



The Impact of CO₂ Laser Treatment and Acidulated Phosphate Fluoride on Enamel Demineralization and Biofilm Formation

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Abstract

Introduction: This study evaluated the impact of CO₂ laser treatment and acidulated phosphate fluoride (APF) on enamel demineralization and biofilm formation, using *in vitro* and *in situ* designs.

Methods: Demineralized enamel slabs were distributed among 8 groups: placebo, placebo + continuous CO₂ laser, placebo + repeated CO₂ laser, placebo + ultrapulsed CO₂ laser, 1.23% APF, APF + continuous CO₂ laser, APF + repeated CO₂ laser and APF + ultrapulsed CO₂ laser. In the *in vitro* study, 15 enamel slabs from each group were subjected to a pH-cycling regimen for 14 days. In the cross over *in situ* design, 11 volunteers wore palatal appliances with demineralized enamel slabs for 2 periods of 14 days each. Drops of sucrose solution were dripped onto enamel slabs 8×/day. Biofilms formed on slabs were collected and the colony-forming units (CFU) of *Streptococcus mutans* and *Lactobacillus* were determined.

Results: For both *in vitro* and *in situ* studies, there was no significant difference between treatments ($P > 0.05$). However, all treatments increased microhardness of demineralized enamel ($P < 0.05$). After a further *in situ* cariogenic challenge, with the exception of the placebo, all treatments maintained microhardness values ($P < 0.05$). Microbiological analysis showed no difference in *Streptococcus mutans* ($P > 0.05$) or *Lactobacillus* ($P > 0.05$) counts between groups.

Conclusion: The results suggest that APF gel combined with the CO₂ laser, regardless of the pulse emission mode used, was effective in controlling enamel demineralization, but none of the tested treatments was able to prevent bacterial colonization.

Keywords: Dental caries; Laser; Fluoride; Enamel; Microbiology.

Introduction

Dental caries is a complex and multifactorial disease and is still the most prevalent chronic oral illness worldwide.¹ The preventive and therapeutic potential of the CO₂ laser for the inhibition of caries on enamel has been discussed.²⁻⁹ However, the parameters for its most effective use are still under debate.

The thermal effect of the CO₂ laser promotes chemical and structural alterations in enamel such as reduction of carbonates, fusion areas and re-crystallization of hydroxyapatite crystals, making enamel more resistant to acid attacks.¹⁰ This process decreases the permeability and hampers the acid diffusion, thereby reducing enamel demineralization.¹¹⁻¹³ Some CO₂ parameters, such as

output power^{14,15} and a wavelength of 10.6 μm^{3,12,16-18} have been widely studied to prevent demineralization or increase enamel microhardness. However, different pulse emission modes of the CO₂ laser have not yet been investigated.

In a continuous mode, the laser energy is emitted continuously, and in a pulsed mode, the energy is emitted in short pulses of high intensity at a certain number of pulses per second. The laser beam remains inactive between pulses.¹⁹ The CO₂ laser emitted in an ultra-short pulsed mode can reduce the thermal influence on the target-tissue due to a lower heat delivery when compared to the long pulsed laser.²⁰

Although the literature is still sparse about the effect

of the CO₂ laser on a dental biofilm, the equipment has been tested as an alternative to reduce oral bacterial infections.^{2,4,13,21,22} Studies have shown its effectiveness against microorganisms that cause inflammation and dental caries.^{4,13,21,22} The main mechanism of the bactericidal effect of the laser is the instantaneous vaporization of the intracellular water.²³

Acidulated phosphate fluoride (APF) gel is one of the most common fluoridated agents used to control enamel demineralization. The gel acts by the deposition of calcium fluoride (CaF₂) predominantly on the enamel surface, where it is transformed into fluorapatite crystals.²⁴ The combination of the CO₂ laser irradiation with the APF gel application can increase acid resistance of dental tissues compared to the individual treatments⁶ by promoting a greater fluoride uptake into the enamel and consequently, decreasing the development of cracks.²⁵ Also, the use of the CO₂ laser and the APF gel synergistically reduces enamel solubility by producing spherical precipitates that morphologically resemble calcium fluoride, serving as a fluoride reservoir.²⁶ Moreover, the CO₂ laser increases the incorporation of fluoride into the crystalline structure of hydroxyapatite, forming fluorapatite.²⁷

The aim of this study was to evaluate the impact of CO₂ laser treatment and APF on enamel demineralization and biofilm formation, using *in vitro* and *in situ* designs.

Materials and Methods

Experimental Design

The factor under study was enamel surface treatment at 8 levels: placebo + continuous CO₂ laser, placebo + repeated CO₂ laser, placebo + ultrapulsed CO₂ laser, 1.23% APF, APF + continuous CO₂ laser, APF + repeated CO₂ laser

and APF + ultrapulsed CO₂ laser.

The response variables were: a quantitative evaluation of subsurface microhardness (KHN) at 4 moments: initial (baseline), after demineralization, after treatment (gel, laser or both) and after a further cariogenic challenge. In addition, a quantitative evaluation of total viable *Streptococcus mutans* and *Lactobacillus* colonies was performed in the *in situ* study. The flowchart of the present study is illustrated in Figure 1.

In Vitro Study

The Selection and Preparation of Enamel Slabs

Bovine incisors without cracks, hypoplasia or hypomineralization were selected by inspection under stereomicroscopy (S6 D Stereozoom; Leica Microsystems AG, Swiss). Teeth were sectioned using a slow-speed water-cooled diamond saw (Isomet 1000; Buehler, Lake Buff, IL, USA) and 4 slabs were obtained (3 × 3 × 2 mm) from the labial face of each tooth.

The slabs were fixed with upward-faced subsurfaces and were flattened with 1200-grit silicon carbide paper (Hermes Abrasives Ltd., VA, USA) in a polishing machine (Struers S/A, Copenhagen, Denmark). The baseline surface hardness was determined using a microhardness tester HMV-2000 (Shimadzu Corporation, Kyoto, Japan) with a diamond penetrator for Knoop hardness (KHN) and a load cell of 25 g for five seconds. Three measures were taken on the side of the slabs (subsurface), 30 μm distant from the surface and 100 μm spacing between the measures.²⁸ The three readings were averaged and the outcome was used as the value of the slab. Specimens with microhardness values 20% above or 20% below the average of other slabs were discarded.

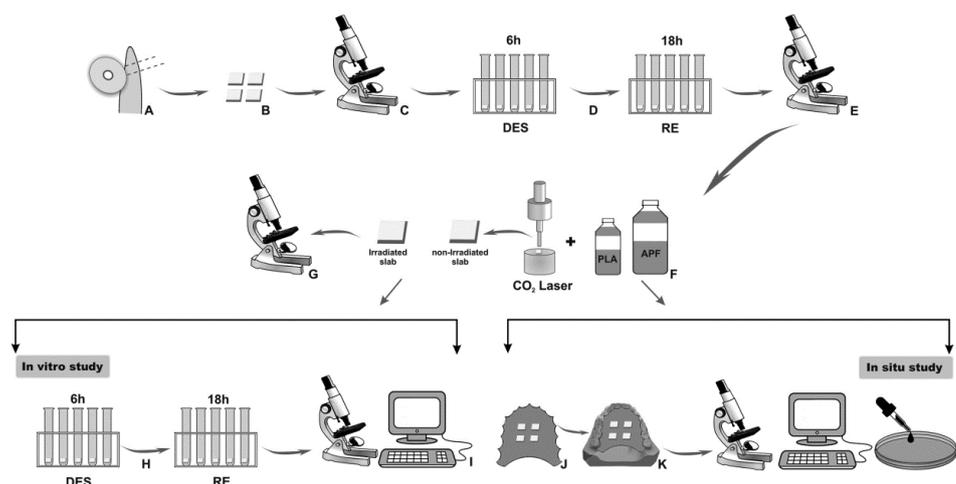


Figure 1. Flowchart of the Study. A- Cross section of the bovine incisor. B- Enamel slabs (3 × 3 × 2 mm). C- Initial microhardness test. D- Initial cariogenic challenge (demineralization and remineralization solutions). E- Microhardness test after demineralization. F- Surface treatment with 1.23% APF or placebo gel + irradiation with the CO₂ laser in the continuous, repeated or ultrapulse modes; or absence of irradiation. *In vitro* study: H- Further cariogenic challenge. I- Microhardness test after further cariogenic challenge and SEM analysis. *In situ* study: J- Acrylic palatal appliances. K- Installation of the devices and intraoral phase. L- Microhardness test after cariogenic challenge, SEM analysis and Microbiological analysis.

A total of 296 enamel slabs were selected; 120 slabs were used in the *in vitro* study and 176 slabs in the *in situ* study. All slabs were sterilized in a microwave oven with 650 W power for three minutes.²⁹

Initial Cariogenic Challenge

The slabs were fixed with melted wax (Kota Ind. Com. Ltda, São Paulo, Brazil) in a teflon matrix. The side surfaces were covered with wax and randomly the enamel surface was exposed to the acidic challenge. Then, slabs were individually immersed in 10 mL of demineralizing (pH 5.0 by 6 hours) and remineralizing (pH 7.0 by 18 hours) solutions at 37° C and cycled during 5 days.³⁰ Initial microscopic white spot lesions were created. After initial demineralization, the microhardness analysis was performed, as described above.

Enamel Surface Treatment

According to a randomized complete block design, for both *in vitro* (n=15) and *in situ* (n=11) studies, the slabs were distributed among 8 groups: placebo + continuous CO₂ laser, placebo + repeated CO₂ laser, placebo + ultrapulsed CO₂ laser, 1.23% APF, APF + continuous CO₂ laser, APF + repeated CO₂ laser and APF + ultrapulsed CO₂ laser.

The placebo gel (Da Terra Farmácia de Manipulação, Ribeirão Preto, SP, Brazil) and 1.23% APF gel (DFL, Jacarepaguá, RJ, Brazil) were applied to the dried enamel surface for one minute using a microbrush applicator (Dentsply Ind. Com. Ltda, Rio de Janeiro, RJ, Brazil). Next, the gel was removed with a piece of absorbent paper. The placebo gel had the same composition of the experimental gel (APF), except by the addition of fluoride.

Subsequently, the irradiation was performed with the 10.6- μ m CO₂ laser (PC015-A; Shanghai JueHua Laser Tech. Development, Shanghai, Japan) using the subablative parameters: 0.5 W output power, a non-contact and unfocused mode, 4 mm irradiation distance and a beam diameter of 0.4 mm.²⁵ In a continuous mode, the CO₂ laser was applied with 1000 mJ energy/second; the repeated pulse was applied with 20 mJ energy/pulse, 0.02 s pulse duration and 0.02 s between time pulses; and an ultrapulsed mode was used with 0.10 mJ energy/pulse, 100 μ s pulse duration and 0.001 s time between pulses. A custom device was used to stabilize the laser pen. The specimens were placed in an automatic scanning device (MPC, ElQuip, Sao Carlos, SP, Brazil) controlled by a microcomputer which moves in all directions (x-y axis) according to previously established commands, allowing the irradiation of the entire area.

After surface treatments, microhardness analysis was performed and then the slabs were subjected to a further cariogenic challenge.

Further Cariogenic Challenge

The slabs were coated again with melted wax (Kota Ind.

Com. Ltda, São Paulo, Brazil) except for their outer surface which was exposed to the acidic challenge. The pH-cycling regimen was performed as described above, but in this time for 14 days, simulating a high cariogenic challenge.³¹ Next, microhardness analysis was performed as previously described.

In Situ Study

Ethical Aspects

This study was approved by the Local Research and Ethics Committee (#11.1.903.58.5). Eleven volunteers aged between 18 and 28 were selected to participate in the study. The inclusion criteria were: a normal salivary flow rate, good general and oral health with no active caries lesions or periodontal treatment needs, an ability to comply with the experimental protocol, no antibiotic use during the 2 months prior to the study, and not using a fixed or removable orthodontic device.

Experimental Period and Intraoral Procedures

Before the bovine slabs being inserted in the oral cavity, they were sterilized and subjected to an initial cariogenic challenge, distributed among 8 groups, and treated as described in the *in vitro* study.

The volunteers were asked to use a dentifrice (Colgate-Palmolive, Osasco, SP, Brazil) and a toothbrush (Oral-B Indicator Plus; Gillette do Brazil Ltda., Manaus, AM, Brazil) supplied by the researchers for 7 days before the intraoral phase began.

Acrylic palatal appliances containing four sites (2 on each side of the appliances) were made and previously demineralized slabs were inserted into each of them. Plastic meshes were fixed over the cavities to protect the enamel surfaces from mechanical attrition, leaving a 1 mm space for biofilm accumulation.³²

The *in situ* study followed a two-period crossover design. Each phase lasted for 14 days with a 7-day washout period between them. The treatment sequence used by each volunteer was determined by a draw.

In the experimental phase 1, part of the volunteers used acrylic palatal appliances containing the slabs treated with the placebo, the placebo + CO₂ laser in a continuous mode, the placebo + CO₂ laser in a repeated mode, the placebo + CO₂ laser in an ultrapulsed mode, while the other part used the slabs treated with the APF, the APF + CO₂ laser in a continuous mode, the APF + CO₂ laser in a repeated mode and the APF + CO₂ laser in an ultrapulsed mode. The volunteers were blind to the group they were using. After 14 days, the acrylic palatal appliances were removed and volunteers were submitted to a washout period of 7 days aiming to eliminate the residual effects of the treatment previously applied. In the experimental phase 2, acrylic palatal appliances were again distributed to volunteers and the treatments were reversed.

No restriction was made concerning the volunteer's diet, but they were instructed to remove the appliances

during meals. Throughout the entire experiment, the volunteers used a dentifrice containing 1100 µg F/g (NaF) and silica as abrasive, and received instructions as previously described.³³

The first cariogenic challenge was performed by the volunteers on the second day of each phase. The volunteers removed the device from the oral cavity and applied one drop of the 20% sucrose solution to each enamel slab to provide a cariogenic challenge during the 14 days, 8 times per day (8:00, 9:00, 10:00, 11:00, 14:00, 15:30, 17:00, 19:00 h). After 5 minutes, the device was re-inserted in the mouth.

On the 14th day of the experiment, approximately 10 hours after the last exposure to treatment solutions, the dental biofilm formed over the enamel slabs was collected, and slabs were subjected to the microhardness test, as previously described.

Microbiological Analysis

The dental biofilm was weighed (Shimadzu AUW 220D; Shimadzu Corporation, Tokyo, Japan), suspended in 1 mL of 0.9 % NaCl solution and sonicated to improve homogenization.³⁴ Aliquots of biofilm suspension were diluted in 0.9% NaCl and serial decimal dilutions were inoculated, in duplicate, in mitis salivarius agar plus 0.2 units of bacitracin/mL and 15% of sucrose (MSB) for the *Streptococcus mutans* group and Rogosa SL agar for the *Lactobacillus* group. The plates were incubated for 48 h at 37° C in an atmosphere of 5% CO₂. The colony-forming units (CFU) were counted and the results were expressed as CFU/mg of biofilm-wet weight.

Statistical Analysis

The statistical analysis of microhardness data, for *in vitro* and *in situ* studies, was performed by analysis of variance (ANOVA) using SPSS 19.0 Windows (SPSS, Chicago, IL), with a significant level of 5%. Data were analyzed by two-way ANOVA with repeated measures, using the same specimen for different comparisons. In the *in situ* study,

the volunteers were considered as statistical blocks. The Bonferroni's test was used for studying the differences between the four microhardness tests performed within each experimental group. The data obtained from the microbiological analysis were evaluated by the Kruskal-Wallis test.

Results

In this study, enamel surfaces were treated with the placebo, APF alone or combined with the CO₂ laser in different pulse emission modes. Microhardness was measured at baseline, after demineralization, after treatment, and after a further cariogenic challenge. For both *in vitro* and *in situ* studies, there was no difference between treatments ($P > 0.05$). However, the enamel condition influenced the microhardness values ($p < 0.05$, Tables 1 and 2).

In the *in vitro* analysis, lower microhardness values were found after demineralization compared to baseline. With the exception of the placebo, all treatments increased the microhardness of demineralized enamel. After a further cariogenic challenge, enamel microhardness was maintained under all protocols, except for the placebo and the APF + ultrapulsed CO₂ laser. Only the combined effect of the APF + continuous CO₂ laser reestablished the baseline microhardness values (Table 1).

In the *in situ* analysis, microhardness decreased after demineralization compared to baseline values. All treatments increased the microhardness of demineralized enamel. After a further cariogenic challenge, all treatments, with the exception of the placebo, maintained the microhardness value. However, the treatments could not reestablish the initial microhardness of the enamel (Table 2).

The microbiological analysis (Table 3) showed no significant difference in the amount of *S. mutans* ($P > 0.05$) or *Lactobacillus* of the enamel slabs treated with the different pulsed CO₂ laser modes, either alone or combined with the APF gel ($P > 0.05$).

Table 1. Means (SD) of Enamel Hardness (Knoop) According to Treatments for In Vitro Study (n = 15)

Groups	Baseline	After Demineralization	After Treatment	After the Cariogenic Challenge
Placebo	280.53Aa (25.07)	186.73Ba (53.16)	213.06Ba (61.71)	189.31Ba (66.76)
APF	295.02Aa (26.13)	192.66Ca (49.35)	228.55Ba (49.35)	198.51BCa (68.39)
Placebo + continuous laser	283.04Aa (34.13)	182.00Ca (70.47)	224.71Ba (50.37)	203.80BCa (61.79)
APF + continuous laser	270.40Aa (27.89)	178.26Ba (60.30)	237.22Aa (89.96)	244.97Aa (47.31)
Placebo + repeated laser	285.62Aa (29.49)	183.91Ca (56.34)	218.17Ba (42.83)	215.24Ba (56.15)
APF + repeated laser	288.24Aa (18.51)	167.64Ca (57.60)	230.22Ba (53.27)	201.75Ba (62.62)
Placebo + ultrapulsed laser	277.62Aa (33.17)	184.95Ca (48.43)	215.11BCa (59.43)	220.86Ba (54.78)
APF + ultrapulsed laser	285.46Aa (29.16)	190.22Ca (71.27)	230.64Ba (72.40)	186.95Ca (57.89)
P value	0.4286	0.9668	0.9666	0.1699

Same capital letters indicate statistical similarity in comparison within treatments (lines) (ANOVA, Bonferroni, $P < 0.05$).

Same lowercase letters indicate statistical similarity in comparison within microhardness measurements (columns) (ANOVA, Bonferroni, $P < 0.05$).

Table 2. Means (SD) of Enamel Hardness (Knoop) According to the Treatments for In Situ Study (n= 11)

Groups	Baseline	After Demineralization	After Treatment	After Cariogenic Challenge
Placebo	282.82Aa (11.08)	174.45Ca (36.38)	217.72Ba (46.17)	191.27Ca (22.37)
APF	276.45Aa (19.55)	180.18Ca (36.96)	208.72Ba (46.53)	195.18BCa (31.23)
Placebo + continuous laser	274.04Aa (15.01)	190.00Ca (40.90)	231.18Ba (43.78)	199.36Ba (28.52)
APF + continuous laser	271.09Aa (20.65)	176.63Ca (28.27)	224.72Ba (28.27)	204.18Ba (22.44)
Placebo + repeated laser	276.62Aa (16.43)	174.45Ca (36.38)	217.72Ba (46.17)	204.45Ba (34.59)
APF + repeated laser	281.00Aa (33.26)	185.64Ca (33.26)	217.36Ba (25.00)	203.54Ba (39.63)
Placebo + ultrapulsed laser	274.36Aa (13.72)	192.63Ca(26.08)	204.72BCa (24.67)	220.36Ba (40.48)
APF + ultrapulsed laser	265.36Aa (20.74)	190.09Ca (42.46)	213.18Ba (31.52)	198.36Ba (39.75)
P value	0.3419	0.8680	0.5045	0.2422

Same capital letters indicate statistical similarity in comparison within treatments (lines) (ANOVA, Bonferroni, $P < 0.05$).

Same lowercase letters indicate statistical similarity in comparison within microhardness measurements (columns) (ANOVA, Bonferroni, $P < 0.05$).

Discussion

Determining the microhardness of dental tissue can provide indirect evidence of mineral gain or loss in the early stages of the carious lesion.³⁵ Therefore, it is one of the most used methods to assess the ability of treatments in inhibiting the progression of caries in enamel. In the present study, microhardness analysis was performed on the same enamel surface to obtain a more reliable result. We also performed both *in vitro* and *in situ* experimental designs to collect complementary data. *In vitro* experiments have the advantage of being faster and allow a proper laboratorial control of variables.^{2,9,15,23,26,36} *In situ* models, in turn, are closer to a clinical situation, reproducing some of the biological and behavioral aspects.^{35,37}

The outcomes of the *in vitro* and *in situ* studies showed no evidence of enamel microhardness increase with the tested treatments. However, all treatments, with the exception of the placebo, were able to conserve microhardness values after a further cariogenic challenge.

Although the efficacy of fluoride compounds in controlling carious lesions has already been reported,³⁸ some studies found that the application of the APF was not sufficient to control enamel demineralization.¹² On the other hand, authors have shown that fluoride application combined with the CO₂ laser had better

results than treatments alone.^{3,25,39-41} Aiming to find an alternative method to control initial white spot lesions, we tested the different surface treatments on previously demineralized enamel samples.

The laser irradiation without fluoride, in different emission modes, failed to induce remineralization but inhibited demineralization in some situations, which is in accordance with a previous investigation.³ Placebo gel application followed by the CO₂ laser in continuous, repeated or ultrapulsed modes increased microhardness values. This can be explained by the fact that during pulsed irradiation, the thermal relaxation time is dependent on the geometry and dimensions of the enamel area exposed to laser irradiation, and then it does not influence on the amount of the propagated heat. Heat propagation is dependent on the ratio between pulse duration and thermal relaxation time values.⁴² Therefore, the temperature increase was probably insufficient to cause chemical and morphological changes in the enamel structure required to increase the acid resistance after a cariogenic challenge.

To increase acid resistance of the enamel using subablative parameters, studies suggest that the energy applied to the target-tissue should increase the temperature to the 100-650°C range.^{43,44} The CO₂ laser is effective in changing the chemical composition and morphology of the irradiated substrate, making it more resistant to acid.^{9,15} This effect is probably related to the temperature increase of the irradiated surface, leading to a decreased permeability, reduced enamel solubility and partial denaturation of the organic matrix.³⁷ The products of the heated organic material can obstruct the pores of the tooth enamel,¹⁸ thereby preventing the penetration of acid ions and decreasing enamel dissolution.^{12,18}

Another relevant aspect is the reduction of the enamel dissolution critical pH to 4.8 after CO₂ laser irradiation. If fluoride is present in the irradiated surface, the critical-pH point is further reduced to 4.3, hampering the formation or progression of caries when both treatments

Table 3. Medians of *Streptococcus mutans* and *Lactobacillus* Counting According to the Treatments for the *In Situ* Study

Groups	<i>Streptococcus mutans</i> (CFU/mg × 10 ³)	<i>Lactobacillus</i> (CFU/mg × 10 ⁴)
Placebo	1.7 A	4.2 A
APF	0.5 A	2.4 A
Placebo + continuous laser	0.3 A	7.6 A
APF + continuous laser	0.16 A	4.6 A
Placebo + repeated laser	0.12 A	5.0 A
APF+ repeated laser	0.2 A	9.9 A
Placebo + ultrapulsed laser	0.34 A	1.0 A
APF + ultrapulsed laser	0.13 A	7.2 A

are combined.⁶

In this study, CO₂ laser irradiation on enamel did not cause a significant decrease in bacterial numbers, which is in agreement with other studies.^{2,23} Further studies using multi-species biofilm designs or *in situ* protocols should be performed to confirm our results. Overall, the results from this study suggest that APF application combined with CO₂ laser irradiation, in different pulse emission modes, was effective in controlling enamel demineralization, but it did not prevent bacterial colonization.

Ethical Considerations

This study was approved by the Local Research and Ethics Committee (#11.1.903.58.5).

Conflict of Interests

The authors declare that they have no conflict of interest.

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