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Effects of Two Remineralizing Agents in Combination with Er:YAG and CO_2 Laser Irradiation on Microhardness of Demineralized Enamel: A Preliminary In Vitro Study

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ABSTRACT

Objectives: This study assessed the effects of two remineralizing agents namely MI Paste Plus containing casein phosphopeptide amorphous calcium phosphate fluoride (CPP-ACFP) and Remin Pro containing hydroxyapatite, fluoride and xylitol (HFX) with/without erbium-doped yttrium aluminium garnet (Er:YAG) and CO_2 laser irradiation on demineralized enamel microhardness.

Materials and Methods: In this in vitro study, 70 sound human premolars were mesiodistally sectioned, demineralized at a pH of 4.6 for 8 hours, and randomly divided into 7 remineralization groups (n=10): of (I) MI Paste Plus (CPP-ACFP), (II) Remin Pro (HFX), (III) MI Paste Plus+CO_2 laser (0.7 W power, 50 Hz), (IV) Remin Pro+CO_2 laser, (V) MI Paste Plus+Er:YAG laser (1 W power, 10 Hz), (VI) Remin Pro+Er:YAG laser, and (VII) negative control. The Vickers hardness number of specimens was then measured. The groups were compared by one-way ANOVA and Tukey's test (α=0.05).

Results: The mean microhardness was 319.8±49.9, 325.3±44.6, 359.4±35.7, 296.4±33.7, 319.9±58.1, 358.9±28.4, and 240.0±41.6 kg/mm2 in groups 1 to 7, respectively. The difference in microhardness was significant among the groups (P<0.0001). Pairwise comparisons revealed significant differences in microhardness between all groups (P≤0.03) except between groups 1 and 2, 1 and 5, 2 and 5, and 3 and 6 (P>0.05).

Conclusion: Both Remin Pro (containing HFX) and MI Paste Plus (containing CPP-ACFP) can cause enamel remineralization. MI Paste Plus+CO_2 laser irradiation and Remin Pro+Er:YAG laser irradiation were significantly more effective than the application of each remineralizing agent alone.

Keywords: Lasers, Gas; Lasers, Solid-State; Casein Phosphopeptide-Amorphous Calcium Phosphate Nanocomplex; Hydroxyapatites; Sodium Fluoride; Tooth Remineralization

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INTRODUCTION

Dental caries deserves attention as it is the most frequent chronic disease in children worldwide [1,2]. Detection of incipient lesions and application of remineralizing agents are essential to prevent the progression of caries and stop the demineralization process before cavitation [3]. White spot lesions can be stopped by using high-calcium remineralizing agents to prevent them from progressing to cavitation [3-6]. Calcium phosphate-based remineralizing agents have shown promising results in non-invasive management of incipient carious lesions [5,6]. Different materials and methods have been proposed for this purpose [4,5,7-14]. Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) is likely to prevent demineralization, enhance remineralization, or do both [5,10]. CPP-ACP is a sulfate-based calcium compound with a high concentration of calcium phosphate [15]. The role of CPP-ACP is to buffer the activity of free calcium and phosphate ions and create a supersaturated state to reduce demineralization and promote remineralization [15]. CPP also fixes calcium and phosphate in a quasi-stable solution and provides high concentrations of Ca^{2+} and PO_4^{3-} ions to diffuse into enamel lesions [15]. A combination of CPP-ACP and fluoride can have a synergistic effect on enamel remineralization [11,12]. MI Paste Plus (GC Corp., Tokyo, Japan) is a commercial combination of 10% CPP-ACP and 900 ppm fluoride (CPP-ACFP), which is used to enhance remineralization.

Hydroxyapatite and its combination with fluoride and other caries-preventive materials can also enhance remineralization [4]. It has been suggested that hydroxyapatite fills the superficial enamel lesions and small defects caused by erosion, and fluoride has been proven to seal dentinal tubules [13]. An aqueous-based remineralizing agent known as Remin Pro (VOCO GmbH, Cuxhaven, Germany) comprising of hydroxyapatite, 1450 ppm fluoride, and xylitol was also introduced for this purpose (xylitol is also known for its cariostatic properties) [13]. This product is claimed to be suitable for management of dentin hypersensitivity, and inhibition of enamel demineralization and progression of subsurface enamel lesions [4,13].

Laser irradiation is another approach proposed as a supplement for prevention of dental caries [7-9]. Some studies investigated the potential preventive effects of laser irradiation on enamel and dentin [14,16]. Carbon dioxide (CO_2) and erbium-doped yttrium aluminum garnet (Er:YAG) lasers have been shown to be effective in prevention of dental caries [4]. These lasers can be absorbed by water and hydroxyapatite crystals and also can modify their crystalline structure and enhance their resistance to demineralization [17-19]. It has been shown that CO_2 laser irradiation can change the chemical structure, solubility, and morphology of enamel and dentin [20]. Hence, it can increase the resistance of enamel to caries and acid attacks, and also enhance the effects of fluoride on enamel composition [20].

Demineralization and remineralization can be evaluated through microhardness tests. Microhardness is classified into two main types: resistance to scratching and resistance to indentation [4,9,14,20-23]. In dental science, indentation resistance is commonly evaluated to assess microhardness [4,9,14,20-24]. Accordingly, the microhardness of a material is calculated based on the small (about a few micrometers) indentation created on the surface by the indenter [4,9,14,20-23]. The Knoop, Vickers, Brinell, Bacrall, and Shore A hardness tests are most commonly used for this purpose [23,24]. The Vickers hardness test is used for brittle materials such as teeth and restorative materials. In addition, this test is used to measure the microhardness of materials that may have different levels of hardness in different parts, and also when the test sample is small [23,25]. In this test, the indenter is in the form of a diamond pyramid with a square-shaped base, and the angle between its opposite faces is 136 degrees [23,25]. The indenter is penetrated into the material with a certain force, creating a square-shaped indentation [23,25]. The diameters of this square are measured under a microscope [23,25]. The penetrating pyramidal indenter causes stress when in contact with the surface of the material [23,25]. Gradually, as the indenter sinks, the force is more spread out [23,25]. Not all elastic changes are recorded because elastic recovery occurs before the microscopic measurement [23,25]. After determining the diameter of the square, the Vickers hardness number of the material is determined by dividing the magnitude of force by the surface area of the affected region [23-25].

Previous studies examined the effects of laser irradiation or application of remineralizing agents on incipient carious lesions [4,5,14]. Nevertheless, when it comes to the combination of both methods, limited evidence exists regarding the potentially synergistic efficacy of their co-administration for caries prevention, as there are merely very few recent studies in this regard [4,5,14]. Thus, the present study aimed to evaluate the effects of different combinations of two remineralizing agents namely MI Paste Plus (containing CPP-ACFP) and Remin Pro containing hydroxyapatite, fluoride and xylitol (HFX) with/without Er:YAG and CO_2 laser irradiation on the microhardness of demineralized enamel.

MATERIALS AND METHODS

In this in vitro experimental study, a total of 70 intact premolar teeth, extracted for orthodontic treatment, were collected from dental clinics within 2 months. The teeth had been extracted for treatment purposes not related to this study. The study protocol was approved by the university research ethics committee (ethical code: IRAJUMS.REC.1398.262).

Sample size:

Based on the parameters derived from a study by Khamverdi et al, [14] including the mean microhardness values of 361.86 and 192.57 in the two groups, standard deviations of 22.22 and 50.87 in the two groups, and assuming a 90% power at 0.05 level of significance with 95% confidence interval (CI), the sample size for each group was calculated to be 8 specimens. The sample size was increased to 10 specimens per group in order to increase the power.

Specimen preparation:

The teeth were cleaned by a toothbrush and immersed in 5.0% thymol solution (Sigma-Aldrich, St. Louis, MO, USA) until the sample size was reached (about 2 months). The teeth were decoronated at the cementoenamel junction by a long fissure diamond bur (Tizkavan, Tehran, Iran) and a high-speed headpiece (Pana-MAX, NSK, Tokyo, Japan) under water coolant. The crowns were bisected mesiodistally and mounted horizontally in acrylic resin blocks (Acropars, Kaveh, Tehran, Iran) as depicted in Figure 1, leaving a window of buccal enamel (4×4 mm^2) exposed for demineralization and remineralization processes. The teeth were stored in distilled water to prevent dehydration.

Demineralization process:

The specimens were stored in a demineralizing solution (0.05 mM calcium chloride+2.2 mM sodium hypophosphite+50 mM acetic acid) with an adjusted pH of 4.6 for 8 hours. Afterwards, the specimens were stored in artificial saliva (Hypozalix; Biocodex, Gentilly, France) for 1 hour. All surface treatments were standardized since the specimens were first subjected to pH cycling altogether and were then grouped. Finally, the specimens were immersed in a remineralizing solution for 15 hours (20 mM HEPES+1.5 mM calcium chloride+0.9 mM potassium hypophosphite+1 ppm fluoride in the form of sodium fluoride with a pH adjusted at 7). This process was continued for 14 days. The demineralization and remineralization solutions were refreshed daily [14].

Experiments:

Next, the specimens were randomly divided into 7 groups as follows (n=10 each):

Group 1: Application of MI Paste Plus containing CPP-ACFP

Group2: Application of Remin Pro containing HFX

Group 3: Application of MI Paste Plus followed by CO_2 laser irradiation

Group 4: Application of Remin Pro followed by CO_2 laser irradiation

Group 5: Application of MI Paste Plus followed by Er:YAG laser irradiation

Group 6: Application of Remin Pro followed by Er:YAG laser irradiation

Group 7: Negative control group: only demineralization was performed in this group with no remineralization.

Application of remineralizing agents:

MI Paste Plus (GC Corp., Tokyo, Japan) was applied directly on the demineralized enamel surface for 5 minutes according to the manufacturer's instructions and was then washed. Similarly, Remin Pro paste (VOCO GmbH, Cuxhaven, Germany) was applied on the demineralized enamel surface according to the manufacturer's instructions for 5 minutes using a microbrush and was then washed. In the laser-treated groups, the remineralizing agent on the surface of specimens was not rinsed.

CO_2 and Er:YAG laser irradiation:

The specimens in groups 3 and 4 were irradiated by a CO_2 laser device (Smart US 20D; Deka, Florence, Italy) with the following laser parameters: wavelength=10.6 µm, power=0.7 W, pulse frequency=50 Hz, focal spot=0.2 mm, pulse duration=0.4 ms, non-contact mode with 10 mm distance between the hollow tube tip and the tooth surface, spot size=0.4 mm, and scanning time of the target=20 s [14]. The following parameters for Er:YAG laser treatment were used in groups 5 and 6: Er:YAG

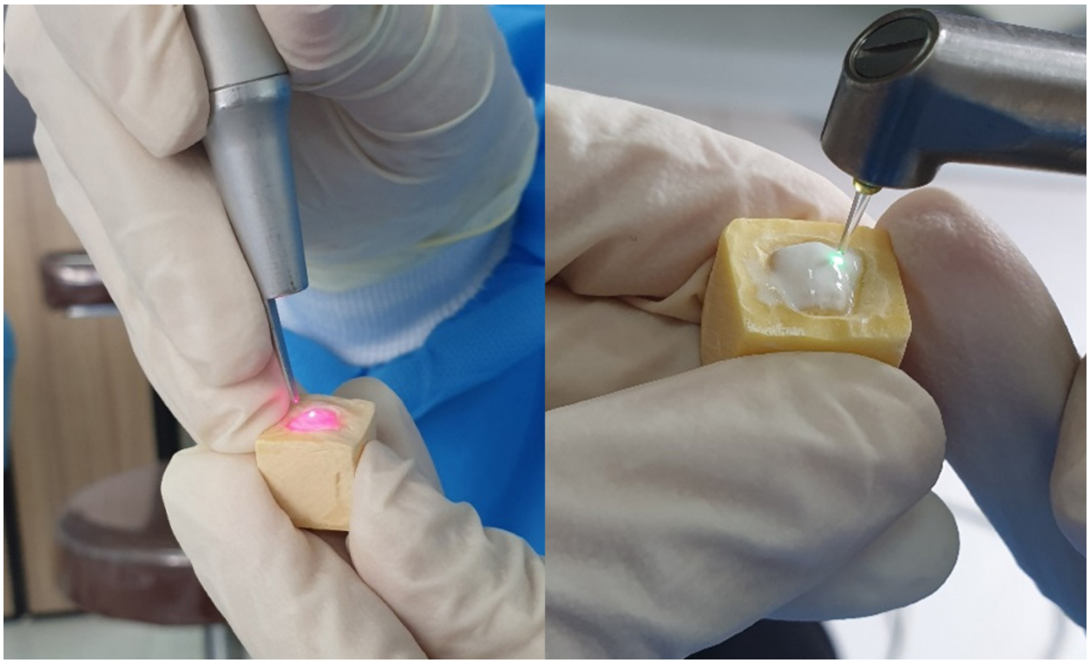

Fig. 1. Mounted teeth undergoing laser irradiation with CO_2 laser (left) and ER:YAG laser (right)

laser (LightWalker AT S; Fotona Medical Lasers, Ljubljana, Slovenia) with a wavelength=2.94 µm, energy: 100 mJ, pulse frequency: 10 Hz, 0% air/water regulations, power=1 W, pulse duration=0.3 ms, tip: conical 600 µm, mode of irradiation: SP (300 µs pulse width), time of scanning of the target: 20 s, and non-contact mode with 1 mm distance between the hollow tube tip and the tooth surface [26]. The Er:YAG laser was equipped with a H14 handpiece (LightWalker AT S Handpiece; Fotona, Ljubljana, Slovenia) holding a conical PIPS tip (600 µm diameter) without water spray. The handpiece was placed perpendicular to the demineralized enamel surface. In order to ensure uniform irradiations and coverage, the specimens were subjected to irradiation from every direction with slow horizontal and vertical motions (Figure 1). The distances for all irradiations were standardized visually by the laser operator who was an expert in dental lasers.

Microhardness test:

The specimens were coded, and the microhardness test was performed by an operator blinded to the group allocation of specimens. The Vickers microhardness test was used to measure the hardness of all treated specimens after storage in distilled water for 48 hours using a microhardness tester (Micrometer 1, Buehler, Lake Bluff, IL, USA) applying 500 g load for 5 seconds to 3 points on the surface. The mean value of the microhardness measured at 3 points was calculated and reported as the microhardness value of each specimen in kg/mm^2.

Statistical analysis:

Descriptive statistics and 95% CIs were calculated for each group. The normality of data distribution was ensured by the Kolmogorov-Smirnov test. The groups were compared in terms of microhardness values by one-way ANOVA followed by the Tukey's post-hoc test. SPSS 25 (IBM, Armonk, NY, USA) was used for all analyses with the level of significance set at 0.05.

RESULTS

Compared to the control group, the experimental groups showed an increase in microhardness by 133.3% (MI Paste Plus), 135.5% (Remin Pro), 149.8% (MI Paste Plus+CO_2 laser), 123.5% (Remin Pro+CO_2 laser), 133.3% (MI Paste Plus+Er:YAG laser), and 149.5% (Remin Pro+Er:YAG laser) (Figure 2, Table 1). Overall comparison of the microhardness values of the groups revealed a significant difference (ANOVA, $P<0.0001$).

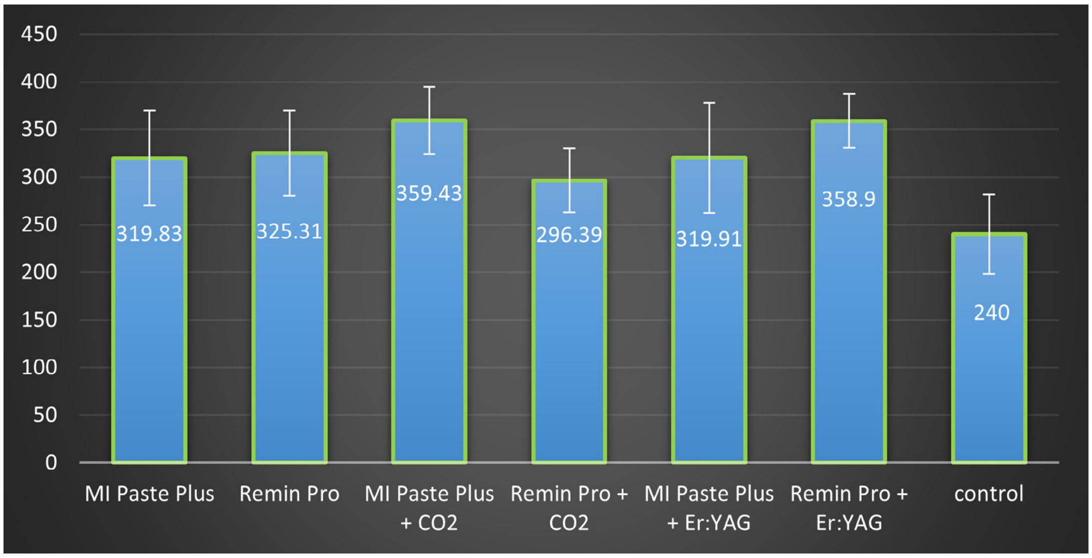

Fig. 2. Mean microhardness of the study groups (kg/mm^2)

Table 1. Microhardness values of the study groups (kg/mm^2)

Group	Mean±Standard deviation	95% Confidence interval	
1. MI Paste Plus (CPP-ACFP)	319.8±49.9 ^{a,b}	304.4	335.3
2. Remin Pro (HFX)	325.3±44.6 ^{a,c}	310.3	340.2
3. MI Paste Plus+CO_2 laser	359.4±35.7 ^{d}	343.9	374.9
4. Remin Pro+CO_2 laser	296.4±33.7	281.6	311.1
5. MI Paste Plus+Er:YAG laser	319.9±58.1 ^{b,c}	305.3	334.4
6. Remin Pro+Er:YAG laser	358.9±28.4 ^{d}	343.4	374.3
7. Control	240.0±41.6	225	254.9

Superscripted letters indicate non-significant pairwise comparisons (Tukey, P≥0.6). The rest of pairwise comparisons were statistically significant (P ≤ 0.03).

Pairwise comparisons by the Tukey's test revealed that the microhardness values of the first two groups (MI Paste Plus versus Remin Pro) were not significantly different (difference=5.48 kg/mm^2, P=0.620, Table 1). Similarly, the microhardness values of groups 1 and 5 (MI Paste Plus versus MI Paste Plus+Er:YAG laser) were not significantly different (difference=0.8 kg/mm^2, P=0.990). The microhardness values of groups 2 and 5 (Remin Pro versus MI Paste Plus+Er:YAG laser; difference=5.4 kg/mm^2, P=0.610) and groups 3 and 6 (MI Paste Plus+CO_2 laser versus Remin Pro+Er:YAG laser; difference=0.53 kg/mm^2, P=0.960, Table 1) were not significantly different either. The rest of pairwise comparisons were all significant (Table 1). The least favorable result was obtained in the control group. All groups showed significantly higher microhardness than the control group (all P-values=0.001). The most promising results belonged to groups 3 and 6 (MI Paste Plus+CO_2 laser and Remin Pro+Er:YAG laser) which yielded quite similar results.

Group 3 (MI Paste Plus+CO_2 laser) showed microhardness values significantly higher than groups 1 (MI Paste Plus, difference=39.6 kg/mm^2, P=0.001), 2 (Remin Pro, difference=34.12 kg/mm^2, P=0.002), 4 (Remin Pro+CO_2 laser, difference=63.04 kg/mm^2, P=0.001), 5 (MI Paste Plus+Er:YAG laser, difference=39.52 kg/mm^2, P=0.001), and 7 (control, difference=119.43 kg/mm^2, P=0.001).

Group 6 (MI Paste Plus+CO_2 laser) showed microhardness values significantly higher than groups 1 (MI Paste Plus, difference=39.7 kg/mm^2, P=0.001), 2 (Remin Pro, difference=33.59 kg/mm^2, P=0.002), 4 (Remin Pro+CO_2 laser, difference=62.51 kg/mm^2, P=0.001), 5 (MI Paste Plus+Er:YAG laser, difference=38.99 kg/mm^2, P=0.001), and 7 (control, difference=118.9 kg/mm^2, P=0.001).

Group 5, MI Paste Plus+Er:YAG laser) showed microhardness values similar to groups 1 and 2, as noted above and in Table 1. The microhardness of group 5 was only significantly higher than that of the control group and group 4 (Remin Pro+CO_2 laser, difference=23.52 kg/mm^2, P=0.030). Group 4 had the lowest microhardness among all the experimental groups and showed a significantly higher microhardness only in comparison with the control group (difference=56.39 kg/mm^2, P=0.001). The microhardness of group 4 was significantly lower than that of groups 1 (difference=23.44 kg/mm^2, P=0.030) and 2 (difference=28.92 kg/mm^2, P=0.007).

DISCUSSION

The present results showed that although the applied remineralizing agents were equally effective, addition of CO_2 laser to MI Paste Plus and addition of Er:YAG laser to Remin Pro significantly improved their remineralizing efficacy. Interestingly, other tested combinations were either added no benefit (MI Paste Plus+Er:YAG laser) or significantly reduced the efficacy of the remineralizing agent (Remin Pro+CO_2 laser). The results of the current study agreed with the findings of two previous studies in some aspects [27,28]. MI Paste Plus can create a supersaturated state of calcium and phosphate over the enamel surface. The fluoride content of MI Paste Plus has a synergistic effect with CPP-ACP; thus, fluoride enhances the remineralizing potential of CPP-ACP [11,29]. Remin Pro contains HFX and can fill superficial enamel defects [30,31]. In this regard, Valizadeh et al. [32] concluded that application of Er:YAG and CO_2 lasers alone could not significantly improve the resistance of dental tissues to caries; nevertheless, they had synergistic effects together with sodium fluoride.

In a study conducted in 2016 [33], fluoride was applied before CO_2 laser irradiation; the results indicated no synergistic effects for the combination of fluoride and CO_2 laser on dental caries and erosion [33]. Silva et al. [34] assessed the effect of some products containing fluoride (i.e., titanium tetrafluoride and stannous fluoride) alone and in combination with CO_2 laser on enamel erosion [34]. Another study revealed that beneficial effects were obtained when acidulated phosphate fluoride was used along with CO_2 laser irradiation [35]. Evidence shows that CO_2 laser irradiation may block the dentinal tubules by dentin melted due to high temperature at the irradiation site. It can remove water, organic compounds, and carbonate ions that have been substituted into the hydroxyapatite structure over time, increasing the mineral composition of enamel [7-9,27].

In the current study, CO_2 laser irradiation after the application of MI Paste Plus had a considerable synergistic effect on enamel microhardness. This synergistic effect might be due to the potential role of CPP-ACFP in MI Paste Plus in formation of persistent tiny calcium phosphate hydrate clusters in the intracellular matrix, which release calcium and phosphate ions [26,36]. These small clusters serve as a reservoir, and are frequently refilled with calcium and phosphate ions, increasing the potential of CO_2 laser to inhibit enamel demineralization [26,36]. Additionally, the change initiated by laser increases the diffusion of CPP-ACFP complex into hydroxyapatite crystals in deeper layers. However, the synergistic effects did not occur in some groups (i.e., MI Paste Plus and Er:YAG laser irradiation) and therefore, further studies are recommended in this regard [26,36].

According to the results of the present study, enamel irradiation by Er:YAG laser after the application of Remin Pro increased the microhardness of enamel surface compared to the application of Remin Pro alone and Remin Pro along with CO_2 laser irradiation. In the present study, low-level Er:YAG laser irradiation combined with MI Paste and Remin Pro resulted in high fluoride uptake in the Remin Pro compared with MI Paste Plus group. Bevilacqua et al. [18] evaluated a wide range of energy densities of Er:YAG laser followed by a fluoride treatment. They reported that laser caused some structural changes in the enamel surface that led to fluoride preservation. The increase in enamel microhardness in the Er:YAG laser+Remin Pro group may be due to the higher fluoride content of Remin Pro [18]. Comparable to the current observation, Ahrari et al. [21] asserted that application of diode laser through photo-absorbing agents comprising of sodium fluoride or MI Paste Plus did not yield synergistic effects to enhance remineralization of white spot lesions [21] but combined use of diode laser and Remin Pro had a synergistic effect on microhardness of white spot lesions [21]. Accordingly, the impact of laser irradiation on the cycle of demineralization may depend on the chemical composition of the applied material. Contrary to the present findings, Nair et al. [37] observed the lowest rate of calcium dissolution and highest acid resistance in using a combination of CPP-ACFP and Er:YAG laser. Fluorapatite is formed in the enamel subsurface where fluoride ions interact with free calcium and phosphate ions. Elimination of water and carbonate causes micro-gaps and micro-fissures between globular granules following laser irradiation, and organic substances of the enamel can trap calcium, phosphate, and fluoride ions released from tooth during the demineralization process [37]. The unfavorable results observed in groups 4 and 5 in the present study may be due to the negative interactions of the applied materials and lasers. Each remineralizing agent has different physical and chemical properties which can affect the dental tissue in a different way; moreover, each laser has a particular topological influence. These effects may reinforce or neutralize each other as it was the case in groups 4 and 5. Further studies with robust methods, inclusion of more intermediate control groups, and use of scanning electron microscopy (SEM) are required to further scrutinize this topic.

Er:YAG laser without coolant may be more beneficial for caries control compared to the use of water spray [19,38]. Due to this fact and in order to achieve adequate temperature rise at the surface to improve crystallographic changes, all irradiations were performed without water coolant in the present study.

This study had some limitations. Due to the COVID-19 pandemic, conduction of SEM assessments was not possible, although none of the relevant previous studies used SEM. The present results and the available literature imply that the effect of laser irradiation on the demineralization cycle may be affected by the chemical composition of materials, the level of energy, air-water cooling status, use of distilled water instead of artificial saliva, wavelength of laser, differences in sample preparation methods, and short or long-term treatments [3,22]. Thus, more studies are required on this topic. Moreover, the present results are generalizable merely to remineralizing agents used and laser specifications adopted in the present study. Future studies are needed to assess different combinations of remineralizing agents and laser protocols. We had some limitations in standardizing the laser parameters of the two laser types. The rationale for the laser parameters used in this study was the previous studies that had suggested these laser parameters [14,26]. Of course, it was not technically feasible to use similar parameters for the two laser types because it is not guaranteed that a specific parameter that works for a particular laser type necessarily works for another laser type as well. A larger sample size could increase the reliability of the results. However, the sample size of 70 specimens was not small, especially noting the fact that each material or each laser type was evaluated in 20 specimens distributed in two groups; not to mention the fact that the controlled conditions of in vitro experimental studies improve the reliability of the results as well. Overall, the significant findings of the present study confirmed the relative adequacy of the current sample size. We also performed demineralization and remineralization in order to simulate the oral environment [14]. But the dynamic oral environment is too complex to be well simulated in vitro. Therefore, future clinical studies are warranted. Another point to keep in mind is the time order of treatments that might affect the outcome [4,17]. We used the method suggested earlier [14] to perform remineralization first followed by laser irradiation. However, future studies can assess a different order for application of remineralizing agent and laser irradiation to find the best possible protocol.

CONCLUSIONS

Application of Remin Pro (10% CPP-ACP and 900 ppm fluoride) and MI Paste Plus (hydroxyapatite, 1450 ppm fluoride, and xylitol) can similarly induce enamel remineralization. This improvement in remineralization may be further enhanced significantly by laser irradiation using certain lasers; i.e., CO_2 laser (but not Er:YAG laser) after using MI Paste Plus and Er:YAG laser (but not CO_2 laser) after the application of Remin Pro. These two combinations were equally effective for enamel remineralization. Interestingly, addition of Er:YAG laser to MI Paste Plus did not yield better results compared to the application of MI Paste Plus alone, while CO_2 laser irradiation after the application of Remin Pro decreased the efficacy of Remin Pro.

CONFLICT OF INTEREST STATEMENT

None declared.

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