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CO_2 Laser and Fluoride on the Inhibition of Root Caries—an in vitro Microbial Model[1]

C. Steiner-Oliveira[a], L. K. A. Rodrigues[b], T. M. Parisotto[a], C. M. Sousa e Silva[a], A. T. Hara[c], M. Nobre-dos-Santos[a, *]

[a] *Piracicaba Dental School, University of Campinas, Av. Limeira 901, 13414-903, Piracicaba, SP, Brazil*
[b] *Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceará, Rua Cap. Francisco Pedro S/N, 60430-170, Fortaleza, CE, Brazil*
[c] *Department of Preventive and Community Dentistry, Oral Health Research Institute, Indiana University School of Dentistry, Indianapolis, IN, USA*
e-mail: nobre@fop.unicamp.br

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Abstract—An increase in the dental caries prevalence on root surfaces has been observed mainly in elderly. This research assessed, in vitro, the effectiveness of a pulsed CO_2 ($\lambda = 10.6$ μm) laser associated or not with fluoride, in reducing human root dentine demineralization in conditions that mimic an oral high cariogenic challenge. After sterilization, root dentine specimens were randomly assigned into 6 groups ($n = 30$), in triplicate. The groups were Control (C), *Streptococcus mutans* (SM), Fluoride (F), Laser (L), Fluoride + laser (FL), and Laser + fluoride (LF). Except for the control group, all the specimens were inoculated with SM and immersed 3 times a day in a 40% sucrose bath. After a 7-day cariogenic challenge, the mineral loss and lesion depth were evaluated by transverse microradiography and fluoride in the biofilm was determined using an ion-selective electrode. Results were statistically analyzed by analysis of variance, at 5% of significance level. For groups C, SM, F, L, FL and LF, the means (standard-deviation) of mineral loss were 816.3 (552.5)^{a}, 3291.5 (1476.2)^{c}, 2508.5 (1240.5)^{bc}, 2916.2 (1323.7)^{c}, 1839.7 (815.2)^{b} and 1955.0 (1001.4)^{b}, respectively; while lesion depths were 39.6 (22.8)^{a}, 103.1 (38.9)^{c}, 90.3 (44.6)^{bc}, 91.7 (27.0)^{bc}, 73.3 (26.6)^{b}, 75.1 (35.2)^{b}, respectively (different superscript letters indicate significant differences among groups). In conclusion, irradiation of root dentine with a pulsed CO_2 laser at fluency of 12.0 J/cm^2 was able to inhibit root surface demineralization only when associated with fluoride. No synergy effect on the inhibition of root dentine mineral loss was provided by the combination of fluoride application and laser irradiation.

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INTRODUCTION

Root caries experience can be expected to increase in future years. The increase of the population's life expectancy associated with the widespread concepts of preventive dentistry both to dentists and patients have contributed to the retention of a great number of teeth in adults and elderly. Epidemiologic studies have revealed that root caries is one of the most frequent types of diseases affecting the adult and elderly population, showing an annual increment of 0.47 [1].

Due to its higher content of water and organic matrix, root surfaces are more susceptible to caries development than enamel in face of a cariogenic challenge [2, 3]. Even though biofilm control along with fluoride treatments are preventive measures used not only for coronary but also for root caries [3–5], they are not capable of totally preventing from occurring. Thus, it is necessary to develop new methods for root caries prevention, targeting this new increasing elderly population [1, 6].

Many studies were performed to examine the effects of lasers on hard dental substrates with several different applications [7–12]. CO_2 lasers are among the methods considered for the caries prevention, acting by modifying the morphology and chemical composition of dental surfaces [13–23]. Moreover, it has also been demonstrated that the demineralization inhibitory effect can be increased when the CO_2 laser treatment is combined with fluoride [19, 24, 25]. As regards to the dentine, some authors have reported demineralization inhibitory effect through melting and subsequent recristalization of the tissue due to the high temperatures achieved with the laser irradiation [14, 15]. Another study has revealed that 10.6 μm CO_2 laser treatment alone and fluoride treatment alone resulted in root caries inhibition of about 30% [22]. When laser was combined with fluoride treatment, a synergistic inhibition of about 85% was achieved [22]. Nevertheless, these authors only tested the mineral content semiqualitatively, through polarized light microscopy and used neutral fluoride gel, which may be less effective than the acidic one in incorporating fluoride into the dental surface. On the other hand,

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Fried et al. [26] did not find any beneficial effect from the laser irradiation, even using a TEA CO_2 laser, which is much more effectively absorbed by the dental tissues than the 10.6 μm CO_2 laser.

Another interesting point to consider is that no studies in the literature have attempted to use a microbiological model to test the CO_2 laser effects on dentine or tested the combined therapy with acidic fluoride and if this association would be more effective when the fluoride is applied before or after the laser treatment.

Thus, the aim of this study was to evaluate, in vitro, using a microbial model, the effects of the CO_2 laser associated with fluoride on the demineralization of root dentin surfaces.

MATERIAL AND METHODS

Experimental Design

This study was approved by the Research and Ethics Committee of the Piracicaba Dental School at the State University of Campinas in Piracicaba, SP, Brazil (Protocol no. 52/2008). The variables under study were laser irradiation and fluoride application on human root dentin. One hundred eighty root dentin specimens were randomly assigned into 6 groups (n = 30). The experimental design was performed in triplicate at different time points to minimize the inherent bias related to microbiological procedures. The groups were Control (C), *Streptococcus mutans* (SM), Fluoride (F), Laser (L), Fluoride + laser (FL), and Laser + fluoride (LF). SM and no-laser treatment were considered as positive and negative controls, respectively. The specimens were treated and submitted to an in vitro caries microbial model, for 7 days with *Streptococcus mutans* in artificial saliva medium. The response variables for this study were root dentin mineral loss and lesion depths, measured after the cariogenic challenge by transverse microradiography analysis.

Specimen Preparation

One hundred eighty specimens (2 × 2 × 2 mm) were obtained from fifty human unerupted third molars that had been stored in 0.1% (v/v) thymol solution at 4°C for 30 days. The specimens were cut using a water-cooled diamond saw and a cutting machine (Isomet 1000; Buehler, Lake, Bluff, IL, USA) and were randomly assigned to the 6 different groups according to the treatments. The specimens were coated with an acid-resistant varnish (Colorama, CEIL Coml Exp. Ind. Ltda., São Paulo, SP, Brazil), leaving a 4.0 mm^2 window of exposed root for the microbial cariogenic challenge.

The root specimens were fixed in the lids of glass containers with orthodontic wire, kept immersed in sterile distilled water, and then sterilized in a gamma radiation chamber (Gammacell 220 Excel, GC-220E; MDS Nordion, Ottowa, Canada) [27].

Laser Treatment

Ninety specimens from the groups L, FL, and LF were irradiated with a pulsed CO_2 laser at 10.6 μm wavelength (Union Medical Engeneering Co. Model UM-L30, Yangju-si, Gyeonggi-Do, Korea). The parameters used were 0.8 W, 10 ms pulse duration, 10 ms of time off, 50 Hz repetition rate and a beam diameter of 0.3 mm. For these conditions, a power meter (Model-201, Coherent Radiation, Palo Alto, CA, United States) indicated a 0.42 W peak power, thus determining an incident fluency of approximately 12.0 J/cm^2 per pulse. A 10-mm distance from the tip of the hand piece to the specimen was maintained during irradiation which was carried out through the scanning of each specimen exposed dentine for approximately 30 s by an X–Y positioning platform, in order to provide a uniform coverage of each window.

Fluoride Treatment

Ninety specimens from the groups F, FL, and LF received a single application of acidulated phosphate fluoride gel (Odahcam, Dentsply, Herpo, Petropolis, RJ, Brazil) containing 1.23% F (NaF) at pH 3.5. Fluoride application was performed on the root specimens for 1 min in the F group, before (FL group) or after the laser treatment (LF group). The gel was wiped off the specimens with paper tissue.

Biofilm Growth

After sterilization, all specimens were removed from the distilled water and immersed in sterile artificial saliva medium [28]. All artificial saliva glass containers (50 ml), except those in the control groups, were inoculated with 0.2 ml ($1–2 \times 10^8$ colony-forming units CFU/ml^{-1}) of an overnight culture of *Streptococcus mutans* UA 159. This procedure was performed only once, and the specimens were transferred into fresh medium every 24 h [29]. Groups were incubated for 7 days at 37°C and a partial 10% CO_2 pressure.

In order to mimic the oral conditions, the specimens were bathed with a 40% sucrose solution 3 times a day for 5 min and after that, they were transferred to a fresh artificial saliva media. Once a day, samples of all group-cultures were streaked onto BHI agar plates and incubated at 37°C in order to check purity.

Mineral Content Analysis

Transverse microradiography (TMR) was performed to determine the mineral content and the lesion depth of all dentine specimens. Each sample

Original means and standard-deviations of dentine mineral losses (ΔZ), dentine lesion depths (LD) and percentage of inhibition of mineral loss and lesion depth

Group	ΔZ (% mineral vol/μm)	% reduction	LD, μm	% reduction
Negative control (C)	816.3 ± 552.5^{a}	–	39.6 ± 22.8^{a}	–
Positive control (SM)	3291.5 ± 1476.2^{c}	Ref.	103.1 ± 38.9^{c}	Ref.
Fluoride (F)	2508.5 ± 1240.5^{bc}	24	90.3 ± 44.6^{bc}	12
Laser (L)	2916.2 ± 1323.7^{c}	11	91.7 ± 27.0^{bc}	11
Fluoride + laser (F + L)	1839.7 ± 815.2^{b}	44	73.3 ± 26.6^{b}	29
Laser + fluoride (L + F)	1955.1 ± 1001.4^{b}	40	75.1 ± 35.2^{b}	27

Note: Different letters indicate significant statistical differences among the groups by the Tukey test.

was cut through the center of the lesion with a Silverstone-Taylor hard tissue microtome (series 1000 Deluxe, Sci Fab, Littleton, CO, USA), in order to obtain two thin sections 160 ± 20 μm thick. All thin sections were mounted on microradiographic X-ray plates (Kodak high-resolution plates) along with an aluminum step wedge. Then they were X-rayed using a nickel-filtered Cu (K) X-ray source (Philips) operated at 20 kV and 30 mA for 65 min. The X-ray plates were processed and the radiographic images taken from the microscope (EOM, Carl Zeiss Inc., Germany) to the computer with a camera (KP-120U, Hitachi Denshi Ltd., Japan). The images were analyzed with specific computer software (TMR, transverse microradiography version 1.26; Inspektor Research Systems BV, The Netherlands). Integrated mineral loss was determined by computing the area obtained by plotting the volume percent mineral profile towards dentine depth in each dentine section, with the sound dentine set as 48 vol % mineral [30] and this enabled the analysis of both mineral loss and lesion depth.

Fluoride Analysis

The fluoride released from the biofilm formed over the root dentine was determined using an Orion 96-09 ion-selective electrode (Orion Research Inc., Boston, MA, USA) and an Orion EA-940 digital ion analyzer that were previously calibrated with various standard solutions (0.025 to 2.00 μg F/ml). The standard solutions were prepared in TISAB II at pH 5.0 (20 g NaOH/1) and 1 M HCl. The readings were expressed in millivolts (mV) and then transformed to μg F/ml through linear regression of the calibration curve. The results were expressed as μg F/mg of dry weight biofilm.

Statistical Analysis

Statistical analysis was performed using SAS software (SAS Institute Inc., version 8.01, Cary, NC, USA), with the significance level set at 5%. The data were evaluated to check the equality of variances and normal distribution of errors. After data transformation (root-squared for ΔZ, LD and fluoride) analysis of variance (ANOVA) was used to check the significance the differences among the treatments was assessed by the Tukey test.

RESULTS

The groups treated with laser and fluoride showed the lowest mineral loss ($p < 0.05$). The sequence of application of the treatments (LF vs FL) did not matter (table). Treatment with fluoride (F) performed separately showed an intermediate result; while laser alone (L) did prevent mineral loss, not differing from the positive control (SM) ($p > 0.05$) (table).

Similarly, the results for lesion depth have evidenced a statistical significant difference between the negative and positive controls ($p < 0.05$) and the shallowest lesion depths were observed for the groups treated with laser and fluoride (FL or LF) (table). Treatment with fluoride (F) and laser (L) alone have shown intermediate results as they did not statistically differ neither from the positive control nor from the combined treatments with laser and fluoride ($p > 0.05$).

Figure 1 shows the mineral profile and the photomicroradiographs of each group. The control group showed no caries lesion; the SM group has developed the deepest dentine lesion; F and L groups have shown similar lesion depths; FL and LF groups have developed the shallowest dentine lesions.

The concentration of fluoride released in the biofilm from each group can be observed in Fig. 2. Laser + fluoride group released the greatest amounts of fluoride ($p < 0.05$) and no statistically significant difference among the other groups was observed.

DISCUSSION

This study was designed to explore the effects of CO_2 laser and fluoride on the initiation of root caries in controlled in vitro conditions in the presence of *S. mutans* and sucrose. The current study used an in vitro caries model that was proved to be cariogenic to human root dentine, since the means of the dentine

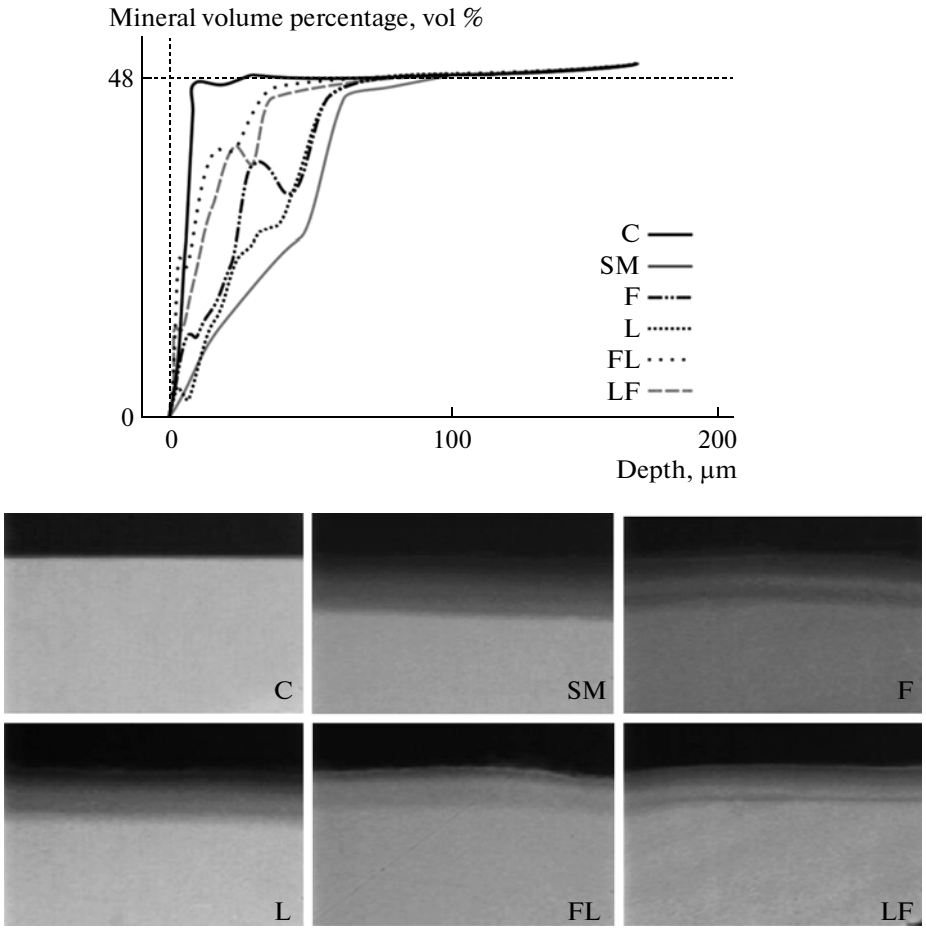

Fig. 1. Mineral profile (vol % X depth in micrometers) accompanied by the respective photomicrographs of the caries lesions formation on the root dentine, according to the groups: negative control (C), positive control *S. mutans* (SM), Fluoride (F), Laser (L), Fluoride + Laser (FL) and Laser + Fluoride (LF).

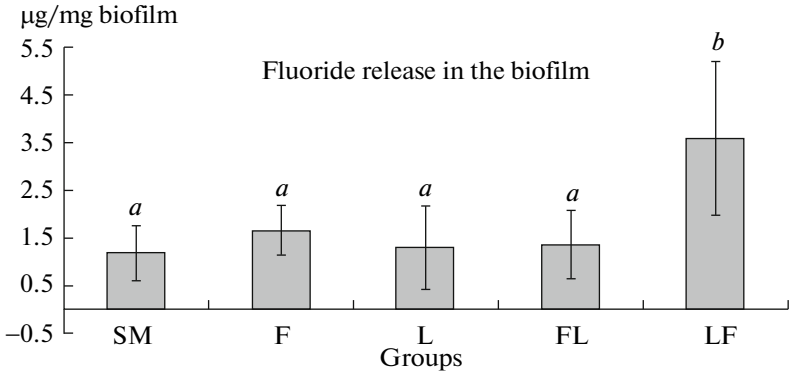

Fig. 2. Concentration of the fluoride released in the biofilm formed over the root dentine.

mineral loss (ΔZ) and lesion depth (LD) for the positive control group significantly differed from the negative control group (table) ($p < 0.05$). Root caries lesions can also be produced by chemical models, although they do not simulate in vivo caries as much as the bacterial model does [31]. In vitro procedures provide a standardized and useful approach, since they allow for greater control of variables [32]. The use the microbiological model was chosen since irradiated dentine presents small cracks that can be preferred sites for bacterial accumulation. This way, due to differences in surface roughness caused by laser irradia-

tion, the presence of these shallow retentive sites may affect bacterial colonization. However, this hypothesis was not confirmed, since lased groups did not show higher mineral loss or lesion depth than SM group, even in the absence of fluoride. In contrast, these values for mineral loss or lesion depth were even lower for the irradiated groups.

The potential of CO_2 laser irradiation to modify the root dentine structure in order to decrease the mineral loss susceptibility has previously been investigated [14–16, 33]. The present study was unable to show the isolated laser and fluoride effects in preventing root dentine caries development, with percentages of demineralization reduction of 11 and 24%, respectively (table). These results agree with those found by Manesh et al. [33] and Featherstone et al. [34] who showed that laser irradiation was not effective in increasing or decreasing the rate of dentine demineralization. It should be emphasized that the referred studies have irradiated the dentine with a CO_2 laser, but with a different wavelength (9.6 μm) and parameters. They have also used chemical models to produce caries lesions. Different results were shown by Gao et al. [22], who found moderate levels of root caries inhibition (about 30%), using a 10.6 μm CO_2 laser and average fluence of 1.14 J/cm^2 *per* shot. Consequently, it can be suggested that the lower fluence used by the latter authors might have produced a dentine surface free of cracks, differently from the present study as well as the study carried out by Manesh et al. [33] who applied 6 and 10 J/cm^2, respectively. It is important to remember that even though CO_2 laser irradiation converts the surface of dentine to a highly mineralized enamel-like composition, the small cracks caused by the contraction due to the removal of the collagen may leave to an increased permeability, thus nullifying the influence of the thin veneer of converted highly mineralized dentine [35]. Besides, since we believe that the microbial model used was not able to mimic the remineralizing phase of the caries process (data not published), a stronger cariogenic challenge was used in the current study. Thus, because of the high cariogenic challenge, laser irradiation might not have been as efficient in our study as it was, when tested by Gao et al. [22].

With regard to anticaries fluoride effect, our results are in contrast to those obtained by Gao et al. [22] and Manesh et al. [33], which demonstrated fluoride preventive effects on dentine demineralization. One possible explanation may be due to experimental differences used in these studies. It could be speculated that the fluoride effect was reduced by the previous cited absence of remineriliz ing phase of the caries model, therefore limiting the remineralization effect of the fluoride. Still, with the use of the microbiological model, the bacteria could have gotten attached more easily to the cracks and fissures created by the laser irradiation, making the L group more susceptible to demineralization and thus explaining the worse results found for this group.

In addition, the absence of statistical difference among the groups F, L+F, and F+L (table) showed that there was no significant synergism between CO_2 laser and fluoride. In the same way, similar results were found by Manesh et al. [33]. Nevertheless, different results were found by Gao et al. [22], which revealed the synergistic effect of fluoride combined with CO_2 laser treatment. However, it should be emphasized that the latter study was performed using a pH-cycling system, which would have highlighted the effect of fluoride and the lesion depth was assessed by polarized light microscopic, which gives limited information about the mineral profile of the samples. The application of fluoride before or after laser irradiation did not provide any additional effect in the inhibition of dentine demineralization. In this respect, even though the dentine from the LF group released higher amounts of fluoride, this ion was trapped in the biofilm and therefore, could have acted reducing the demineralization, since the mineral profile was not different for the groups with fluoride applied before or after the laser irradiation (Fig. 1).

CONCLUSIONS

Irradiation of human root dentine with a pulsed 10.6-μm wavelength CO_2 laser and fluency of 12.0 J/cm^2 was able to inhibit root surface demineralization, but only when associated with high concentration fluoride gel, in an in vitro microbial model. No synergy effect was observed for the combination of fluoride treatment and laser irradiation on the root dentine caries inhibition.

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