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Cariology

Effect of CO_2 laser combined with AmF/NaF/$SnCl_2$ solution on the prevention of human and bovine enamel erosion

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Abstract: This *in vitro* study evaluated the potential of CO_2 laser (10.6 μm) combined with a stannous/fluoride-containing solution for preventing erosion in human/bovine enamel. Forty-eight samples of each substrate were randomly allocated to four groups (n = 12): W – distilled water; E – AmF/NaF/$SnCl_2$ solution; L – CO_2 laser; and LE – CO_2 laser+AmF/NaF/$SnCl_2$ solution. After surface treatments, samples were submitted to a 5-day erosive challenge, alternating immersions in 0.5% citric acid (2 minutes, 6x/day) and in artificial saliva. Optical profilometry (μm) and scanning electron microscopy (SEM) were used to determine surface loss and surface morphology, respectively. Data were statistically analyzed by two-way ANOVA and Tukey's tests ($p < 0.05$). For human enamel, tissue loss was lower in group L (12.37 ± 4.46) than in group W (16.45 ± 2.76), and higher than in the groups treated with AmF/NaF/$SnCl_2$ solution (E-5.44 ± 2.37; LE-5.55 ± 2.31). In group L, SEM images reveled a disorganized surface but fewer projections than in group W and LE showed fewer irregularities than W, E, and L. For bovine enamel, tissue loss in group L (13.90 ± 3.50) did not differ from that in group W (14.10 ± 2.98), and was higher than losses in groups E (5.70 ± 2.12) and LE (8.12 ± 2.56), which were statistically similar to each other. Groups W and L had similar aspects of demineralization, whereas groups E and LE showed homogenous surfaces. Surface-treated samples had no changes in their surfaces. CO_2 laser was able to slightly prevent surface loss only on human enamel surface, but did not enhance the AmF/NaF/$SnCl_2$ effect on the prevention of enamel erosion.

Keywords: Tooth Wear; Lasers, Gas; Tin; Dental Enamel.

Introduction

The tooth wear involving chemical wear of the mineral portion of teeth by extrinsic or intrinsic acids or chelating substances on plaque-free dental surfaces is considered dental erosion.[1,2] This wear can lead to an irreversible loss of enamel and it is important to diagnose this condition as early as possible and to initiate preventive measures to avoid and minimize lesion progression.[3]

Recent studies on the prevention of erosive tooth wear have focused on investigating fluoride compounds combined with metal ions.[4,5] Among them, fluoride combined with tin has been shown to be outstanding by demonstrating remarkable effectiveness in controlling the erosive demineralization process. The most widely accepted mechanism by which tin solutions act is by precipitating a relatively acid-resistant amorphous layer[6] of CaF_2, Sn_2OHPO_4, $Sn_3F_3PO_4$, and $Ca(SnF_3)_2$ salts, depending on the composition of the product applied.[4,7] When the enamel is exposed to erosive challenges, stannous and possibly fluoride ions are incorporated into the surface during the erosive demineralization-deposition process.[6]

Although the use of amine-fluoride-tin solution is considered the "gold standard" for the prevention of erosive demineralization,[8] there are other approaches that can be investigated to optimize the performance of existing treatments, in order to increase patient compliance.[9] With CO_2 laser (9.3 µm to 10.6 µm) irradiation absorbed by mineralized dental tissues,[4,9,10] it would be possible to increase the acid resistance of the substrate. However, the existence of several protocols makes it challenging to choose which one should be used; it is critical to carefully select the variables, *e.g.* wavelength, irradiation mode, power, pulse duration, beam diameter, irradiation time, and dose, to obtain the intended result.[9]

CO_2 laser at the wavelength of 10.6 µm is in the region of absorption close to that of the phosphate, carbonate, and hydroxyl groups of apatite.[11] Among commercially available lasers, 10.6 µm is the wavelength most widely used for medical purposes[10,12] and can penetrate 10 times deeper into the enamel,[13] affecting the thickest enamel layer.[14] Several studies have shown that 10.6 µm CO_2 laser with energy density between 10.0 and 11.5 J/cm^2 is effective in producing morphological and chemical changes that reduce erosive tooth wear.[13,15] The most broadly accepted hypothesis of the mechanism of action of CO_2 lasers is based on changes in the chemical structure of dental enamel, such as protein decomposition, carbonate evaporation, and formation of pyrophosphates transforming carbonated hydroxyapatite into a purer hydroxyapatite, which is less acid soluble.[10,11,16-19] These changes are due to temperature increase[11,17,18] and fluence between 1.5 J/cm^2 and 11.5 J/cm^2 was not able to cause thermal injury to the pulp.[20] The combination of CO_2 laser and fluoride demonstrated a synergistic effect,[4,12,21] and is based on the possibility of laser increasing the diffusion of fluoride ions into the enamel, which could decrease the dissolution rate of the substrate.[4,21] However, it is still not clear whether the firmly bound or the loosely bound fluoride plays a major role in the laser-induced increase of fluoride uptake; furthermore, only few studies have used fluoride solution combined with tin.[4,13]

Studies in the literature have stated that bovine enamel could be a possible substitute for human substrate in dental erosion/abrasion models. In this study, bovine enamel was chosen, mainly due to its easy availability, uniform composition and orientation of prisms corresponding to those of human enamel, with a percentage by weight of calcium equivalent to that of human enamel.[22,23] However, it has not yet been established in the literature whether laser irradiation interacts with bovine dental enamel in the same way as it does with human enamel.

Considering the promising results of CO_2 laser for the reduction of enamel erosion, the aim of this *in vitro* study was to evaluate CO_2 laser potential (10.6 µm), combined with AmF/NaF/$SnCl_2$, to prevent erosion in human and bovine enamel.

Methodology

Experimental design

This randomized *in vitro* study was approved by the Research Ethics Committee of the School of Dentistry of the University of São Paulo (Protocol n. 1.235.283) and by the Ethics Committee on Animal Use of the same Institution (Protocol n.025/2015). The experimental units consisted of 96 samples of dental enamel, of which 48 were of human and 48 of bovine origin. Two factors were involved in this study: *substrate type*, at two levels (human and bovine), and *surface treatment of the enamel*, at four levels (n = 12): W – distilled water (negative control); E – AmF/NaF/$SnCl_2$ solution (positive control); L – CO_2 laser; and LE – CO_2 laser + AmF/NaF/$SnCl_2$ solution. The response variable was the loss of mineral tissue (in µm), analyzed quantitatively by means of non-contact

optical profilometry and surface morphology, assessed qualitatively by scanning electron microscopy (SEM).

Sample size, preparation, and selection

A sample size calculation was conducted and for a repeated-measures two-way ANOVA, a minimum effect size of 0.4, and a significance level of 0.05 (α), it was suggested that 12 samples per group of each substrate would be necessary to achieve a power of 80% (β).

In total, 50 bovine incisors and 30 freshly extracted unerupted human third molars were used to obtain enamel samples measuring approximately 5.0 x 5.0 x 2.0 mm and 4.0 x 4.0 x 2.0 mm, respectively, by sectioning the teeth with a high precision cutting machine (Isomet 1000, Buehler Ltd., Lake Buff, Illinois, USA). The samples were sterilized with gamma radiation and included in acrylic resin (SamplKwickResin, Buehler Ltd, Lake Buff, USA) with the aid of quadrangular silicone matrices and their surfaces were flattened and polished with #1200-, #2400-, and #4000-grit Al_2O_3 sandpapers (Buehler Ltd., Lake Bluff, USA). After each polishing procedure, the samples were sonicated for 8 min in distilled water.

The enamel surface curvature (baseline) was determined by evaluating the samples by means of an optical profilometer (3D Proscan 2100, Scantron, Taunton, UK). Samples with surface curvatures exceeding 0.3 µm were excluded.[24] Thereafter, surface microhardness tests were performed on the remaining samples (three indentations, 100 µm distance apart, 0.49 N, 20 s, Microdurometer HMV-2000, Shimadzu, Kyoto, Japan),[23] and the mean surface microhardness values were considered for selection. All samples with a mean microhardness 10% greater than or less than the average of all samples obtained were excluded from the study. Based on the mean microhardness data, 48 samples of each substrate were selected, for bovine (342 ± 34.2 Kg/mm^2)[25] and human enamel (350 ± 35 Kg/mm^2),[25,26] and randomly distributed into the experimental groups. To ensure homogeneous distribution, this randomization was analyzed statistically by one-way ANOVA, and no statistical difference was found between the baseline groups ($p < 0.05$).

Two thirds of the experimental area of each sample were covered with two UPVC tapes (Graphic Tape; Chartpak, Leeds, USA), and this area served as reference for profilometric analysis, leaving an exposed central window of 4.0 x 1.0 mm for human and 5.0 x 1.0 mm for bovine samples.

Surface treatment

The samples of the L and LE groups were irradiated once with a pulsed CO_2 laser (Union Medical Engineering, Model UM-L30, Yangju-si, Gyeonggi-Do, Korea) before the erosive cycling procedures[4,27]. The irradiation, performed for 10 s, followed these parameters: wavelength of 10.6 µm, pulse duration of 10 µs, repetition rate of 50 Hz, output power of 0.8 W, energy density of 11.3 J/cm^2,[12,20] energy per pulse of 0.016 J, beam diameter 0.3 mm. The laser tip was positioned 10 mm from the enamel sample and irradiation was performed manually in a zigzag pattern, from right to left, until the line ended, and the next lower line was irradiated from left to right.[28] The metal matrices were used to protect the edges and the UPVC tape. The power output was measured by a power meter before the first irradiation and after every five irradiated samples.[4]

Before the erosive cycles, the samples of the negative control group (W) were subjected to surface treatment, which consisted of immersion in 5 mL of distilled water (Vent Filter MPK01, Merck KGaA, Darmstadt, Germany) for 2 min, twice a day, for 5 days. In groups E and LE, the samples were immersed in 5 mL of AmF/NaF/$SnCl_2$ solution [pH 4.5, 500 ppm F^- (125 ppm F^- from amine fluoride, 375 ppm F^- from sodium fluoride, 800 ppm Sn^{-2}) (ElmexErosion®, GABA International, Therwi, Switzerland), for 2 min, under a static condition, twice a day.[8,29] In group LE, immersion in the solution was performed after laser irradiation. After treatments, the samples were washed with distilled water for 10 s and carefully dried for 5 s with an oil-free air jet. All treatments were performed at predetermined times: the first immersion was performed at the beginning of the cycle and the second one 30 minutes after the last daily immersion in citric acid.

Erosive cycling

Throughout the 5-day experimental period, the enamel samples were subjected to erosive cyclic

demineralization and mineral deposition, including multiple daily erosive acid challenges, exposure to the test solutions, and storage in artificial saliva (pH 6.74–4.08 mM H_3PO_4, 11.90 mM $NaHCO_3$, 20.10 mM KCl and 1.98 mM $CaCl_2$, chemicals from Merck KGaA).[30] Samples were individually immersed in 5 mL of citric acid (0.05 M citric acid monohydrate, pH 2.3, Merck KGaA, Darmstadt, Germany), 6x/day, 2 min each, at room temperature, under constant agitation using an orbital shaker (frequency of 35 rpm)[31] (Figure 1). All citric acid solutions were renewed at each immersion and the pH was monitored. The time between cycles was 1.5 h, and during the remaining time, samples were stored in artificial saliva, which was changed at the beginning and end of each day.[4,29]

Tissue loss analysis

The profilometric analysis was performed on an optical profilometer (3D Proscan 2100, Scantron, Taunton, UK). After removing the UPVC tapes from the edges of the sample, the sensor was programmed to scan 2.0 x 1.0 mm [200 steps (0.01 mm) on the X axis and 10 steps (0.1 mm) on the Y axis] with scanning time of 28 s. The images were analyzed by a blinded examiner using a specific software program (Proscan Application software version 2.0.17), which calculated the average height of the two reference areas and subtracted this value from the height of the experimental area, in µm (3-pt step height).

Surface morphology analysis

After the profilometric analysis, three samples of each group, subjected to erosive cycles, and three other samples, subjected only to surface treatments, were randomly selected and prepared for observation by SEM (FEI, QUANTA FEG 650, Thermo Fisher Scientific, Waltham, USA), operating at 15 kV at 1.500x magnification[4]. The SEM images were taken of the central region of the samples.

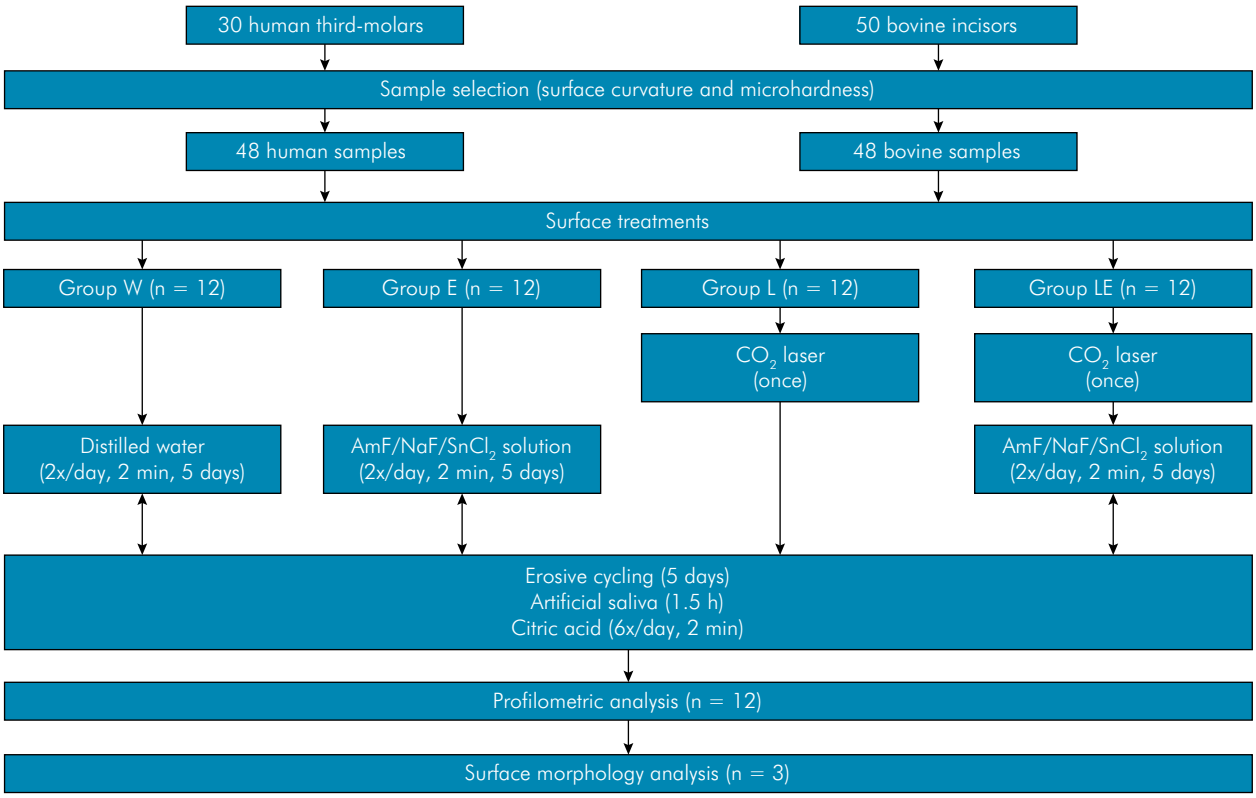


Figure 1. Experimental design flowchart.

Statistical analysis

The data were tested and normal distribution was verified. Differences in mean values of tissue loss between groups were analyzed by two-way ANOVA followed by Tukey's tests at a 5% significance level. The data were analyzed using Sigma Plot 12.0 (Systat Software Inc, San Jose, USA).

Results

Surface loss

Mean surface loss (μm) and standard deviation for the different treatments are shown in Table. Statistical analysis revealed a significant interaction between *substrate* and *treatment* (p = 0.016). For the human enamel, the mean tissue loss in group L (12.37 ± 4.46) was lower than that of the negative control group (W), which presented the highest surface loss. The groups treated with tin solutions (E and LE) had the lowest tissue loss and did not differ statistically from each other. For the bovine enamel, tissue loss in group L (13.90 ± 3.50) did not differ from that of W (14.10 ± 2.98), and surface loss was higher than that observed in groups E and LE, which were statistically similar to each other. When the different substrates were compared, only group LE showed statistically significant difference, with the bovine enamel (8.12 ± 2.56) showing higher tissue loss than the human enamel substrate (5.55 ± 2.31).

SEM findings

The human (Figure 2) and bovine enamel (Figure 3) samples subjected only to surface treatments presented no change in surface morphology after the proposed treatments. The surfaces showed a homogenous and uniform surface layer, with some irregularities due to the preparation of the samples.

After erosive cycling in the human substrate (Figure 4), group W revealed an irregular and rough structure corresponding to that expected from an eroded surface after exposure to aggressive erosive cycling. Group E suggested an image with fewer projections when compared with group W. Group L appeared to have a disorganized surface layer, however, with a less erosive pattern than found in groups W and E. Group LE showed a lower level of erosive wear compared to that of groups E and L.

For the bovine substrate (Figure 5), samples from the negative control group had an irregular, demineralized surface with grooves. Group E revealed a reduction in grooves and presence of a more homogeneous and continuous surface when compared with group W, suggesting a lower degree of surface demineralization. Group L showed a more disorganized surface when compared with group E, and an aspect similar to that of the image obtained in group W, with projections and pits. Group LE produced a more homogenous and uniform surface layer when compared with groups W and L; however, in deeper areas, SEM revealed an irregular pattern of the irradiated samples.

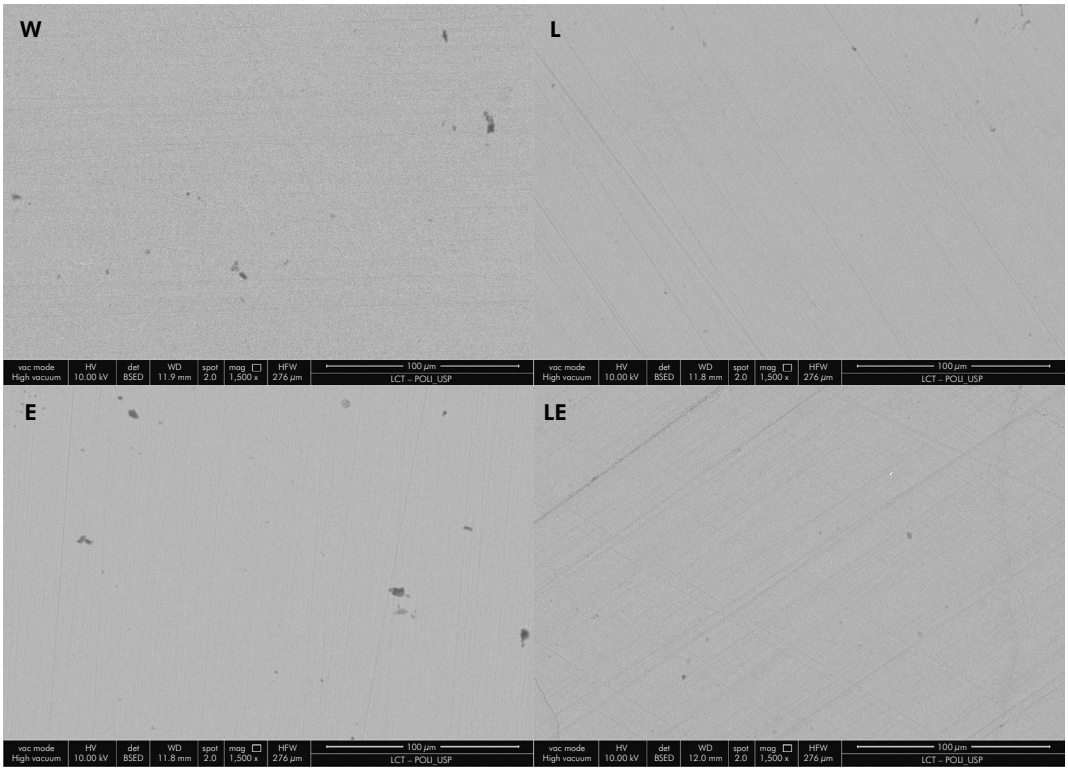
Discussion

Several therapies have been proposed for the treatment of dental erosion, but as erosive tooth wear cannot be prevented totally with the recommended strategies, other approaches that have the potential to increase protection have been studied.[32,33] In the present study, AmF/NaF/$SnCl_2$ solution, either

Table. Mean surface loss (μm) and standard deviation for the different treatments.

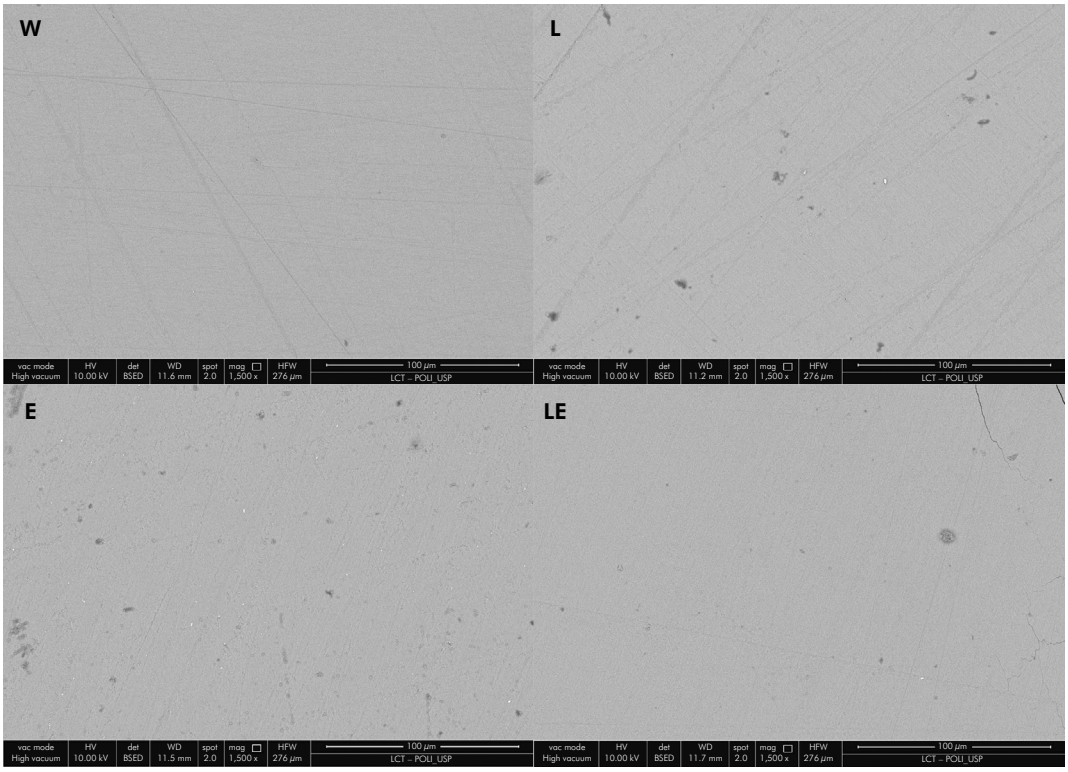
Treatments	Tissue loss (μm)	
	Human enamel	Bovine enamel
Distilled water (W)	16.25 ± 2.76 Ca	14.10 ± 2.98 Ba
AmF/NaF/$SnCl_2$ solution (E)	5.44 ± 2.37 Aa	5.70 ± 2.12 Aa
CO_2 Laser (L)	12.37 ± 4.46 Ba	13.90 ± 3.50 Ba
CO_2 Laser + AmF/NaF/$SnCl_2$ solution (LE)	5.55 ± 2.31 Aa	8.12 ± 2.56 Ab

*Capital letters indicate differences among distinct treatments and lowercase letters between different substrates; **Different letters represent statistically significant difference.



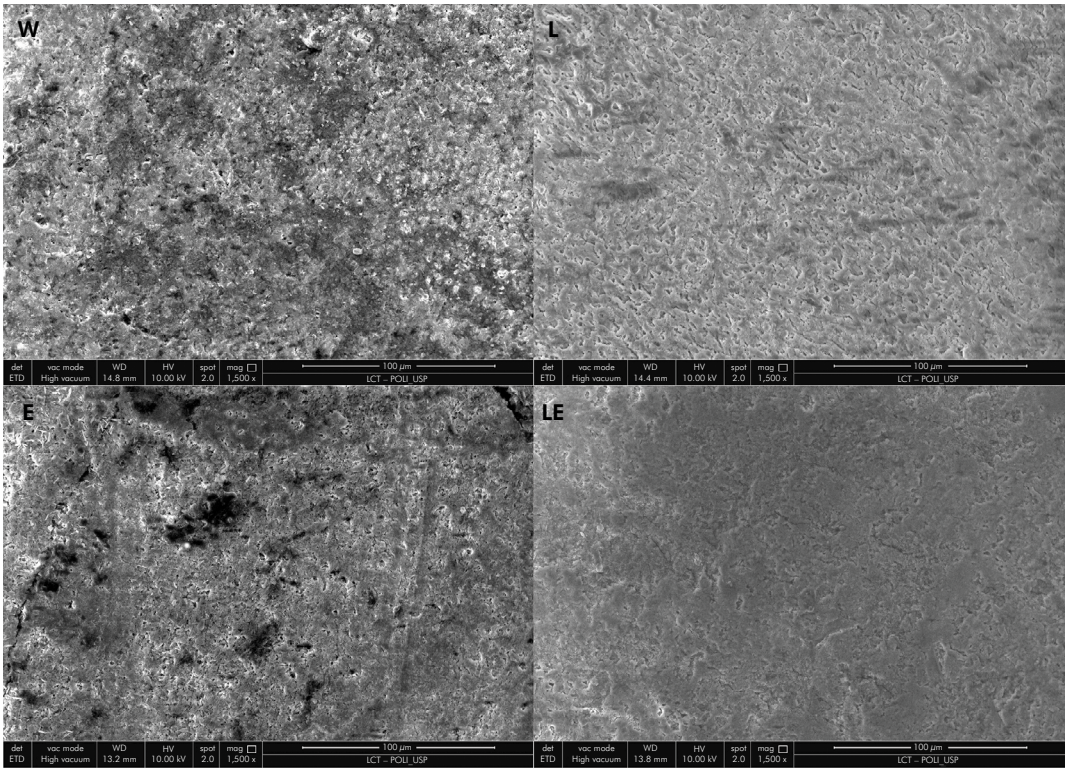
W: distilled water; E: AmF/NaF/$SnCl_2$ solution; L: CO_2 laser; LE: CO_2 laser + AmF/NaF/$SnCl_2$ solution.

Figure 2. Scanning electron microscopy image of human enamel after surface treatment.



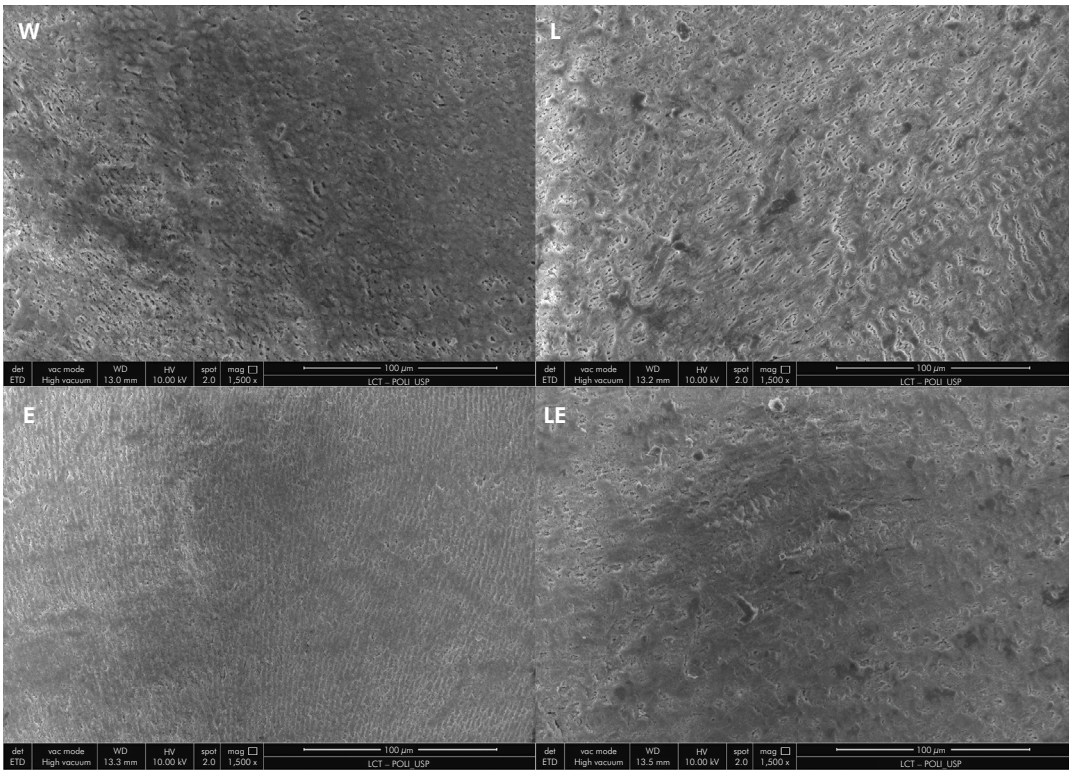
W: distilled water; E: AmF/NaF/$SnCl_2$ solution; L: CO_2 laser; LE: CO_2 laser + AmF/NaF/$SnCl_2$ solution.

Figure 3. Scanning electron microscopy image of bovine enamel after surface treatment.



W: distilled water; E: AmF/NaF/$SnCl_2$ solution; L: CO_2 laser; LE: CO_2 laser + AmF/NaF/$SnCl_2$ solution.

Figure 4. Scanning electron microscopy image of human enamel after surface treatment and erosive cycling.



W: distilled water; E: AmF/NaF/$SnCl_2$ solution; L: CO_2 laser; LE: CO_2 laser + AmF/NaF/$SnCl_2$ solution.

Figure 5. Scanning electron microscopy image of bovine enamel after surface treatment and erosive cycling.

combined with high-power laser or not, was able to decrease mineral surface loss more effectively when compared with CO_2 laser alone. These results, found for both substrates, corroborated the findings of other studies in the literature that obtained similar results using this solution.[8,29] Sn/F-containing solution showed the potential to reduce the loss of enamel mineral tissue by up to 65% to 78%.[34] The human enamel subjected to AmF/NaF/$SnCl_2$ solution and laser combined with the solution demonstrated a reduction in surface loss by 66% and 65%, respectively, when compared with the negative control group; the bovine enamel exposed to AmF/NaF/$SnCl_2$ solution and combined with laser had a limited reduction by 59% and 42%, respectively, compared with the negative control group.

In human enamel, CO_2 laser application, following the parameters considered for irradiation, demonstrated a limited ability to control mineral loss when compared with the AmF/NaF/$SnCl_2$ solution. However, the reduction in mineral loss was not effective as demonstrated by Steiner-Oliveira et al.,[20] in caries research, which may indicate that the parameters used were not appropriate to cause the structural changes necessary to control dental erosion. These parameters could have been more effective if used with CO_2 laser operating at a wavelength of 9.3 µm and 9.6 µm.[4,18]

Unlike human enamel, CO_2 laser was not able to reduce the loss of mineral tissue in the bovine substrate when compared with the negative control. The difference between the results of the laser groups for bovine and human enamel may be related to a distinct response of these substrates.[35] Many *in vitro* studies have used bovine enamel as if it were human enamel, even with a more porous surface, larger hydroxyapatite crystals,[36] and an ample interprismatic region.[37] Ortiz-Ruiz et al.[38] showed a strong correlation between organic material concentration, crystal size, and thermal capacity. It can be hypothesized that the temperature increase caused by CO_2 laser acted differently on each substrate, and because bovine enamel is less mineralized[39] and has a smaller crystal size when compared to human enamel, it made the substrate more susceptible to mineral loss in the face of erosive challenges. In addition, laser irradiation may have widened the interprismatic spaces in the bovine substrate, exposing the prisms and favoring mineral loss, unlike the laser used in the human substrate group.[35]

Comparing the results of this study with those of Steiner-Oliveira et al.,[40] who investigated the combination of CO_2 laser (10.6 µm, 3 W, 10 Hz, 1 J / cm^2) with fluoride gel in bovine enamel and found that fluoride gel and the combination of treatments were not effective in inhibiting mineral surface demineralization, even in milder erosive cycling. The use of the AmF/NaF/$SnCl_2$ solution, twice daily, during the aggressive 5-day cycling, inhibited surface demineralization on both substrates. Daily use of the solution may have promoted deposition and storage of the loosely bound fluoride released during subsequent acid challenges.[41] In both energy densities, the laser was not able to increase the absorption of strongly bound fluoride by dental substrates, but it was applied only once, before the erosive cycling.

The present *in vitro* study corroborated the findings of Rocha et al.,[35] given that the groups presented similar mineral loss when compared with the same treatment for human and bovine enamel. However, only the groups in which AmF/NaF/$SnCl_2$ was combined with laser showed different results for the substrates, showing less surface loss for the human substrate. One theory that may explain this difference is that even if a similar mineral loss were expected between groups, the low energy density probably would not have been sufficient to promote the necessary thermal and morphological changes on the enamel surface to increase its acid resistance, and the protocol would therefore be unsuitable for the prevention of tooth erosion.

Regarding the mechanism of action of amine-fluoride-tin, it produces salts on the dental surface when it reacts with hydroxyapatite, thus forming a protective layer that is less susceptible to acid demineralization, and the deposition depth of 10-20 µm produces an acid-resistant subsurface.[6] As samples were subjected to twice-daily immersions in AmF/NaF/$SnCl_2$ solution, the deposition of salts on the surface and the incorporation of ions into the subsurface[8] were probably optimized resulting in higher degree of resistance to mineral loss and better

protection of the substrate, even against frequent and aggressive acid challenges.[6]

In the present study, CO_2 laser was incapable of preventing dental erosion. It is demonstrated that irradiation with high-power laser is able to produce changes in the morphology of the dental substrate, due to the growth of crystals, forming new and more acid-resistant compounds and causing changes in the crystal lattice.[10,41] CO_2 laser did not promote surface changes in either substrate, as shown by SEM images, being at odds with what Steiner-Oliveira et al.[20] reported. As erosive cycling can be considered aggressive because it simulates the diet of an individual with a high risk for dental erosion,[42] it could be hypothesized that the surface treated by CO_2 laser was removed during this relatively severe cycling.[43] Zuerlein et al.[19] demonstrated that CO_2 laser (10.6 µm) irradiation generates absorption depth of 12 µm in the layer of modified enamel,[41] and the pulse duration interferes with the depth of penetration.[19] Besides, pulse duration is associated with thermal relaxation time of enamel (90 µs).[19,41] Consequently, the short pulse used may have promoted treatment only in the outer layers of the enamel.

CO_2 laser was applied once, prior to erosive cycling. There is no consensus in the literature about the exact moment of laser irradiation,[10] but in this study, the choice was to perform it before application of the fluoride solutions[4,34] to simulate a standard clinical procedure of a single professional application, which could be combined with AmF/NaF/$SnCl_2$ rinse solution, twice a day, to simulate patient home care. Esteves-Oliveira et al.[13] showed differences in mineral loss when CO_2 laser (10.6 µm, pulse 5 µs, 226 Hz, 0.3 J/cm^2) was applied once (at the beginning of the erosive cycle) or twice (at the beginning and on the 6th day of the erosive cycle), but they did not indicate differences when CO_2 laser was combined with amine-fluoride-tin solution. Similar to the procedure performed in the present study, the erosive cycle lasted 5 days and one irradiation session was considered sufficient.[4,10]

The group in which CO_2 laser was combined with the tin solution did not show better results when compared with the fluoride solution alone, indicating that there was no synergistic effect between them.[12,26,34] Due to the increase in temperature caused by the laser before the samples were immersed in AmF/NaF/$SnCl_2$ solution[26], there would be morphological and structural changes, creating fluorapatite and better deposition of ions on the surface and on the subsurface of the enamel,[6] which would make surfaces even more resistant to erosive demineralization[16]. However, results were similar to those of the AmF/NaF/$SnCl_2$ solution, showing no antagonistic effect or impairment of the mode of action of the solution.[26]

Conclusion

Within the limitations of this *in vitro* study, it was possible to conclude that CO_2 laser (10.6 µm) did not increase the effect of AmF/NaF/$SnCl_2$ solution, demonstrating the absence of a synergistic relationship between treatments. Laser irradiation was able to slightly prevent surface loss only in human enamel. The AmF/NaF/$SnCl_2$ solution was the best strategy for preventing dental erosion when performed twice daily.

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