Original Article

Effect of casein-phosphopeptide amorphous calcium phosphate and fluoride with/without erbium, chromium-doped yttrium, scandium, gallium, and garnet laser irradiation on enamel microhardness of permanent teeth

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ABSTRACT

Background: Laser therapy, along with the use of fluoridated compounds is a novel technique suggested for caries prevention. Casein phosphopeptide amorphous calcium phosphate (CPP-ACP) is another product suggested for this purpose. This study compared the effect of CPP-ACP and fluoride with/without Erbium, chromium-doped yttrium, scandium, gallium, and garnet laser irradiation on enamel microhardness of permanent teeth.

Materials and Methods: This *in vitro* experimental study evaluated 35 extracted third molars. The teeth were decoronated, and the crowns were split into buccal and lingual halves. The samples were randomly divided into seven groups ($n = 10$) of GC Tooth Mousse, MI Paste Plus, laser, fluoride varnish, laser + GC Tooth Mousse, laser + MI Paste Plus, and laser + fluoride varnish. The baseline microhardness was measured before the intervention. After the intervention, the samples were kept in artificial saliva for 1 h and were then immersed in the demineralizing solution for 3 h followed by 21 h of immersion in the remineralizing solution for a total period of 12 days. Finally, the teeth were kept in the remineralizing solution for 2 more days. The secondary microhardness of the teeth was then measured. Data were analyzed using the Shapiro–Wilk test, two-way ANOVA, and Tukey's Honestly Significant Difference test.

Results: The fluoride varnish (14.31%) and laser + fluoride varnish (18.79%) groups experienced minimum reduction in microhardness, while the GC Tooth Mousse group experienced maximum reduction in microhardness (91.64%) ($P < 0.001$). Laser irradiation before the application of remineralizing agents increased the microhardness only in laser + GC Tooth Mousse group ($P < 0.001$).

Conclusion: Fluoride varnish increased the enamel microhardness, while GC Tooth Mousse had no such effect. Laser therapy before the application of remineralizing agents did not significantly enhance enamel resistance to demineralization.

Key Words: Casein phosphopeptide-amorphous calcium phosphate nanocomplex, fluorides, lasers, solid-state, tooth demineralization, tooth remineralization

INTRODUCTION

Dental caries remains one of the most common infectious diseases of the childhood and adolescence

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period.[1-3] There is a general consensus that prevention is better than cure. This also applies to caries prevention as well.[4] Several methods have been suggested for caries prevention. Fluoride therapy has a special place in caries prevention strategies.[5] The application of fluoride is a confirmed method of caries prevention. Fluoride reinforces the enamel surface by the formation of fluorapatite crystals and confers resistance against demineralization. Fluoride also enhances enamel remineralization,[6,7] and has cariostatic activity.[8] Application of fluoride along with a method to increase its absorption and uptake, can guarantee caries prevention.[1,3,9] Laser therapy, along with the use of fluoridated compounds, is a novel technique suggested for this purpose.[3]

Casein phosphopeptide amorphous calcium phosphate (CPP-ACP) is another product derived from the casein protein of milk, which has been suggested for caries prevention.[10,11] ACP provides amorphous and more accessible form of phosphate and calcium for the tooth structure, compared with calcium and phosphate ions present in the saliva.[12] When applied topically, CPP-ACP regulates the activity of phosphate and calcium ions and decreases demineralization and enhances remineralization as such.[6,10,11,13] CPP-ACP is available in the dental market under different commercial brands. GC Tooth Mousse is a CPP-ACP compound commonly available in the dental markets worldwide. It changes the concentration gradient of the tooth surface to increase the uptake of phosphate and calcium ions and enhances the absorption of fluoride into the tooth structure.[12,13] CPP binds to calcium and phosphate through the phosphoserines present in its chemical formulation and allows the formation of small calcium phosphate clusters (ACP). Highly insoluble calcium and phosphate are solubilized in the presence of CPP. Furthermore, CPP binds to the tooth surface and serves as a reservoir for calcium and phosphate ions.[4,10]

Recently, fluoride varnishes with added CPP and ACP were introduced to the market,[6] and some reports are available regarding the cariostatic activity of CPP-ACP paste/solution and its synergistic effect with fluoride.[14]

Laser therapy is another novel modality recently suggested to confer caries resistance to the tooth structure. Considering the reportedly optimal efficacy of laser and fluoride therapy for reinforcement of enamel structure, researchers studied the synergistic effects of these two treatment modalities, and some reported favorable results.[3,7] However, for caries prevention, it is important that the tooth surface is not traumatized by laser while it undergoes morphological or chemical changes.[9]

Erbium laser is commonly used for dental prophylaxis.[15] It ablates the hard tooth structure with minimal trauma to the pulp and the surrounding structures.[15] Erbium, chromium-doped yttrium, scandium, gallium, and garnet (Er, Cr: YSGG) laser is extensively used for cavity preparation, caries removal, endodontic treatment, and surgical procedures.[1] It is used along with air/water spray, which aids in ablation and also cools the area to prevent thermal damage.[16] Several theories have tried to explain the mechanism of enhancement of enamel resistance by laser irradiation. The suggested mechanisms include: (I) decreasing the enamel permeability by melting the enamel crystals and their recrystallization, (II) decreasing the enamel solubility by the formation of less soluble products such as tetracalcium di-phosphate monoxide, and (III) decreasing the enamel solubility by minute structural changes such as decreasing the water and carbonate content of the enamel and increasing its hydroxyl ion content and formation of pyrophosphate.[17]

Application of fluoride compounds along with laser irradiation may result in formation of a more resistant enamel structure and, at the same time, minimize the unfavorable changes caused by laser irradiation. It seems that the physical, chemical, and kinetic alterations following laser irradiation can increase the penetration depth and substantivity of fluoride in the enamel structure.[3] Simultaneous application of fluoride and laser to enhance enamel resistance has been previously studied. However, the results on this topic are controversial. Some studies reported that laser irradiation enhanced the uptake of fluoride by the tooth structure.[18-20]

Thus, this study was aimed to compare the effects of CPP-ACP and fluoride with/without Er, Cr: YSGG laser irradiation on enamel microhardness of permanent teeth.

MATERIALS AND METHODS

This *in vitro* experimental study was evaluated 35 extracted sound third molars. Teeth with caries, cracks, or hypo-calcification were excluded from the studys.

The sample size was calculated to be ten samples in each group (a total of 70 for 7 groups) according to a previous study,[18] considering alpha = 0.05, beta = 0.2, and study power of 80%.

After collection, the teeth were dried with air spray and inspected visually to ensure the absence of white spot lesions, hypoplasia, hypo-mineralization, and fluorosis. The teeth were also visually inspected for enamel cracks. Transillumination was used for this purpose as well. The teeth were immersed in 0.1% thymol solution for 24 h and were then kept in saline at room temperature until the experiment. The saline was refreshed daily. All teeth had been extracted within 3 months before the onset of the study.

The crowns were cleaned with pumice powder and rubber-cup attached to a low-speed handpiece. Next, the roots were cut at 5 mm below the cementoenamel junction using a diamond disc (Yuanda jgs, Huaxian Gaoping YanDa Diamond Grinding Plant, China) under water spray. The crowns were then fixed with sticky wax in a cutting machine and split into buccal and lingual halves (Hamco machine, NY, USA). The buccal and lingual enamel surfaces were polished using 400, 600, 800, and 1000-grit silicon carbide papers (Matador, Germany) to obtain a smooth enamel surface. Next, a sticker measuring 3 mm × 3 mm was placed on the surface to create a window and the surrounding areas were coated with nail varnish (Golden Rose, Erkul Kozmetik Sanayi ve Ticaret, Turkey). To simulate the oral environment, all samples were immersed in artificial saliva (2 g methyl-p-hydroxybenzoate, 10 g sodium carboxymethyl cellulose, 0.625 g Kcl, 0.059 g $MgCl_2.6H_2O$, 0.166 g $CaCl_2.2H_2O$, and 0.326 g KH_2PO_4) for 1 h before the experiment. Next, they were randomly divided into seven groups (n = 10):
- Group 1: CPP-ACP (GC Tooth Mousse; GC Corporation, Tokyo, Japan)
- Group 2: MI Paste Plus (GC Corporation, Tokyo, Japan)
- Group 3: Fluoride varnish (FluoroDose; Centrix Company, CT, USA)
- Group 4: Er, Cr: YSGG laser (Waterlase, Biolase Technology, San Clement, CA, USA)
- Group 5: Er, Cr: YSGG laser + GC Tooth Mousse
- Group 6: Er, Cr: YSGG laser + MI Paste Plus
- Group 7: Er, Cr: YSGG laser + fluoride varnish.

The samples were coded from 1 to 70 using black nail polish. Before the intervention, the teeth underwent Vickers hardness test to measure their baseline microhardness. For this purpose, the tooth surface received three vertical strokes at points 12 mm apart with 300 g load for 10 s, applied by a pyramid diamond indenter. The diameter of the created indentation after each stroke was measured, and the mean value was calculated and reported as the baseline microhardness of the respective sample. In Groups 1–3, the respective remineralizing agents were applied according to the manufacturers' instructions. In Group 4, laser therapy was performed using Er, Cr: YSGG laser with 2780 nm wavelength, 100 mJ energy, 10 Hz frequency and 8 J/cm² energy density with 35%–40% water and 50% air at 1 mm distance for 30 s according to our pilot study (Waterlase, Biolase Technology, San Clement, CA, USA). In Groups 5–7, the laser was first irradiated, and then the remineralizing agents were applied according to the manufacturers' instructions.

In Group 1, CPP-ACP (GC Tooth Mousse) was applied and remained on the tooth surface for 3 min. Next, it was cleaned, and the samples were immersed in artificial saliva for 1 h. The composition of the artificial saliva was as follows: 2 g methyl-p-hydroxybenzoate, 10 g sodium carboxymethyl cellulose, 0.625 g Kcl, 0.059 g $MgCl_2.6H_2O$, 0.166 g $CaCl_2.2H_2O$, 0.326 g KH_2PO_4.

In Groups 2 and 3, the respective remineralizing agents were applied on the surface with a swab and remained there for 3 min according to the manufacturers' instructions. They were then removed, and the teeth were immersed in artificial saliva for 1 h.

For pH cycling, the samples were separately immersed in a demineralizing solution comprising of 2.0 mmol/L calcium and 2.0 mmol/L phosphate in 75 mmol/L acetate buffer with a pH of 4.3 for 3 h daily followed by 21 h of immersion in a remineralizing solution with the composition of 1.5 mmol/L calcium, 0.9 mmol/L phosphate, and 150 mmol/L KCL in 20 mmol/L cacodylate buffer with a pH of 7.4. Between the two cycles, the samples were rinsed with distilled water, dried with paper towel, and then subjected to a new cycle. This process was repeated for 12 days, and then, the samples were kept in the remineralizing solution for 2 more days. The entire process was performed in an incubator at 37°C. Next, the samples were rinsed with saline for 10 s, and their secondary microhardness was measured using a Vickers hardness tester, as explained earlier. The mean secondary microhardness value was recorded for each sample.

Data were analyzed using SPSS version 21 (SPSS Inc., Chicago, IL, USA). Normal distribution of data was evaluated using the Shapiro–Wilk test. Homogeneity of variances was evaluated using the Levene's test, which revealed that the assumption of homogeneity of variances was met in all groups ($P < 0.001$). Pairwise comparisons were carried out using Tukey's Honestly Significant Difference (HSD) test.

RESULTS

Table 1 presents the mean microhardness values before and after the intervention and the percentage of microhardness reduction in the seven groups. As shown, minimum percentage of reduction in microhardness (14.31%) was noted in the fluoride varnish group. Maximum percentage of reduction in

microhardness (91.64%) was noted in the GC Tooth Mousse group.

All the interventions had significant effects on enamel microhardness ($P < 0.001$). A significant difference was noted in the mean change of microhardness among the seven groups ($P < 0.001$).

Thus, pairwise comparisons were carried out using the Tukey's HSD test [Table 2]. Significant differences were noted between MI Paste Plus + laser and laser + fluoride varnish ($P = 0.019$), MI Paste Plus + laser and fluoride varnish ($P = 0.004$), fluoride varnish + laser and GC Tooth Mousse ($P = 0.00$), laser and GC Tooth Mousse ($P = 0.001$), GC Tooth Mousse and MI Paste Plus ($P = 0.001$), and GC Tooth Mousse and fluoride varnish ($P = 0.001$) groups.

Table 1: Mean microhardness values before and after the intervention and the percentage of reduction in the 7 groups (*n*=10)

Group	Mean±std. deviation of microhardness at baseline	Mean±std. deviation of microhardness after the intervention	Difference in microhardness	Percentage of reduction
GC Tooth Mousse+laser	371.40±17.896	278.30±37.491	93.1±41.92	36.12
MI varnish+laser	339.70±35.603	217.30±42.264	122.4±67.64	63.85
Fluoride varnish+laser	339.70±25.312	292.10±40.567	47.6±48.55	18.79
Laser	340.90±25.762	283.70±27.305	57.2±32.32	20.98
GC Tooth Mousse	343.70±25.351	188.80±38.352	154.9±59.38	91.64
MI varnish	342.30±24.336	286.00±34.400	56.3±32.75	20.91
Fluoride varnish	342.80±22.958	306.40±44.754	36.4±50.40	14.31

Table 2: Pairwise comparisons of the mean microhardness of the groups

Group 1	Group 2	Mean difference	Std. error	*P*
GC tooth mousse+Laser	MI varnish+Laser	-29.30000	21.95773	0.833
	Fluoride varnish+Laser	45.50000	21.95773	0.382
	Laser	35.90000	21.95773	0.661
	GC tooth mousse	-61.80000	21.95773	0.088
	MI varnish	36.80000	21.95773	0.634
	Fluoride varnish	56.70000	21.95773	0.149
MI varnish+Laser	Fluoride varnish+Laser	74.80000*	21.95773	0.019
	Laser	65.20000	21.95773	0.061
	GC tooth mousse	-32.50000	21.95773	0.755
	MI varnish	66.10000	21.95773	0.055
	Fluoride varnish	86.00000*	21.95773	0.004
Fluoride varnish+Laser	Laser	-9.60000	21.95773	0.999
	GC tooth mousse	-107.30000*	21.95773	0.000
	MI varnish	-8.70000	21.95773	1.000
	Fluoride varnish	11.20000	21.95773	0.999
Laser	GC tooth mousse	-97.70000*	21.95773	0.001
	MI varnish	0.90000	21.95773	1.000
	Fluoride varnish	20.80000	21.95773	0.963
GC tooth mousse	MI varnish	98.60000*	21.95773	0.001
	Fluoride varnish	118.50000*	21.95773	0.001
MI varnish	Fluoride varnish	19.90000	21.95773	0.970

DISCUSSION

This study compared the effect of CPP-ACP and fluoride varnish with/without Er, Cr: YSGG laser irradiation on enamel microhardness of permanent teeth. Our results showed that the fluoride varnish (14.31%) and laser + fluoride varnish (18.79%) groups experienced a minimum reduction in microhardness, while the GC Tooth Mousse group (91.64%) experienced a maximum reduction in microhardness. Laser irradiation before the application of remineralizing agents increased the microhardness only in Group 5 (laser + GC Tooth Mousse).

Reynolds *et al.*[14] reported that MI Paste Plus significantly enhanced enamel remineralization, which was in agreement with our findings since MI paste plus group in our study experienced 91% reduction in microhardness.[17] Since fluoride cannot completely stop the progression of caries, synergistic effects of laser and fluoride have also been studied for more effective caries prevention.[19,21] Tagomori and Morioka[22] discussed that laser-modified enamel had higher uptake of acidulated phosphate fluoride (APF), especially when laser therapy was performed before fluoride therapy. Hossain *et al.*[23] reported that CO_2 laser irradiation, combined with the application of sodium fluoride was more effective than CO_2 laser irradiation alone.

Er, Cr: YSGG laser operates at 2.78 μm wavelength, which is suitable for the ablation of hard tooth structure with minimal damage to the pulp and surrounding tissues. It can effectively ablate the enamel since it has high absorption in water and the hydroxyl radicals present in the structure of hydroxyapatite.[24] Although the use of sub-ablative laser energy has been suggested for caries prevention, there is still controversy regarding the exposure parameters and their efficacy for the reduction of enamel solubility.[24]

Mathew *et al.*[25] evaluated the enamel demineralization of permanent teeth using atomic absorption spectrometry and showed that application of APF alone and in combination with laser decreased enamel demineralization, which was in line with our findings. However, they showed that the application of CO_2 laser along with APF resulted in higher enamel resistance than the application of APF alone, which was different from our results. This difference is probably due to the use of different laser types since they used CO_2 laser while we used Er, Cr: YSGG laser. They also showed that the results in the use of Er: YAG laser were similar to the application of fluoride alone, which was in line with our findings probably because Er: YAG laser is somewhat similar to Er, Cr: YSGG laser. Anaraki *et al.*[19] used atomic absorption spectrometry to assess the enamel resistance of molar teeth and showed that demineralization in the CO_2 laser group was lower than that in the APF alone and APF + Er, Cr: YSGG laser groups. They demonstrated that although CO_2 laser conferred further resistance to the enamel, Er, Cr: YSGG laser had no such effect when used along with fluoride. This finding was in line with our findings. Ana *et al.*[1] used the Knoop microhardness test and found no significant difference in the application of Er, Cr: YSGG laser + APF compared with APF alone.

Controversy exists regarding the sequence of laser therapy and fluoride therapy. Considering the role of laser in the preservation of fluoride ions close to the enamel, we first laser-irradiated the samples and then applied fluoride, which was similar to the methodology adopted by some previous studies.[1,24,25] Some other studies found no significant difference in the sequence of application of laser and fluoride regarding their effect on enamel resistance.[21,22] Thus, differences in the results of studies can be due to some other factors such as laser settings and methods of the assessment of enamel resistance.

Subramaniam and Pandey[26] assessed the effect of CPP-ACP and Er, Cr: YSGG laser on primary teeth and reported that laser treatment before the application of CPP-ACP significantly increased the surface microhardness, which was different from our findings. This difference may be due to the use of different types of teeth and different microhardness tests since they used the Brinell test while we used Vickers hardness test. Furthermore, they immersed the samples in 1% citric acid for 30 min before laser treatment while it was not performed in our study. However, they indicated that laser irradiation, compared with the application of CPP-ACP alone, increased the enamel microhardness, which was in line with our findings. Vitale *et al.*[27] reported that the application of diode laser accompanied by topical application of fluoride yielded superior results compared with the use of fluoride gel alone, which was different from our results. This difference can be due to the use of different laser types with different mechanisms of

action. Apel *et al.*[28] reported that the use of erbium laser along with fluoride did not enhance enamel resistance to acid attacks, which was in agreement with our findings. However, Hossain *et al.*[29] showed the optimal efficacy of Er, Cr: YSGG laser in increasing the enamel resistance to acid attacks with no adverse thermal effects. Scanning electron microscopic observations revealed that laser-irradiated areas had been melted, and the thermally degenerated surface of enamel and dentin remained unchanged after demineralization. However, the melted surface may not be necessarily required to confer resistance against acid attacks. Enamel resistance can also be achieved by chemical changes such as reduction in carbonate content of the superficial enamel or degradation of part of the organic matrix. Chin-Ying *et al.*[30] reported that the erbium laser significantly decreased the enamel porosities and prevented enamel demineralization.

In this study, laser treatment before fluoride therapy had no significant effect on enamel resistance to acid attacks. In this study, the reduction in microhardness in the laser group had no significant difference with that in MI Paste Plus and fluoride varnish groups. However, the reduction in microhardness in the GC Tooth Mousse group was significantly higher than that in laser, MI Paste Plus, and fluoride varnish groups. Furthermore, our results showed that laser irradiation before the application of GC Tooth Mousse increased the enamel microhardness, but not significantly. Moreover, laser irradiation before the application of fluoride varnish and MI Paste Plus increased the microhardness, but not significantly. Bahrololoomi and Lotfian[31] concluded that diode laser in combination with fluoride varnish was not more effective than fluoride alone for increasing the enamel resistance to demineralization, which was in line with our results.

In vitro design was a limitation of this study, which limits the generalization of results to the clinical setting. Future studies using different laser types with different exposure settings are required to assess their effect on enamel resistance when used in combination with different remineralizing agents. Furthermore, the effect of laser irradiation on the pulp should be investigated in future studies.

CONCLUSION

Fluoride varnish enhanced the enamel microhardness and its resistance to demineralization while GC Tooth Mousse had no such effect. Laser therapy before the application of remineralizing agents did not significantly enhance enamel resistance to demineralization.

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Conflicts of interest
The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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