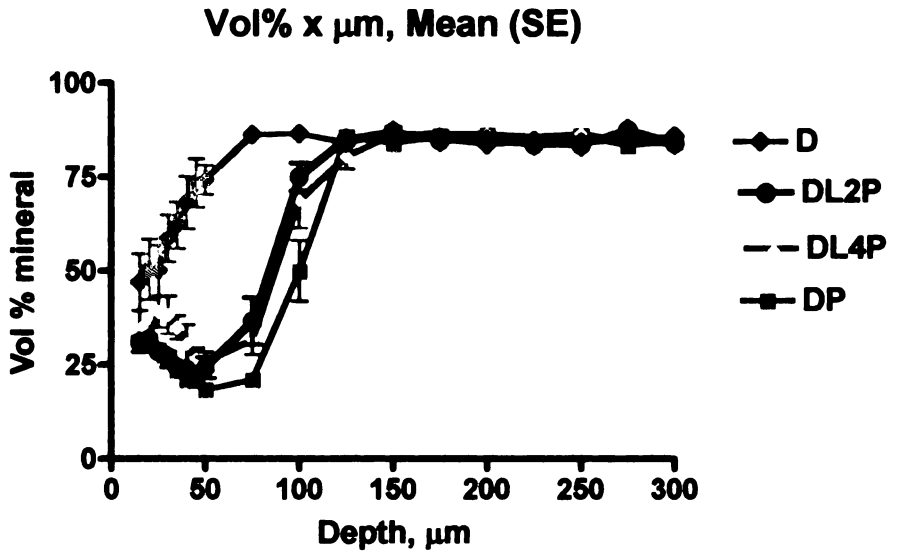

Figure 9: Volume percent mineral profiles for group D and DP versus 2 and 4 J/cm^2 laser treated groups followed by pH cycling.

The volume percent mineral profile for the DL2P group was almost identical to that of the DP group up to a depth of approximately 50 μm. At this point, there was a near doubling of the volume percent mineral at depths of 75 and 100 μm (Figure 10).

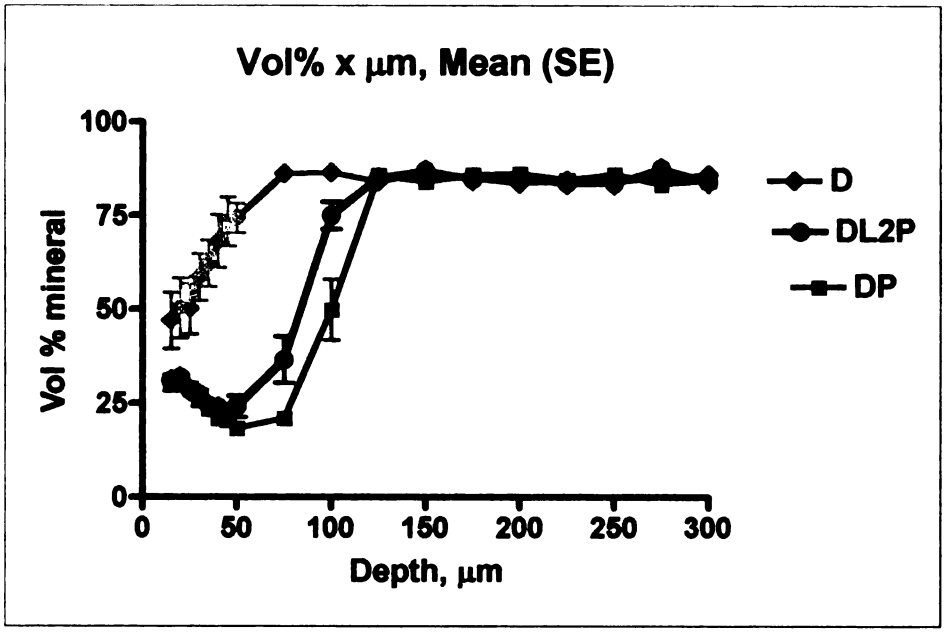

Figure 10: Volume percent mineral profiles for group D and DP versus the laser (2 J/cm^2) only treated group.

The volume percent mineral profile for the DL4P showed a surface layer of increased volume percent mineral from a depth of 25-35 μm (Figure 11). The volume percent mineral profiles for the DL4P and DP groups show a similar pattern from 50 μm to 100 μm. However, the DL4P group did have an increased effect on volume percent mineral from 8% to 10% to 19% greater, when compared with the DP group, for depths of 50, 75 and 100 μm, respectively (Figure 11).

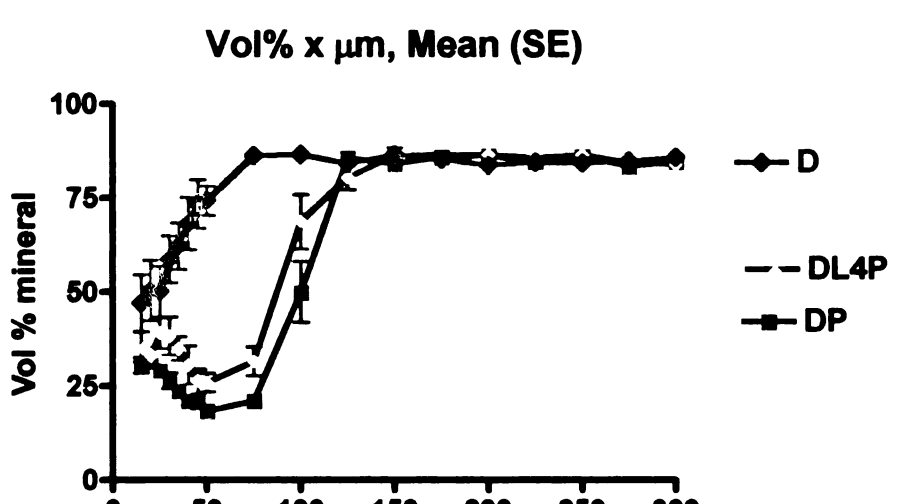

Figure 11: Volume percent mineral profiles for group D and DP versus the laser (4 J/cm^2) only treated group.

A marked increase in inhibition of lesion progression occurred with the DFP group when compared to the DP group while a slight amount of remineralization occurred as indicated by comparing the DFP group to the D group (Figure 12).

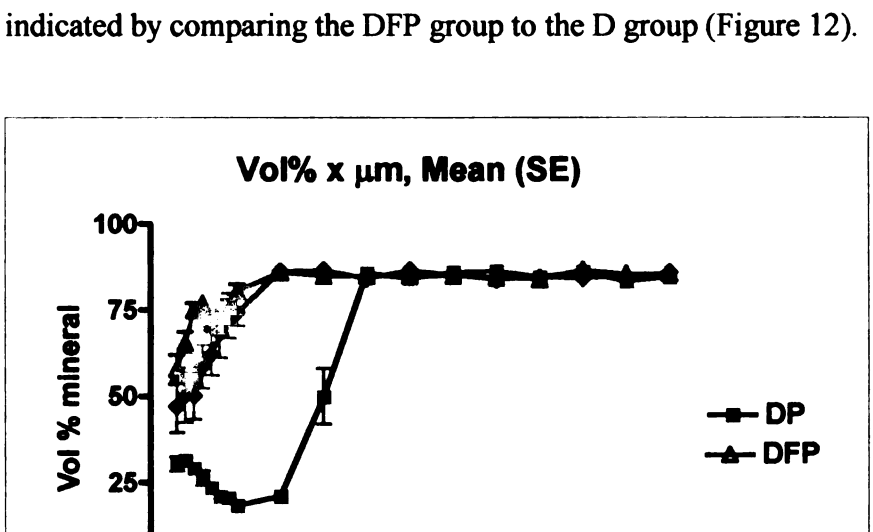

Figure 12: Volume percent mineral profiles for group D, DP and DFP.

A significant increase in inhibition of lesion progression occurred with the DFP group achieving almost complete inhibition at a depth of 50 μm (Figure 13). As shown in Figure 13 below, there is a 30 μm surface layer that had a marked increase in volume percent mineral that overlies a slightly demineralized region at a depth of 35-45 μm.

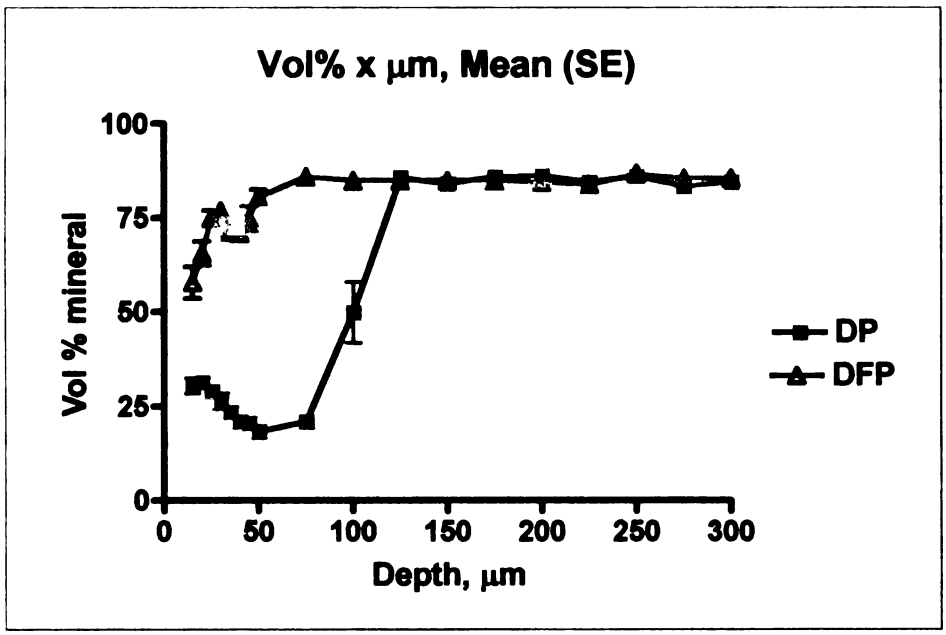

Figure 13: Volume percent mineral profiles for group DP versus DFP.

The combination therapy groups, despite sequence of treatment, showed nearly identical volume percent mineral profiles, as shown in Figure 14.

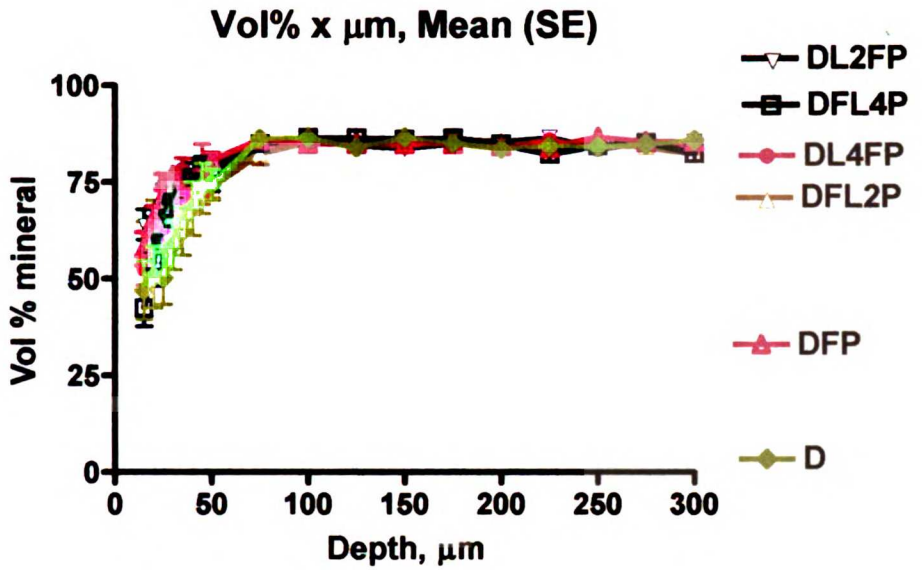

Figure 14: Volume percent mineral profiles for groups DL2FP, DFL2P, DL4FP, DFL4P, D and DFP.

Groups that received any laser or fluoride treatment showed a relatively higher volume percent mineral at a depth of 15 µm. Those groups that received only laser treatment had lower volume percent mineral values that were in the range of 30-35% (Figure 15).

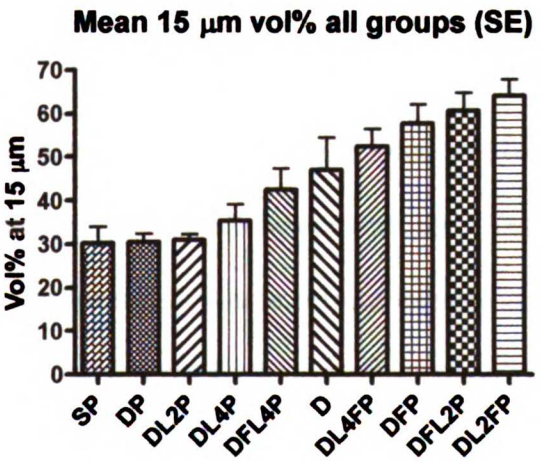

Figure 15: Volume percent mineral profiles for each group at a depth of 15 µm.

At a depth of 20 μm, there is a clear separation of treatment groups into two distinct sets. Those groups receiving only laser treatment had almost identical volume percent mineral values as the DP and SP groups. Any groups receiving fluoride treatment had a range of volume percent mineral, at a depth of 20 μm, of approximately 55 to 65% (Figure 16).

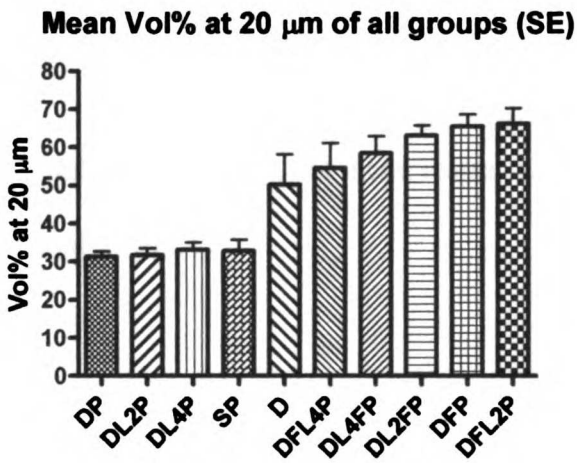

Figure 16: Volume percent mineral profiles for each group at a depth of 20 μm.

At a depth of 25 μm, a similar trend was found where there was a clear separation of treatment groups into two distinct sets. Those groups receiving only laser treatment had almost identical volume percent mineral values as the DP and SP groups, with the exception that the DL4P group had an approximately 10% greater volume percent mineral. At a depth of 25 μm, any groups receiving fluoride treatment had a similar range of volume percent mineral (approximately 65 to 75%) to those observed at a depth of 20 μm (Figure 17).

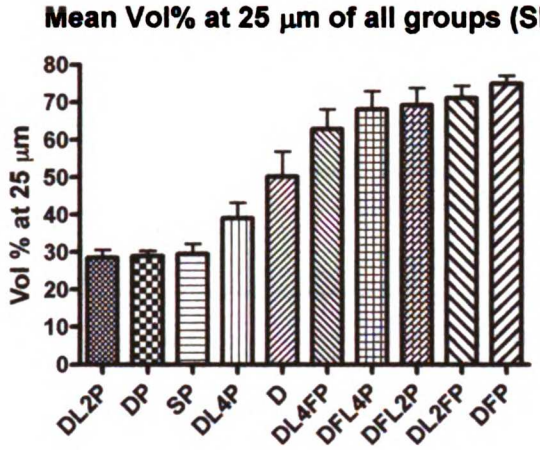

Figure 17: Volume percent mineral profiles for each group at a depth of 25 μm.

Table 1 summarizes the mean values of the volume % mineral at depths of 15 μm, 20 μm, and 25 μm.

Table 1: The means of the volume % mineral at depths of 15 μm, 20 μm, and 25 μm were examined by an ANOVA with a post ANOVA Tukey's multiple comparison test ($p<0.05$) to determine the statistically significant differences between groups.

15 Microns			20 Microns			25 Microns		
Group	Mean (SD)	Significance	Group	Mean (SD)	Significance	Group	Mean (SD)	Significance
SP	30.26 (11.45)		DP	31.28 (4.43)		DL2P	28.48 (6.97)	
DP	30.44 (6.47)		DL2P	31.75 (5.74)		DP	28.92 (4.21)	
DL2P	30.97 (4.39)		DL4P	33.01 (6.20)		SP	29.44 (8.21)	
DL4P	35.32 (11.81)		SP	32.77 (8.72)		DL4P	39.05 (12.47)	
DFL4P	42.47 (15.33)		D	50.33 (25.15)		D	50.14 (21.33)	
D	46.98 (23.68)		DFL4P	54.57 (20.84)		DL4FP	62.82 (16.93)	
DL4FP	52.36 (12.93)		DL4FP	58.51 (14.34)		DFL4P	68.17 (14.95)	
DFP	57.77 (13.58)		DL2FP	63.16 (8.72)		DFL2P	69.39 (14.17)	
DFL2P	60.70 (13.03)		DFP	65.50 (10.24)		DL2FP	71.24 (10.20)	
DL2FP	64.01 (12.48)		DFL2P	66.31 (12.51)		DFP	74.95 (6.52)	

In the present study, the relative mineral loss was significantly lower in groups receiving laser and fluoride treatment, regardless of sequence of treatment at surface depths of 15 to 25 μm ($p<.05$). In groups receiving laser only treatment, the relative mineral loss was significantly greater when compared to groups receiving both laser and fluoride treatment, with the exception of the DFL4P group at a depth of 15 μm ($p<.05$).

Relevant pair wise comparisons of the relative mineral loss, ΔZ, were made by t-test with the level of significance adjusted by the Bonferroni correction ($p<.00294$). Figure

18 illustrates the relative mineral loss was significantly lower for groups receiving fluoride only treatment and both laser and fluoride treatment, in any sequence when compared with the control group with demineralized samples that underwent pH cycling ($p<.00294$). Additionally, the groups that received only laser treatment at 2 and 4 J/cm^2

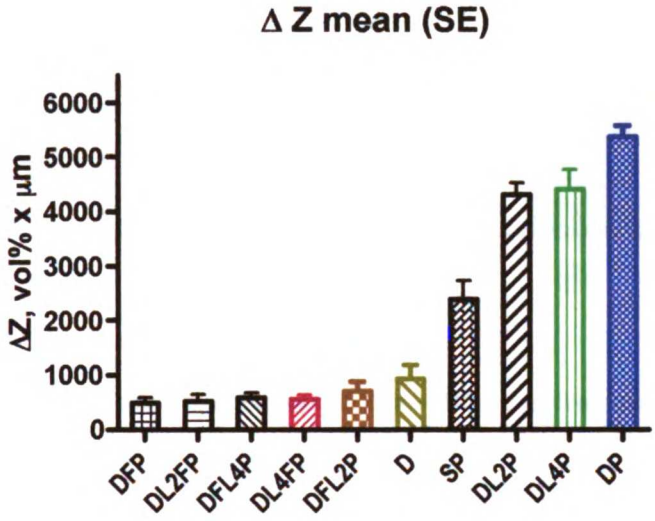

Figure 18: Relative mineral loss ($\triangle Z$ in vol% x μm) from microhardness analysis of all groups.

showed there was no protective effect from this treatment displaying mineral loss values that were not statistically different from demineralized samples that underwent pH cycling ($p<.00294$).

Upon inspection of the relative mineral loss ($\triangle Z$) values for the demineralized control group it is clear that an adequate amount of artificial caries-like lesions were produced in the samples using the methods outlined above (Figure 19). It is also interesting to note that the amount of lesion progression was significantly greater in those samples that had demineralized surfaces when compared those with a sound enamel surface ($p<.00294$), as shown in Figure 19.

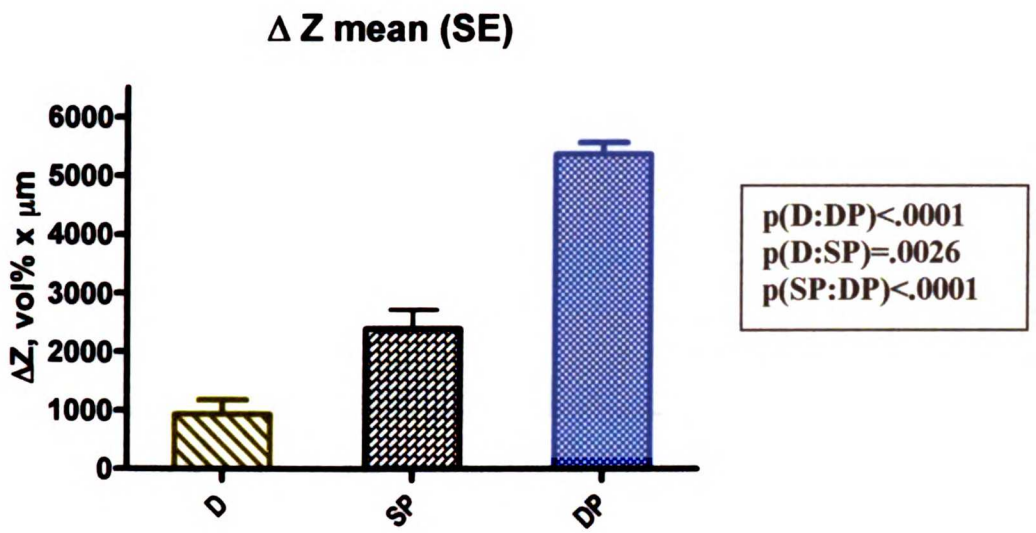

Figure 19: Relative mineral loss (△Z in vol% x μm) from microhardness analysis for groups D, SP and DP.

No sequence effect of treatment order using laser and fluoride treatment was present, as illustrated in Figure 20 and 22. This was true for laser treatment at fluences of 2 and 4 J/cm^2 where there was no significant difference between groups DL2FP and DFL2P; and DFL4P and DL4FP ($p<.00294$). There was a significant amount of protection against further lesion progression when samples were treated with both laser and fluoride when compared with demineralized samples that underwent pH cycling, as seen in Figure 20 and 22 ($p<.00294$). However, as depicted in Figure 21 and 23, it is important to note that there was no significant difference between the groups exposed to any combination of fluoride and laser treatment when compared to the group that received only fluoride gel treatment ($p<.00294$).

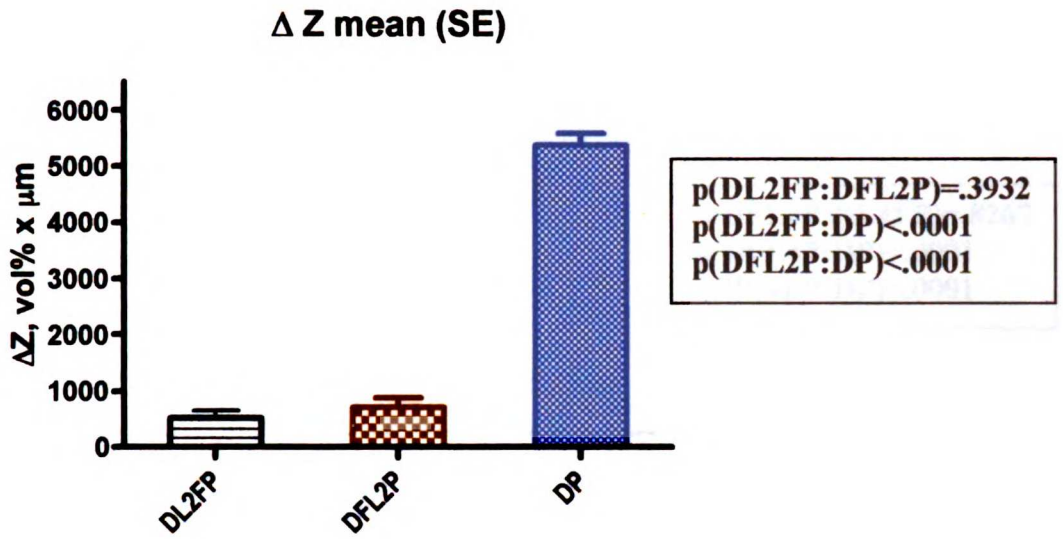

Figure 20: Relative mineral loss (△Z in vol% x μm) from microhardness analysis for groups DL2FP, DFL2P and DP.

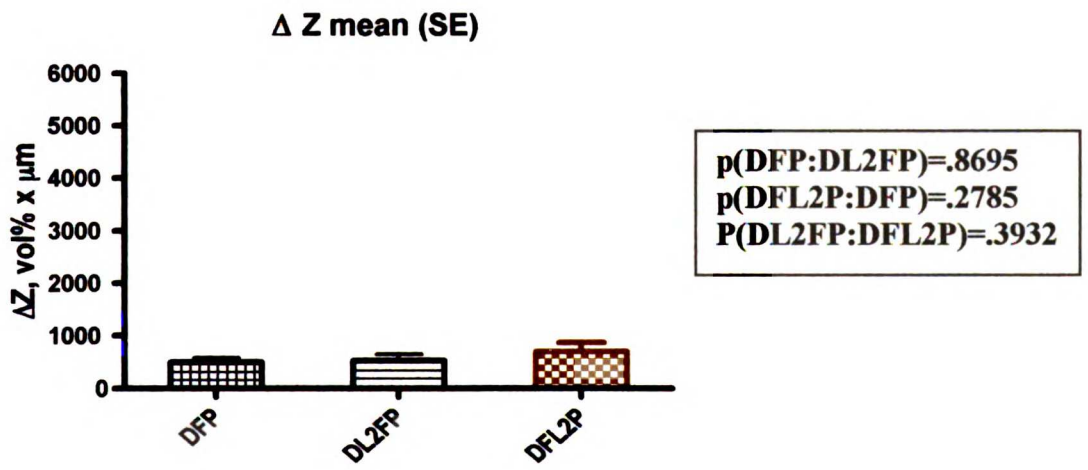

Figure 21: Relative mineral loss (△Z in vol% x μm) from microhardness analysis for groups DFP, DL2FP and DFL2P.

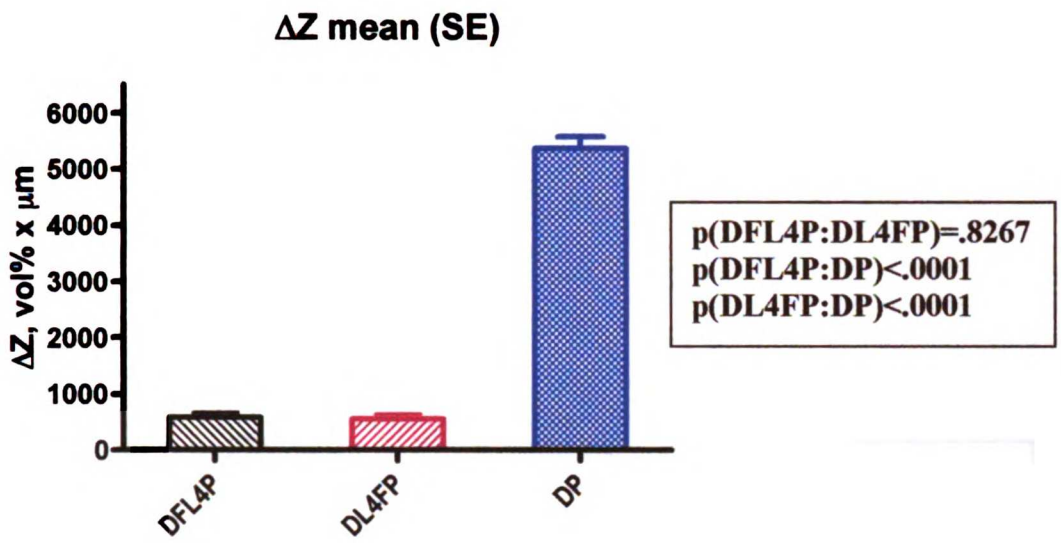

Figure 22: Relative mineral loss (△Z in vol% x μm) from microhardness analysis for groups DFL4P, DL4FP and DP.

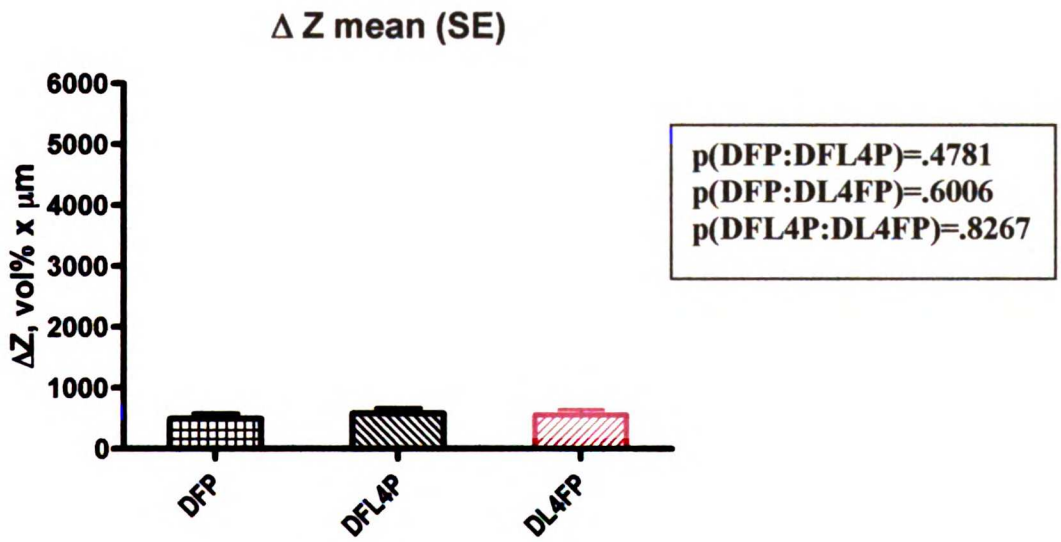

Figure 23: Relative mineral loss (△Z in vol% x μm) from microhardness analysis for groups DFP, DFL4P and DL4FP.

In the present study, there was no significant difference in treatment effect when using fluences of 2 or 4 J/cm^2 resulting in relative mineral loss values in the range of 4000 vol % x μm ($p<.00294$), as seen in Figure 24 .

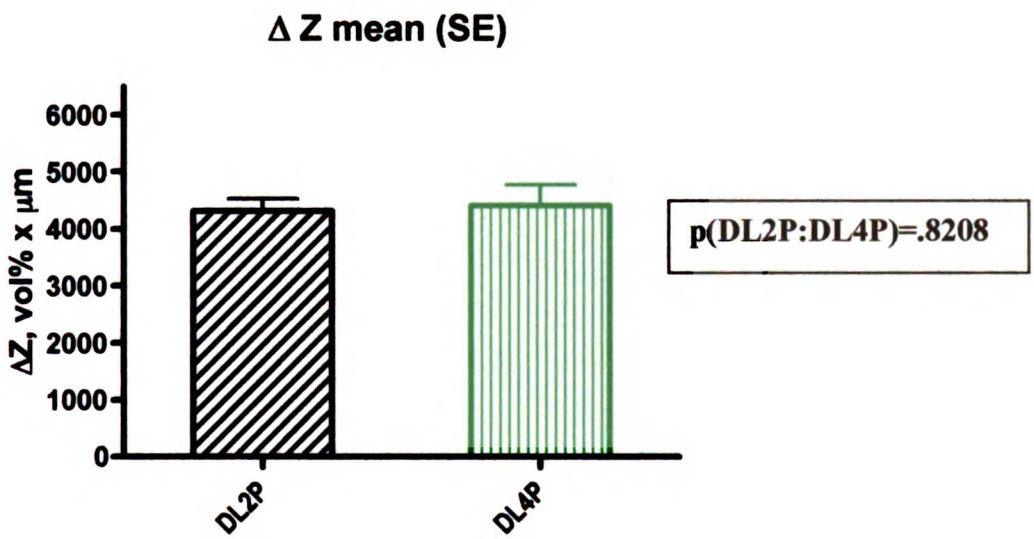

Figure 24: Relative mineral loss (△Z in vol% x μm) from microhardness analysis for groups DL2P and DL4P.

As illustrated in Figure 25, there appears to be a small but, significant protective effect of laser only treatment at 2 J/cm^2 when compared to the demineralized group that underwent pH cycling ($p<.00294$).

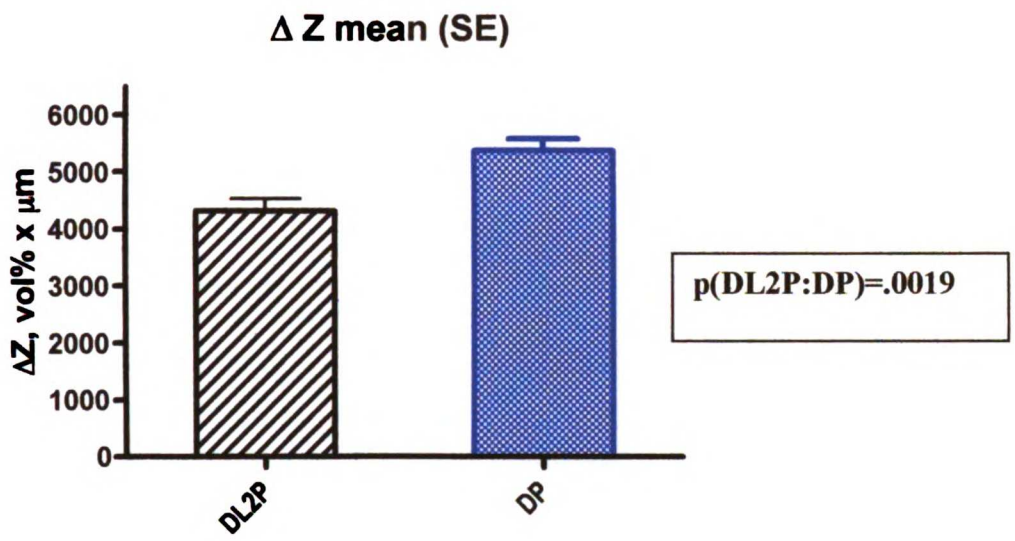

Figure 25: Relative mineral loss ($\triangle Z$ in vol% x µm) from microhardness analysis for the DL2P and DP groups.

It appears that laser treatment using a fluence of 4 J/cm^2 did not result in a significant protective effect against further lesion progression during pH cycling, as shown in Figure 26 ($p<.00294$).

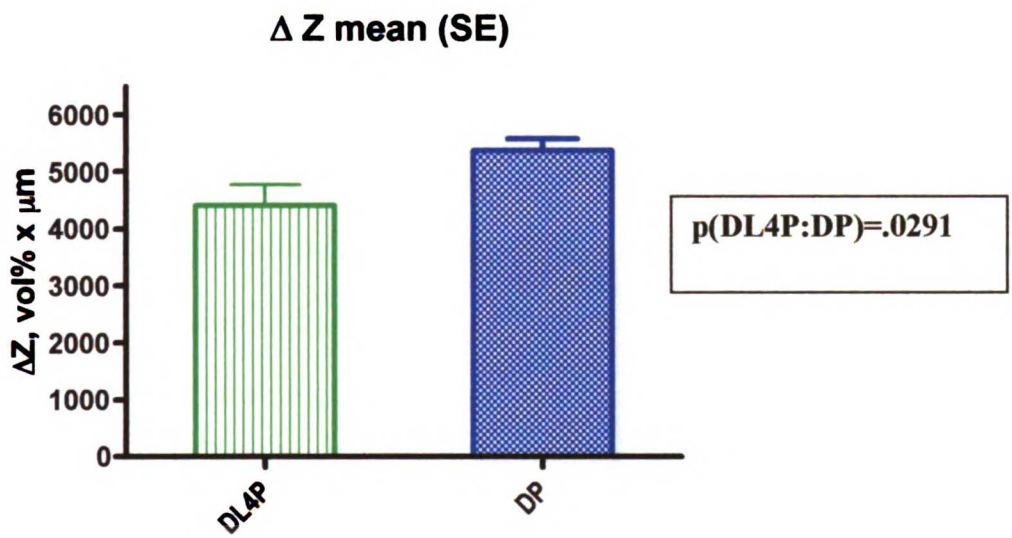

Figure 26: Relative mineral loss (△Z in vol% x µm) from microhardness analysis for the DL4P and DP groups.

The best inhibition of mineral loss was observed when the demineralized enamel was treated with a 5-minute topical application of fluoride gel, as illustrated in Figure 27. The relative mineral loss was significantly less for the fluoride treated group when compared with the demineralized group that underwent pH cycling indicating a significant protective effect existed for this treatment group ($p<.00294$).

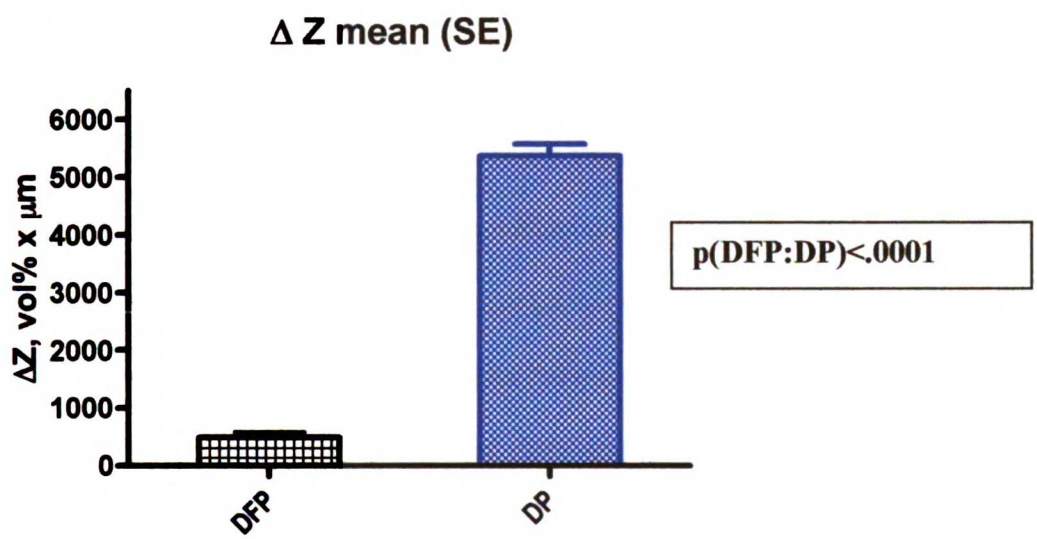

Figure 27: Relative mineral loss ($\triangle Z$ in vol% x μm) from microhardness analysis for the DFP and DP groups.

Discussion

The laser parameters chosen for this study were based on results of previous experiments carried out in our lab and the literature that currently exists that has documented successful inhibition of enamel demineralization.[14,18,21,24,28] The low energy densities used together with the efficient absorption of infrared carbon dioxide laser irradiation by enamel decreases the probability of damage to the dental pulp and surrounding soft tissues.[44] The use of pulsed lasers permit an increase in the peak power density while maintaining a constant pulse energy density allowing the laser to influence the surface of enamel without adversely affecting the underlying dentin and pulp.[22] Comparing the current results to the existing body of laser literature proves challenging due to the various types of lasers used and the myriad of parameters that are altered during experimentation. This study used three control groups and various laser and fluoride treatment conditions so valid comparisons could be made within this study design to evaluate if enamel solubility could be altered using a new 9.6 μm carbon dioxide laser with the following parameters: 20 pulses per spot, 20 μs pulse duration, 20 Hz repetition rate and incident fluences of 2.0 and 4.0 J/cm^2 per pulse.

The three control groups chosen for this experiment included a demineralized enamel group, a sound enamel group that underwent pH cycling and a demineralized enamel group that underwent pH cycling. As seen in Figure 4, the methods employed to demineralize enamel proved successful decreasing the volume percent adequately to a depth of approximately 50 μm producing a shallow early caries-like lesion. This validates the observed increase in mineral content with subsequent treatments since this

control group indicates that demineralization did indeed occur with the methods used. A pH cycling model was used to mimic the oral environment that leads to carious lesion progression. This proved to be successful, in this study, as can be observed by the further demineralization that occurred in the sound enamel group that underwent pH cycling (Figure 4). It is interesting to note that the DP group experienced a marked reduction in volume percent mineral when exposed to the pH cycling conditions used in this experiment, more than was expected upon examination of the SP group's response to pH cycling (Figure 4). It appears that an enamel surface that is damaged and has lost considerable mineral content becomes highly susceptible to lesion progression when exposed to a further acid challenge. This finding is important to observe when evaluating subsequent treatment effects since the absence of volume percent mineral decreases would indicate resistance to enamel demineralization during pH cycling. In addition, increases in volume percent mineral are even more substantial when evaluated in the context of a lesion with a volume percent mineral profile that hovers around 25% up to a depth of 75 μm.

Laser Only Effects

The laser-only treatments (DL2P and DL4P) using the parameters outlined above appeared to have a protective effect against further lesion progression of the compromised enamel (DP) as illustrated in Figure 9. However, the protective effect was only significant for laser treatment at 2 J/cm^2 but not at a fluence of 4 J/cm^2, as shown in Figures 25 and 26. The volume percent mineral profile for the DL2P group was almost identical to that of the demineralized enamel that had undergone pH cycling (DP) up to a depth of 50 μm (Figure 10) indicating that laser treatment had no positive influence on

the demineralized sample to this depth. However, the inner part of the profile from 50 μm to 125 μm showed a beneficial affect for the laser treatment (Figure 10). The volume percent mineral profile for the DL4P group was greater than that found for the DL2P group within the first 50 μm however; the overall relative mineral loss between these two groups was not statistically different (Figures 9 and 24). The relatively low (approximately 20%) inhibition of demineralization using these two laser conditions is in contrast to previous studies. For example, the lack of laser inhibition of enamel demineralization conflicts with those results obtained by Featherstone et al. (2001) who used an intra-oral model and found 84% inhibition of further demineralization using laser only treatment (9.6 μm, 100 μs pulse duration, 5 Hz repetition rate, 4 J/cm^2, 25 pulses per spot).[11]

The volume percent mineral profile for the DL4P group included a surface region of remineralization that was not present in the profile for the DL2P group or the DP group (Figure 11). This finding is consistent with one study that explored the laser irradiation effects on sound human enamel where wavelengths in the 9.32 μm range preferentially influenced surface enamel. These changes included producing a harder surface and altering the surface properties of enamel that inhibited lesion formation.[23] The mechanisms at work in this reported study appear to match the observed mineral profile for the DL4P group within the first 50 μm. Although this similarity exists, it is important to note that the present study used demineralized samples while the reported study used sound enamel. Almost all of the previously reported studies that showed marked inhibition of demineralization as a result of carbon dioxide laser treatment were done starting with sound enamel, rather than enamel with artificial caries-like lesions, as used

in the present study. Even though there was a small increase in mineral content at depths of 75 and 100 μm using both laser fluences of 2 and 4 J/cm^2 this finding was not statistically significant when the overall mean △Z values of the groups were compared. The higher fluence resulted in a higher temperature increase at the outer surface, which apparently altered the mineral sufficiently to inhibit demineralization in this region when exposed to further acid challenge during pH cycling. Similarly, Nelson et al., found that using a carbon dioxide laser produced beneficial effects by creating a localized increase in temperature with rapid melting and recrystallization of apatite in a region < 5 μm from the enamel surface.[23] The temperature increase below this depth is adequate to influence the denaturation of organic matrix to approximately 10 μm.[45] These authors attributed the observed decrease in enamel solubility to changes in the permeability and hydration of the enamel surface that occurred with laser treatment changing the physical and chemical properties of enamel.[23] It appears that these mechanisms were at work in the present study, when using a higher fluence of 4 J/cm^2.

In the current study, it appears that using laser parameters at both 2 and 4 J/cm^2 did not have a significant beneficial effect on artificial caries-like lesions when comparing the observed relative mineral loss values (△Z). Similarly, another study by Featherstone et al. found no protective effect of laser treatment (CO_2 laser at 9.32 μm, 200 pulses, 200 ns pulse duration, 15 mJ per pulse) on caries-like lesions of enamel under conditions of lesion progression using a pH cycling model.[9] Hsu et al. found a similar reduction in beneficial effects of laser treatment when using enamel samples without an organic matrix.[30] Laser treatment (10.6 μm CO_2 laser, .346 W, 5 ms/pulse, 20 Hz repetition rate) of sound enamel resulted in 98.7% reduction in mineral loss while the same laser

treatment of organic matrix depleted enamel resulted in a 74% reduction in mineral loss. Although a reduced effect of laser treatment was found in these studies, it is important to note that the laser treatments, in the Hsu et al. study, resulted in $\triangle z$ values, for non-organic irradiated enamel and irradiated sound enamel, of 1191 vol % x μm and 52 vol % x μm, respectively. In these studies, the laser effect was reduced in demineralized enamel however, in the present study; the laser effect was minimal with $\triangle z$ values in the 4000 vol% x μm range for both laser groups at 2 and 4 J/cm^2.

The absence of an observed protective effect of laser treatment alone may have been due in part because the enamel being treated was partially demineralized. Phan et al., found a reduced laser effect when treating sound and carious bovine enamel.[36] These authors found mean dissolution rates for calcium and phosphate were reduced by 73% and 81%, respectively for laser (CO_2 at 9.6 μm, 1 J/cm^2, 2 μs pulse duration and 1 Hz repetition rate) treated sound enamel while laser treatment of carious enamel groups resulted in a 65% and 76% reduction in the calcium and phosphate dissolution rates, repectively.[36] Dissolution rate reductions of 35%, 65% and 75% were observed for APF only, laser only (CO_2 at 9.6 μm, 25 pulses per spot, 2 μs pulse duration, 1 J/cm^2 and 1 Hz repetition rate), and APF followed by laser treatment, respectively, in carious enamel.[36] It is possible that the absorption properties of demineralized enamel are altered in such a way that the optimal laser parameters are different for sound and carious enamel.

The lack of a protective laser effect on demineralized enamel observed in this study stands out against a growing body of literature that argues otherwise. Nobre dos Santos et al. found a CO_2 laser treatment produced a significant protective effect against lesion progression by 49% and 42% inhibition using 1.0 and 1.5 J/cm^2, respectively.[25]

Featherstone et al., found that laser treatment (CO_2 at 9.6 μm, pulse durations of 5 or 20 μs) at a low fluence of 1.0 J/cm^2 can be used for caries prevention without producing soluble surface phases that were found at higher fluences (2.0-3.0 J/cm^2).[14] Although the present experiment used fluences in this range, high soluble surface phases were not observed. Other studies with laser parameters (CO_2 at 9.6 μm, 1-3 J/cm^2, 20 μs pulse duration) that closely matched the ones used in this study showed that they may be useful for reducing enamel solubility and caries prevention.[14,15] Unfortunately, the current results of this study do not support the findings of that study.

Combination of Fluoride and Laser Treatment Effects

There was no significant difference found between groups DL2FP and DFL2P (Figure 5). The volume percent mineral more than doubled for each group from 15 μm to 50μm and reached levels present in sound enamel at a depth of 75 μm. This would lead one to believe that a significant improvement in the acid resistance of enamel occurred when exposed to these experimental conditions. However, as seen in Figure 7, a major component that appears to be producing this effect is the 5-minute topical fluoride gel. An interesting finding is the difference in lesion profile between the fluoride only group and that of the group undergoing laser and fluoride therapy. An area of decreased mineralization is present in the fluoride only group from 35 to 45 μm. This zone is absent in the lesion profile of the laser and fluoride treated groups that appear to have a more uniform and gradual increase in volume percent mineral, as shown in Figure 5. Borggreven et al., found that enamel permeability increased when low energy densities were used.[46] Perhaps this increased access of the fluoride ions may be one explanation for the more uniform appearance to the mineral profiles in samples treated with both the

laser and fluoride gel. Similar patterns of results were found where there were no significant difference found between DL4FP and DFL4P (Figure 6). The volume percent mineral increased quite markedly within the first 50 μm however, it appears that the fluoride treatment was the key treatment producing the observed effect, as seen in Figure 8. Again, it is important to note that the combined laser (4 J/cm^2) treatment with fluoride resulted in a uniform increase in volume percent from the enamel lesion surface to a depth where sound enamel is present (Figure 6).

The results obtained in this study when using a combination of laser and fluoride treatment vary greatly when compared with other studies in the literature. Rodrigues et al. (2006) found a 76% inhibition in mineral loss when using both carbon dioxide laser treatment (9.6 μm, 5 μs pulse duration, 10 Hz repetition rate, 1.5 J/cm^2, 25 pulses per spot) in combination with a fluoride dentifrice *in situ*.[35] Featherstone et al. found a combination of laser treatment (CO_2 laser at 9.32 μm, 200 pulses, 200 ns pulse duration, 15 mJ per pulse) with a 5-minute APF gel led to complete inhibition of lesion progression with the mean ΔZ values for this group not being statistically different from caries-like lesions that did not undergo pH cycling.[9] Another study, found laser treatment increased fluoride uptake into enamel.[47]

Sequence Effects of Laser and Fluoride Therapy

According to the results of the current study, there was no benefit to rendering treatment in a particular order. There was no significant difference in the groups that reversed the order of laser and fluoride treatment using fluences of both 2 and 4 J/cm^2. This finding contradicts the study by Nobre dos Santos et al., who found a significantly higher percent inhibition in carious enamel treated first with fluoride (5-minute APF gel)

followed by laser treatment (CO_2 at 9.6 μm, 25 pulses per spot, 5 μs pulse duration, 10 Hz repetition rate, 1.0 and 1.5 J/cm^2).[25] There was a 62% and 76% inhibition of lesion progression for carious enamel samples treated first with fluoride followed by laser treatment at fluences of 1.0 and 1.5 J/cm^2, respectively. This is statistically different than that found for samples that were treated in the reverse order, producing a reduced effect of 49% and 53% inhibition of lesion progression at fluences of 1.0 and 1.5 J/cm^2, respectively.

Fluoride Only Treatment

The results of this study indicate that demineralized lesions that undergo further acid challenge using a pH-cycling model benefit most from a five-minute topical fluoride treatment only (Figure 12). Other studies have documented beneficial effects from fluoride treatments however, these showed only a 45% mineral loss inhibition, using an *in situ* model including three times daily use of a fluoridated dentifrice.[35] The results of the current study are supported by a study that evaluated a fluoride dentifrice in an intra-oral model where demineralization was inhibited and remineralization was enhanced.[11] The organic matrix of enamel has been shown to be depleted early on during demineralization.[48] The positive fluoride effect observed in this study is similar to that observed by Hsu et. al. (2001) who evaluated the effect of fluoride treatment on enamel that had its organic matrix removed.[30] In this study, the fluoride treated groups had a significant reduction in mineral loss of 38% when compared with a control group. Some have attributed this to a greater available surface area when the organic matrix is compromised allowing greater access to the fluoride ions.[30,49] Hsu et al. found a four minute sodium fluoride gel treatment was more effective in reduction in mineral loss

when the samples being treated had their organic matrix removed.[30] Fluoride only treatment of sound enamel resulted in a 17% reduction in mineral loss while this same treatment of non-organic enamel resulted in a significant 38.4% reduction in mineral loss. Another study found that 5-minute treatment with an APF gel on caries-like lesions of dental enamel decreased lesion progression by approximately 60% with a $\triangle Z$ value of 1300 μm x vol%.[9] The current study found an even greater protective effect of fluoride with a $\triangle Z$ of 488 μm x vol % being obtained following a 5-minute APF gel treatment of demineralized enamel. This observed significant increase in acid resistance surpassed that obtained under all other combinations of fluoride and laser treatment, regardless of treatment order and laser only treatments at both 2 and 4 J/cm^2 (Figure 3). Carious enamel provides greater access to fluoride ions due to its increased porosity and permits a reduction in the dissolution rates of calcium and phosphate thereby inhibiting enamel demineralization.[36]

Mechanism of Action of Fluoride Treatment

Mechanism of action for fluoride therapy in the present model appear to include: inhibition of demineralization in enamel during the acid challenge of pH cycling model, enhancement of remineralization of carious enamel and deposition of a fluoroapatite-like coating during remineralization that is more resistant to caries.[50,51] Rolla and Saxegaard (1990) attributed the high fluoride uptake into dental hard tissues to the low pH and high fluoride concentration by production of a CaF_2-like layer on the enamel surface that may act as a reservoir during periods of acid susceptibility.[52] Fluoride therapy does not appear to produce its effect through the loss of carbonate from apatite. Phan et al. found that fluoride treatment (1-minute 1.23% APF gel) did not influence carbonate loss.[36]

However, it may be possible that the application time of 1 minute was not sufficient considering the current recommendation is a four-minute application time.[1]

Mechanism of Action of Laser Treatment

Enamel surface melting and recrystallization have been reported to occur under conditions involving a pulsed laser with short laser interaction times.[23] The laser parameters used in this experiment, satisfy these criterion and inspection of the laser-irradiated surface of enamel indicated melting did indeed occur. However, these findings did not correspond with a reduction in the solubility of the enamel surface. Studies have reported that using the appropriate laser fluences created temperatures at the enamel surface where carbonate is lost rendering a more acid resistant hard tissue.[33,53] Featherstone et al., found total loss of carbonate, using a 9.6 μm laser treatment at 4 J/cm^2, that markedly reduced caries progression after reaching a peak surface temperature of 800^0C.[32] The microhardness analysis of the current study on demineralized enamel did not find a comparable protective effect despite having the same laser parameters except for a reduced pulse duration of 20 μs from their 100 μs that was used on sound enamel. Similarly, Phan et al., used laser irradiation (CO_2 at 9.6 μm, 25 pulses per spot, 2 μs pulse duration, 1 J/cm^2 and 1 Hz repetition rate), that resulted in a significant amount of carbonate loss from enamel in both sound (85%) and carious bovine (92%) enamel rendering the samples resistant to acid dissolution.[36] These investigators attributed this finding to thermal modification of the enamel structure where surface temperatures reached a critical threshold[54] decomposing the carbonated apatite into carbon dioxide and hydroxyapatite.[32,36] It is possible that the laser parameters, used in this study, did not

reach this critical threshold required for an adequate amount of carbonate loss leaving the remaining enamel susceptible to further lesion progression during pH cycling.

Conclusion

A new TEA 9.6 μm carbon dioxide laser (20 pulses per spot, 20 μs pulse duration, 20 Hz repetition rate, at 2.0 and 4.0 J/cm^2) failed to produce a marked protective effect against lesion progression in demineralized enamel. Laser only treatment at 2 J/cm^2 provided a small but significant protective effect against further acid challenge during pH cycling. When laser therapy was combined with fluoride treatment, a significant protective effect was observed. However, it appears that a large part of the inhibition of lesion progression was attributed to the five-minute fluoride application. The results of this study indicate that demineralized lesions that undergo further acid challenge using a pH-cycling model benefit most from a five-minute topical fluoride treatment only. Fluoride therapy not only inhibited further lesion progression when samples underwent pH cycling but some remineralization of the original artificial caries-like lesion occurred. This observed significant increase in acid resistance surpassed that obtained under all other combinations of fluoride and laser treatment, regardless of treatment order. Future studies are required to further explore the specific set of optimal laser conditions for this new TEA carbon dioxide laser that may be used clinically for the prevention and potential reversal of carious lesions. It is essential that control groups, specifically using fluoride treatment alone, be used as part of the experimental design to ensure accurate interpretation of the data.

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APPENDICES

Appendix A: Normalized Volume % Mineral and ΔZ values for each Group

D ce	Normalized Volume % Mineral 1	2	3	4	5	6	7	8	9	10	Overall Average Teeth 1-10	Overall Std. Dev. Teeth 1-10
	34.01	89.31	87.99	48.20	52.52	33.87	23.29	39.37	35.40	25.84	46.98	23.68
	35.95	94.01	80.26	46.55	82.74	31.53	26.73	35.74	37.85	31.89	50.33	25.15
	46.64	95.27	80.26	52.69	39.69	28.94	32.51	43.33	46.04	36.05	50.14	21.33
	62.57	89.31	85.92	78.12	36.85	38.99	37.31	51.71	56.85	48.45	58.61	19.86
	63.08	95.27	84.92	79.96	40.79	43.55	36.83	61.56	61.01	54.46	62.14	19.54
	59.63	91.60	83.86	74.69	103.18	39.75	31.74	73.87	63.60	59.68	68.16	22.10
	67.50	90.44	81.98	81.89	106.96	45.46	35.97	75.21	70.75	76.92	73.31	20.55
	74.00	89.31	75.29	71.54	73.09	47.85	62.90	88.14	86.26	74.61	74.30	12.47
	79.34	88.21	85.92	80.91	91.36	93.51	80.74	93.45	89.34	78.54	86.13	5.87
	82.75	89.31	83.94	84.96	83.53	83.14	91.58	89.15	87.26	88.83	86.45	3.16
	84.57	84.06	86.94	84.96	87.71	85.70	87.29	80.79	83.34	74.60	83.99	3.89
	85.51	84.06	89.06	86.03	81.97	88.50	84.19	95.75	77.36	92.12	86.46	5.21
	84.57	88.21	91.29	87.13	81.97	83.14	80.55	79.14	86.25	89.90	85.22	4.03
	85.51	76.83	77.69	82.88	80.46	81.46	88.45	86.18	89.34	87.78	83.66	4.47
	84.57	82.13	81.15	87.13	87.71	88.50	82.50	79.96	81.57	87.78	84.30	3.23
	86.47	85.06	85.92	82.88	85.15	84.85	85.34	77.56	87.26	81.10	84.16	2.91
	85.51	87.13	82.06	87.13	85.15	84.85	85.29	91.25	84.32	76.14	84.88	3.89
	85.51	88.21	86.94	81.89	91.36	84.85	79.80	85.23	88.29	86.76	85.88	3.29
	1329.24	383.65	123.53	719.93	403.80	1620.99	2114.88	792.75	940.92	1699.30	936.17	770.88

roup L2FP stance µm)	Normalized Volume % Mineral 1	2	3	4	5	6	7	8	9	10	Overall Average Teeth 1-10	Overall Std. Dev. Teeth 1-10
15	55.17	74.93	66.54	67.67	61.74	69.11	84.40	37.50	58.10	64.91	64.01	12.48
20	60.06	79.26	56.72	74.76	54.54	64.22	69.13	52.97	58.55	61.37	63.16	8.72
25	73.02	83.26	68.35	77.73	50.89	76.40	83.43	59.17	70.59	69.58	71.24	10.20
30	60.02	83.45	62.32	79.30	58.33	73.28	82.48	62.07	72.73	77.92	71.19	9.70
35	75.97	82.46	61.79	76.96	57.06	80.56	74.82	65.85	83.27	72.84	73.16	8.89
40	81.85	72.00	67.85	80.10	87.58	84.39	76.38	64.74	85.14	75.54	77.56	7.63
45	80.07	82.26	68.51	84.39	74.57	80.64	81.55	70.13	86.14	66.05	77.43	7.09
50	75.24	80.39	53.64	88.21	79.28	86.39	82.46	68.24	85.11	62.85	76.18	11.28
75	82.76	84.82	86.48	85.32	81.87	86.40	86.35	78.09	87.95	90.62	85.07	3.48
100	86.61	79.52	85.41	84.41	80.99	88.40	87.42	89.11	89.19	85.64	85.67	3.28
125	85.62	89.39	78.64	83.51	82.77	87.44	77.21	84.16	88.17	88.56	84.55	4.15
150	78.41	86.25	85.43	87.09	85.59	81.55	89.61	82.33	75.64	84.71	83.66	4.22
175	88.67	85.11	78.64	80.93	86.58	84.39	87.35	89.11	84.12	84.71	84.96	3.28
200	81.85	83.31	89.68	89.20	86.58	81.55	87.42	82.33	81.38	89.58	85.29	3.54
225	88.67	86.81	87.59	89.21	84.63	86.40	88.46	82.31	89.06	81.18	86.43	2.85
250	84.64	81.27	84.39	80.93	85.59	86.40	79.72	89.11	87.09	76.40	83.55	3.88
275	88.67	86.01	88.71	85.33	83.67	86.40	84.39	81.45	83.22	85.64	85.35	2.30
300	81.85	87.32	86.50	84.39	88.61	82.47	83.42	85.10	87.12	88.56	85.54	2.45
ΔZ	454.27	131.16	1096.37	187.86	888.35	148.82	247.11	1254.95	192.96	552.83	515.47	420.84

Group DFL2P Distance (µm)	Normalized Volume % Mineral 1	2	3	4	5	6	7	8	9	10	Overall Average Teeth 1-10	Over Std Dev Teet 1-1(
15	39.72	68.64	85.82	44.93	65.02	68.21	63.12	53.12	58.40	59.99	60.70	13.0
20	61.57	69.91	79.07	68.93	50.55	72.94	41.35	77.11	78.94	62.71	66.31	12.5
25	65.39	82.97	91.40	75.18	45.94	80.08	58.50	53.61	75.62	65.20	69.39	14.1
30	53.54	82.97	64.94	66.35	65.58	85.67	54.93	76.51	81.63	63.21	69.53	11.5
35	54.33	77.80	77.34	68.27	66.16	62.97	60.47	71.87	83.53	67.83	69.06	8.8
40	58.18	74.75	70.79	74.73	71.16	72.23	58.03	66.20	73.30	65.95	68.53	6.2
45	63.69	74.75	91.40	62.28	60.31	87.70	70.55	67.33	87.61	63.27	72.89	11.8
50	71.04	84.83	77.17	72.41	72.52	83.72	59.47	74.64	82.57	59.32	73.77	9.0
75	84.93	82.97	78.18	90.75	87.70	88.76	58.91	79.30	85.52	87.80	82.48	9.2
100	83.02	80.32	80.63	89.75	78.55	88.76	86.70	86.51	87.61	90.21	85.21	4.2
125	87.96	90.96	81.74	86.17	86.69	84.68	83.67	87.50	86.55	87.80	86.37	2.5
150	87.96	80.28	85.77	86.42	85.69	88.76	85.66	90.63	87.61	83.36	86.21	2.8
175	86.92	85.79	92.53	87.54	87.70	88.76	85.66	78.48	84.51	84.43	86.23	3.6
200	85.91	89.72	86.56	77.01	87.68	81.86	94.68	80.99	83.53	87.80	85.57	4.9
225	81.19	78.54	82.52	87.22	85.69	84.68	88.84	86.51	83.53	82.32	84.10	3.1
250	86.92	88.60	85.19	84.30	84.73	81.86	76.55	90.63	84.51	83.36	84.67	3.8
275	83.02	82.02	84.30	86.42	83.72	84.68	86.70	80.99	85.52	84.43	84.18	1.8
300	82.09	88.77	85.77	80.16	84.55	80.96	76.55	82.75	81.63	81.30	82.45	3.3
ΔZ	1021.19	429.06	544.84	331.59	758.63	75.75	1932.79	901.69	189.52	847.23	703.23	534.5

Group DL4FP Distance (μm)	Normalized Volume % Mineral 1	2	3	4	5	6	7	8	9	10	Overall Average Teeth 1-10	Overall Std. Dev. Teeth 1-10
15	56.81	50.33	59.08	66.18	62.15	31.25	40.40	54.44	34.83	68.13	52.36	12.93
20	66.08	59.81	79.22	66.18	65.19	30.09	50.30	45.37	51.54	71.31	58.51	14.34
25	71.93	53.66	66.39	52.30	81.81	25.34	66.37	58.80	84.69	66.93	62.82	16.93
30	85.74	48.01	83.99	61.32	70.33	38.24	81.51	68.69	84.70	68.12	69.06	16.18
35	84.73	60.31	83.99	73.33	80.99	81.86	92.64	69.95	83.70	70.00	78.15	9.52
40	71.09	81.99	73.38	55.49	67.94	74.06	65.19	84.48	84.68	73.37	73.17	9.11
45	83.75	93.56	65.77	85.07	56.46	92.13	101.25	59.75	83.70	80.32	80.18	14.92
50	79.13	84.85	65.15	85.70	72.89	69.64	91.83	69.99	85.71	87.71	79.26	9.20
75	82.79	82.92	81.06	83.68	86.21	84.64	83.14	93.04	82.71	86.71	84.69	3.39
100	83.75	79.32	89.38	83.68	85.30	97.19	72.46	92.92	85.72	87.71	85.74	6.88
125	81.85	89.92	87.14	86.74	84.40	81.89	85.85	86.47	78.18	89.77	85.22	3.70
150	86.78	87.86	86.07	83.68	81.81	77.58	81.38	84.48	84.70	87.71	84.21	3.23
175	85.74	90.09	87.14	85.70	88.11	86.29	83.98	84.48	86.77	86.71	86.50	1.75
200	82.79	81.04	85.02	79.92	80.08	74.95	88.73	91.87	94.86	82.94	84.22	6.04
225	87.83	87.86	81.06	82.71	79.38	89.34	91.83	81.66	94.86	82.05	85.86	5.20
250	85.74	75.17	82.99	85.68	95.45	87.57	82.25	80.73	81.78	79.48	83.68	5.45
275	83.75	86.87	86.07	91.19	91.09	82.66	87.75	80.73	78.18	83.85	85.21	4.22
300	86.78	86.87	80.13	85.70	79.38	87.53	90.77	81.67	79.94	84.78	84.36	3.87
ΔZ	476.61	484.17	593.41	595.64	518.96	1216.68	309.49	647.93	381.48	311.36	553.57	260.72

Group DFL4P Distance (µm)	Normalized Volume % Mineral 1	2	3	4	5	6	7	8	9	10	Overall Average Teeth 1-10	Overall Std. Dev. Teeth 1-10
15	45.26	54.26	37.69	65.77	32.27	23.64	30.06	35.86	67.74	32.11	42.47	15.33
20	43.07	87.19	65.71	70.70	71.25	26.66	29.93	69.43	46.76	34.99	54.57	20.84
25	67.97	83.34	83.43	78.29	62.04	36.97	79.60	75.34	60.41	54.34	68.17	14.95
30	76.65	71.75	75.74	73.07	75.74	74.29	76.25	76.14	71.42	61.80	73.29	4.46
35	77.48	80.57	74.99	72.27	64.83	74.33	71.69	78.69	68.19	66.34	72.94	5.29
40	65.51	83.16	66.89	65.12	85.27	78.17	71.72	87.37	79.65	71.55	75.44	8.37
45	85.73	91.51	68.75	87.69	75.69	77.46	74.68	85.32	73.97	71.02	79.18	7.77
50	64.92	80.60	85.35	77.37	72.68	88.60	75.42	79.57	72.49	88.55	78.55	7.61
75	83.79	85.41	87.35	85.40	86.68	84.57	83.17	84.31	86.29	82.45	84.94	1.58
100	90.07	86.39	88.37	88.80	82.72	82.59	81.21	89.64	85.28	89.66	86.47	3.34
125	83.79	85.36	84.36	91.34	97.25	85.51	79.48	83.31	88.40	84.39	86.32	4.95
150	79.19	85.36	88.40	90.09	82.72	88.65	86.21	85.32	88.40	87.47	86.18	3.25
175	88.93	85.39	82.51	85.35	85.68	87.60	92.84	83.31	85.28	86.42	86.33	2.94
200	88.96	78.77	81.60	86.53	91.10	82.66	86.02	85.32	82.37	85.38	84.87	3.64
225	84.77	85.39	86.34	80.20	77.38	84.53	82.99	86.36	83.32	72.45	82.38	4.48
250	83.79	83.34	88.39	80.20	82.72	88.63	83.06	82.32	86.29	86.36	84.51	2.77
275	91.21	87.48	81.60	83.25	82.72	86.54	86.96	85.10	83.30	85.39	85.35	2.84
300	74.29	87.50	83.43	79.24	82.72	78.28	86.23	84.30	82.37	87.47	82.58	4.26
ΔZ	633.72	124.58	487.48	411.92	429.92	684.78	847.93	388.74	659.48	1145.01	581.36	281.76

up DFP stance µm)	Normalized Volume % Mineral 1	2	3	4	5	6	7	8	9	10	Average Teeth 1-10	Std. Dev. Teeth 1-10
15	72.06	56.50	63.11	78.22	46.26	43.31	57.78	42.46	73.62	44.40	57.77	13.58
20	72.78	64.78	65.39	85.26	57.75	51.40	72.19	60.32	71.30	53.85	65.50	10.24
25	81.67	73.68	74.06	87.17	75.15	67.53	72.78	65.03	79.69	72.75	74.95	6.52
30	80.78	75.66	76.94	83.39	79.42	67.53	77.17	78.52	76.90	71.23	76.75	4.56
35	79.90	74.41	67.64	82.48	80.31	53.39	70.28	66.57	77.71	67.04	71.97	8.83
40	83.53	69.58	62.49	79.88	71.31	63.17	73.57	72.28	70.75	62.17	70.87	7.13
45	82.59	69.58	74.76	87.21	85.23	72.36	68.45	66.06	85.23	56.05	74.75	10.19
50	88.56	70.23	76.40	85.00	80.33	87.19	79.41	74.32	87.09	75.98	80.45	6.28
75	84.49	85.40	83.43	88.02	87.36	81.03	88.44	86.70	89.34	83.39	85.76	2.66
100	85.47	89.46	86.35	88.02	75.96	87.19	77.18	89.50	85.18	83.39	84.77	4.73
125	84.49	83.50	80.64	91.32	85.23	88.31	83.74	86.70	79.51	84.42	84.79	3.45
150	83.53	88.41	87.45	80.73	88.46	85.04	85.48	85.81	84.18	78.60	84.77	3.20
175	85.47	85.40	82.48	81.59	89.60	88.31	85.48	83.24	78.01	88.81	84.84	3.60
200	83.52	77.48	93.04	84.31	75.96	84.00	84.62	84.08	89.33	87.67	84.40	5.05
225	79.90	86.38	88.51	75.88	84.20	81.03	87.42	86.70	86.22	82.39	83.86	4.00
250	89.64	82.58	83.43	83.39	90.76	81.03	87.38	84.93	95.03	87.67	86.59	4.34
275	85.47	85.40	81.55	87.22	85.23	84.00	87.38	83.24	90.40	85.48	85.54	2.44
300	87.50	86.38	81.55	92.54	89.60	86.10	86.32	80.81	77.14	86.56	85.45	4.48
ΔZ	256.21	631.35	738.61	75.02	361.74	861.99	307.65	596.43	165.24	886.58	488.08	292.64

oup _2P ance m)	Normalized Volume % Mineral 1	2	3	4	5	6	7	8	9	10	Overall Average Teeth 1-10	Overall Std. Dev. Teeth 1-10
15	33.20	32.79	34.58	25.87	30.28	28.16	22.88	31.73	38.09	32.08	30.97	4.39
20	30.43	37.95	32.22	25.32	31.83	31.03	19.64	37.81	34.86	36.41	31.75	5.74
25	17.64	37.35	21.16	33.30	31.66	34.72	18.83	27.31	30.23	32.63	28.48	6.97
30	39.95	28.00	23.30	26.49	31.98	22.92	26.64	23.98	21.16	26.66	27.11	5.45
35	24.18	24.80	32.70	23.90	27.59	23.07	14.97	22.22	23.95	26.31	24.37	4.46
40	26.30	25.90	35.13	33.30	20.65	17.97	15.02	18.72	19.63	25.31	23.79	6.63
45	20.76	24.25	40.97	19.57	14.52	18.03	21.34	21.32	17.47	21.14	21.94	7.20
50	23.41	28.24	38.78	40.09	20.69	17.40	14.40	18.92	16.34	23.31	24.16	8.99
75	66.23	40.67	78.08	22.55	28.42	25.50	23.79	24.73	21.89	33.71	36.56	19.81
100	86.19	66.09	46.23	72.68	79.92	82.51	78.58	77.90	77.82	82.41	75.03	11.56
125	88.06	83.17	90.05	86.22	77.45	86.59	87.52	89.42	82.83	79.21	85.05	4.26
150	85.27	86.07	88.21	84.56	90.51	88.78	84.32	89.42	85.60	87.71	87.05	2.17
175	86.19	86.07	77.88	83.76	86.35	89.92	86.43	88.41	79.42	81.59	84.60	3.88
200	86.19	86.07	85.59	86.22	82.56	87.67	84.32	81.12	81.96	84.99	84.67	2.14
225	81.83	82.25	87.32	87.94	80.78	87.67	84.32	79.48	85.60	79.21	83.64	3.38
250	81.83	83.17	83.12	86.22	79.08	72.30	84.32	86.47	93.95	84.98	83.55	5.54
275	87.11	85.08	83.93	87.94	90.51	85.53	86.43	83.71	92.82	91.67	87.47	3.22
300	83.52	88.12	83.89	77.14	92.75	81.55	82.32	81.97	77.82	90.65	83.97	5.12
ΔZ	3044.46	4278.27	3132.34	4490.30	4704.73	4534.91	4925.35	4719.86	4837.30	4442.29	4310.98	672.52

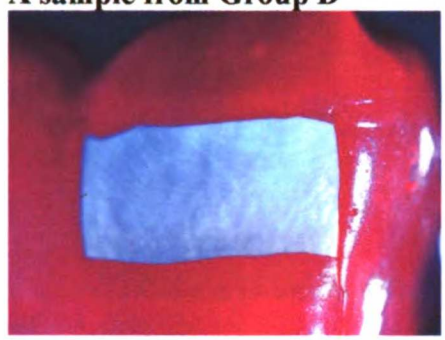
roup)L4P stance µm)	Normalized Volume % Mineral 1	2	3	4	5	6	7	8	9	10	Overall Overall Average Teeth 1- 10	Overa Overa Std. Dev Teet 1-1(
15	28.75	61.01	41.77	27.90		31.22	33.96	42.77	20.83	29.69	35.32	11.8
20	29.65	32.19	38.76	23.86		32.84	31.62	42.77	26.30	39.08	33.01	6.2(
25	37.30	52.17	41.32	24.50		25.61	38.61	63.40	38.33	30.23	39.05	12.4
30	40.04	47.58	39.60	17.44		23.86	42.14	70.07	33.68	29.20	38.18	15.2
35	36.13	36.73	36.72	22.05		25.19	38.39	54.49	33.65	30.25	34.84	9.2(
40	29.16	56.04	24.47	12.48		21.83	20.95	54.80	33.97	20.87	30.51	15.3
45	32.10	28.54	24.61	13.28		18.74	21.08	48.07	26.86	23.35	26.29	9.87
50	31.07	29.67	24.55	14.48		19.31	22.23	39.34	23.55	28.28	25.83	7.2(
75	24.36	32.47	37.86	15.77		22.47	29.34	54.08	25.92	40.91	31.46	11.4
100	27.94	84.06	85.39	39.21		70.48	62.17	77.42	79.19	91.54	68.60	21.7
125	64.66	90.07	88.33	74.79		88.33	83.37	79.97	66.72	86.97	80.35	9.6(
150	87.97	82.24	96.06	88.47		71.82	88.39	84.56	85.79	90.23	86.17	6.6(
175	86.00	88.98	88.33	86.37		86.34	87.33	86.55	88.91	80.89	86.63	2.4
200	93.20	81.35	73.86	91.83		88.31	87.33	85.54	91.22	85.89	86.50	5.9
225	91.08	80.49	84.45	84.35		88.28	86.28	86.46	86.82	84.79	85.89	2.9(
250	93.26	92.27	86.35	87.41		80.05	83.36	82.66	87.89	84.69	86.44	4.3
275	76.80	88.95	80.89	83.39		85.39	82.43	89.71	87.89	82.82	84.25	4.17
300	87.04	75.65	81.73	83.38		91.46	81.51	84.56	84.78	83.72	83.76	4.2
ΔZ	5608.42	3486.64	3804.09	6153.24		4892.32	4626.37	2583.60	4640.65	3860.02	4406.15	1099.

ip DP ance m)	Normalized Volume % Mineral 1	2	3	4	5	6	7	8	9	10	Overall Average Teeth 1-10	Overal Std. Dev. Teeth 1-10
5	30.16	32.24	41.02	24.41	17.76	30.07	26.26	32.47	34.67	35.36	30.44	6.47
20	28.47	27.88	36.33	32.90	26.64	24.26	31.74	32.47	33.73	38.38	31.28	4.43
25	31.62	26.39	33.76	36.62	23.75	23.14	27.87	27.52	29.23	29.27	28.92	4.21
30	25.45	23.62	30.28	44.62	22.14	20.22	24.48	24.79	24.67	23.03	26.33	6.93
35	21.54	22.89	27.44	32.90	22.13	22.25	18.44	19.33	25.54	23.33	23.58	4.20
40	24.02	18.63	23.69	25.54	17.84	15.99	18.92	24.06	18.65	22.34	20.97	3.31
45	23.27	19.93	24.61	29.63	18.29	15.91	19.40	18.77	17.19	18.39	20.54	4.14
50	19.30	19.93	21.19	25.71	15.64	15.49	15.92	16.48	15.15	17.84	18.27	3.35
75	26.43	21.37	29.34	22.31	19.52	15.54	18.16	15.84	16.35	24.96	20.98	4.77
00	38.23	36.00	84.77	82.59	89.60	21.07	46.21	31.68	37.10	31.79	49.90	25.5
25	82.26	82.40	87.69	84.39	90.63	87.96	89.04	86.60	84.37	80.56	85.59	3.30
50	83.09	86.23	82.93	82.59	80.46	87.96	84.90	87.77	87.35	76.76	84.00	3.59
75	89.49	83.32	86.70	85.31	83.00	84.77	89.04	90.21	83.43	82.19	85.75	2.95
00	83.09	88.28	87.69	86.26	84.78	86.87	84.88	87.77	84.35	88.45	86.24	1.87
25	83.09	87.24	86.70	85.31	83.00	80.88	81.13	86.60	81.57	89.42	84.49	2.96
50	86.63	90.43	88.71	85.31	87.61	87.95	81.13	81.19	84.37	86.56	85.99	3.06
75	85.72	80.60	74.80	88.22	83.88	75.66	82.97	84.35	87.26	90.41	83.39	5.12
00	86.63	81.49	84.77	82.59	86.64	87.96	86.92	75.52	87.30	85.65	84.55	3.79
VZ	5366.50	5714.48	4402.42	4377.70	4922.35	6429.06	5585.13	5659.82	5855.36	5471.59	5378.44	644.3

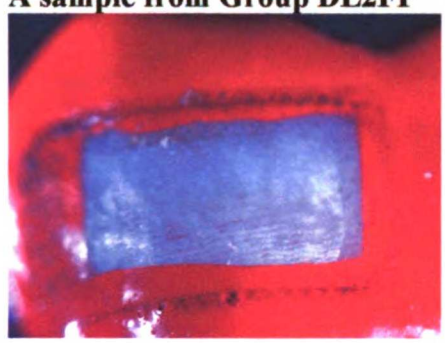
ıp SP tance ım)	Normalized Volume % Mineral 1	2	3	4	5	6	7	8	9	10	Average Teeth 1-10	Std. Dev. Teeth 1-10
15	30.58	50.67	25.38	18.96	40.64	33.45	36.72	80.24	21.11	14.83	35.26	19.14
20	26.46	44.53	42.99	22.14	39.48	29.42	39.00	53.24	27.67	23.21	34.81	10.46
25	28.16	45.08	40.14	23.66	32.02	26.38	25.51	100.30	20.80	23.21	36.53	23.71
30	23.76	44.26	73.40	25.42	40.84	23.07	25.28	81.96	22.29	40.06	40.03	21.57
35	22.39	41.75	67.91	28.55	40.85	36.82	30.78	76.23	18.68	55.60	41.95	19.12
40	24.55	53.63	60.55	30.74	56.96	24.72	25.35	78.58	27.66	23.62	40.64	19.93
45	30.91	60.23	53.03	30.19	70.91	48.47	51.82	92.93	37.30	32.69	50.85	20.03
50	40.14	78.17	49.43	24.86	78.96	42.58	52.83	95.28	31.38	32.55	52.62	23.71
75	79.37	79.01	85.49	45.62	86.86	87.50	80.29	87.62	78.80	34.93	74.55	18.59
100	85.18	82.56	88.62	82.21	83.72	88.66	81.90	81.09	87.44	59.65	82.10	8.38
125	87.31	86.44	85.49	75.49	89.09	87.51	87.12	87.62	86.39	81.72	85.42	3.99
'50	83.15	80.75	82.58	84.73	86.86	81.18	91.00	86.62	87.44	86.03	85.03	3.18
175	81.22	88.53	84.50	85.60	84.74	85.29	81.09	89.68	85.36	83.39	84.94	2.74
200	87.31	85.44	85.49	88.33	76.36	87.50	86.21	83.77	89.63	91.83	86.19	4.15
225	85.18	82.56	81.65	83.03	87.96	82.17	85.30	84.70	86.39	87.88	84.68	2.29
250	83.15	81.64	88.62	83.03	78.08	83.18	94.13	85.65	76.28	89.81	84.36	5.39
275	85.18	87.47	85.49	89.28	90.24	84.22	78.75	85.65	86.39	91.83	86.45	3.65
300	87.31	89.61	82.58	93.30	87.96	85.29	79.51	80.24	79.67	92.87	85.83	5.23
ΔZ	2627.08	1416.83	1356.13	4052.36	1068.88	2171.56	2288.98	137.66	2590.59	3982.22	2141.70	1290.4

Appendix B: Representative Sample Photo for Each Group

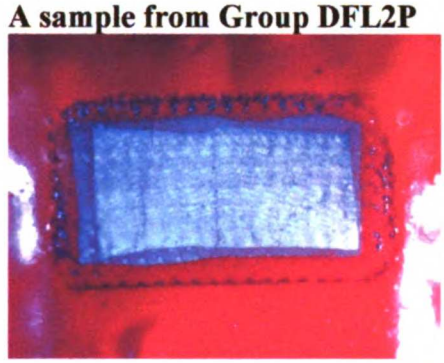
A sample from Group D



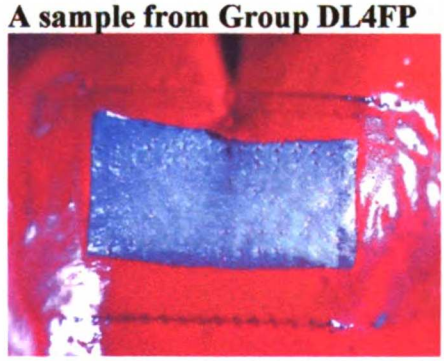
A sample from Group DL2FP



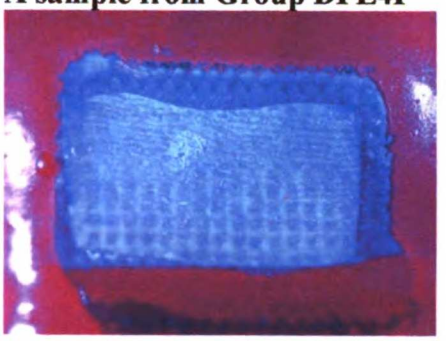
A sample from Group DFL2P



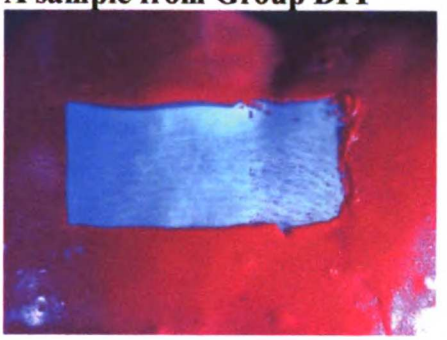
A sample from Group DL4FP



A sample from Group DFL4P

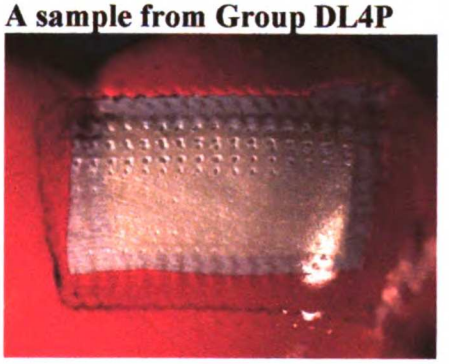


A sample from Group DFP



A sample from Group DL2P

A sample from Group DL4P



A sample from Group DP

A sample from Group SP