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Dentine caries inhibition through CO_2 laser (10.6 μm) irradiation and fluoride application, *in vitro*

M. Esteves-Oliveira [a,*], D.M. Zezell [b], P.A. Ana [b], S.S. Yekta [a], F. Lampert [a], C.P. Eduardo [c]

[a] Department of Operative Dentistry, Periodontology and Preventive Dentistry (ZPP), RWTH Aachen University, Pauwelsstr. 30, 52074 Aachen, Germany

[b] Centro de Lasers e Aplicações, Instituto de Pesquisas Energéticas e Nucleares IPEN/CNEN-SP, Av Prof. Lineu Prestes, 2242 – Cidade Universitária 05508-000, São Paulo, SP, Brazil

[c] Restorative Dentistry Department of the School of Dentistry of the University of São Paulo (FOUSP), Av Prof. Lineu Prestes, 2227 – Cidade Universitária 05508-000, São Paulo, SP, Brazil

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ABSTRACT

Objective: The purpose of the study was to investigate whether dentine irradiation with a pulsed CO_2 laser (10.6 μm) emitting pulses of 10 ms is capable of reducing dentine calcium and phosphorus losses in an artificial caries model.

Design: The 90 dentine slabs obtained from bovine teeth were randomly divided into six groups ($n = 15$): negative control group (GC); positive control group, treated with fluoride 1.23% (GF); and laser groups irradiated with 8 J/cm^2 (L8); irradiated as in L8 + fluoride 1.23% (L8F); irradiated with 11 J/cm^2 (L11); irradiated as in L11 + fluoride 1.23% (L11F). After laser irradiation the samples were submitted to a pH-cycling model for 9 days. The calcium and phosphorous contents in the de- and remineralization solutions were measured by means of inductively coupled plasma optical emission spectrometer – ICP-OES. Additionally intrapulpal temperature measurements were performed. The obtained data were analysed by means of ANOVA and Tukey's test ($\alpha = 0.05$).

Results: In the demineralization solutions the groups L11F and GF presented significantly lower means of calcium and phosphorous losses than the control group; and in L11F means were significantly lower than in the fluoride group. Both irradiation parameters tested caused intrapulpal temperature increase below 2 °C.

Conclusion: It can be concluded that under the conditions of this study, CO_2 laser irradiation (10.6 μm) with 11 J/cm^2 (540 mJ and 10 Hz) of fluoride treated dentine surfaces decreases the loss of calcium and phosphorous in the demineralization process and does not cause excessive temperature increase inside the pulp chamber.

1. Introduction

The effectiveness of topical fluoride application, water fluoridation and the advances in minimally invasive restorative techniques have lead to a great decrease in the number of decayed teeth in the young population and to an increase in the number of retained teeth in the mouths of adults.[1] Additionally, a significant increase in the proportion of elderly population has been observed all around the world, so that at present, a large number of patients present a much higher number of teeth at risk for caries development. In the USA population the persons at higher risk for root caries are adults with low incomes and the elderly.[2] In Europe, it is supposed that the increase in immigration and the decrease in birth rates will increase the root caries prevalence in adults.[3]

* *Corresponding author*. Tel.: +49 241 80 88111; fax: +49 241 80 82468.
E-mail address: mestevesoliveira@ukaachen.de (M. Esteves-Oliveira).
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Considering that adults and the elderly will constitute the major portion of future societies in many industrialized countries, it makes sense to reflect now on new methods for preventing this type of caries lesions, which mainly affects dentine.

CO_2 laser irradiation has been shown to be highly effective in inhibiting caries progression in enamel. The greatest advances have been made by the research group of Featherstone and collaborators in the last 12 years, and levels of caries inhibition as high as 81% have been observed.[4–6] An *in situ* investigation has shown that CO_2 laser treatment inhibits enamel mineral loss in a high-caries-challenge situation and a controlled trial *in vivo* also showed a 46% reduction in mineral loss in comparison with teeth brushed twice daily with fluoridated dentifrice (1100 ppm F).[6,7]

As high percentages of demineralization inhibition have been observed for CO_2 laser-irradiated enamel, it seems reasonable to speculate that such effect may also be achieved using laser irradiation in dentine.[4,6,8–11] However, there are still very few studies investigating the preventive effect of CO_2 laser irradiation in dentine and there is no consensus between the different authors about which parameters would cause inhibition of demineralization. Both increase and decrease in the dentine acid dissolution rate have been observed in different investigations.[12,13] Continuous CO_2 laser ($\lambda = 10.6\ \mu m$) irradiation of dentine with 1 W caused a significant decrease in calcium acid solubility in the study of Hossain et al.[14]and the opposite (increase in acid dissolution) in the study of Featherstone et al.[13] using the same power and the same laser.

Moreover, of the few published studies investigating the caries preventive effect of the 10.6 μm wavelength in dentine, over half of them were performed using the continuous-wave emission mode.[13–16] As it is known that continuous irradiation significantly increases the chances of thermal damage to the hard and soft dental tissues, this irradiation mode has been not recommended for clinical treatment.[17,18] On the other hand, studies testing irradiation with the pulsed-mode presented inhibition of demineralization and increase in fluoride uptake, but failed to report several irradiation parameters.[19,20] Consequently, this makes it difficult to reproduce these investigations and hampers more complex, direct *in situ* or *in vivo* investigations from being conducted.

Considering that pulsed irradiation decreases the risks of irreversible damage to the dental pulp and could be more indicated for a future clinical trial, the purpose of the study was firstly, to investigate whether dentine irradiation with a pulsed CO_2 laser (10.6 μm) emitting pulses of 10 ms is capable of influencing mineral loss in an artificial caries model. Secondly, to verify whether these irradiation conditions promote pulp chamber temperature increase within the safe range.

2. Materials and methods

2.1. Samples

Ninety bovine incisors that had been stored in a 0.1% thymol solution (pH 7.0) directly after extraction were used. The roots were separated from the crowns using a diamond saw under water cooling and slabs measuring 4 mm × 4 mm (2 mm thick) were obtained from their cervical thirds. The outer surface of the samples was serially flattened with 240-, 400- and 600-grit Al_2O_3 abrasive papers and polished with polishing cloths and 6 μm alumina paste. Between every polishing step the samples were submitted to a 30-s sonication bath. The samples were observed under a stereomicroscope (Nikon SMZ 1000, Nikon Corporation, Tokyo, Japan) and those presenting surface structural defects or cracks were discarded. All the slabs were completely covered with acid-resistant varnish except for a rectangular window measuring 2 mm × 4 mm on the external surface.

The samples were randomly divided into six groups ($n = 15$): 2 control groups, one negative, not treated (GC) and one positive, treated with fluoride gel (GF); and 4 laser-treated groups, two of them laser irradiated with 8 J/cm^2 of energy density, one without fluoride application (L8) and one with (L8F), and two groups laser irradiated with 11 J/cm^2 of energy density, one without fluoride application (L11) and one with (L11F).

For groups G8F and G11F, the fluoride treatment was performed before the laser irradiation using an acidulated phosphate fluoride (APF) gel (DFL Ltd., Rio de Janeiro, Brazil) containing 1.23% of fluoride, pH 3.5, applied for 4 min. After application, samples were washed with distilled water and dried with absorbent paper.

2.2. Laser irradiation

A pulsed CO_2 laser emitting at 10.6 μm wavelength (UM-L30, Union Medical Engineering Co., USA) was used. The focal distance was adjusted in order to result in a beam diameter of 2.5 mm at the irradiation position and the other irradiation parameters were determined in a pilot study, ensuring that no visible ablation or carbonization was caused. A complete description is given in Table 1. Before the experiments began and after every 5 irradiations, the energy emitted was controlled with an energy meter (Coherent FieldMaster GS + Detector LM45; Coherent, USA).

To standardize the irradiation conditions, the mirror arm of the laser was fixed onto a laboratory apparatus support and the samples were fixed onto an XY micropositioner mounted on a linear motorized stage (Newport Klinger MT160-250 Linear Stage, New York, USA). The motor was moved at a speed of 7.5 mm/s and two lines of irradiation were enough to irradiate the entire exposed area. For each irradiation line 3 pulses were overlapped per spot.[21]

2.3. pH cycling

After the irradiations all the samples were individually placed in plastic tubes (Falcon Tubes™, BD, Franklin Lakes, USA) and subjected to the following pH-cycling model[22] for 9 days (8 + 1 day remineralization bath) at 37 °C:

1. 4 h in 50 ml demineralization bath (1.4 mM de calcium nitrate, 0.91 mM sodium dihydrogen phosphate, 0.05 M acetate buffer, 0.06 μg F/ml, pH 5.0).
2. The specimens were individually rinsed thoroughly for 10 s in distilled water and smoothly dried with absorbent paper, in order to avoid dilution of the individual baths.

Table 1 – Description of the groups and the laser parameters used for the laser irradiation in each group.

	GC	GF	L8	L8F	L11	L11F
Wavelength	Negative control	Positive control (fluoride)	10.6 μm			
Repetition rate			10 Hz			
On time			0.01 s = 10 ms			
Off time			0.09			
Beam diameter			2.5 mm			
Irradiation distance			10 cm			
Average Power			3.8 W	3.8 W	5.4 W	5.4 W
Energy Density			8 J/cm^2	8 J/cm^2	11 J/cm^2	11 J/cm^2
Energy per Pulse			383 mJ	383 mJ	540 mJ	540 mJ
Fluoride before irradiation			No	Yes	No	Yes

3. 20 h in 25 ml remineralization bath (1.5 mM de calcium nitrate, 0.9 mM sodium dihydrogen phosphate, 150 mM de potassium chloride, 0.1 M Tris buffer, 0.05 μg F/ml, pH 7).
4. After 8 days of cycling the blocks remained in the remineralization solution for 24 h.

The proportions of the de- and remineralization solutions per area of exposed enamel were 6.25 and 3.12 ml/mm^2 respectively. The plastic tubes containing the samples were maintained at 37 °C and under a constant agitation of 200 rpm throughout the entire cycling period. After completion of cycling procedure, before and between the further investigations, the specimens were stored on wet cotton fabric at room temperature and at a constant relative humidity of 100%.

2.4. *Calcium and phosphorous analyses*

After the end of the pH-cycling procedures, the samples were removed from the plastic tubes and the amount of calcium and phosphorous released into the two solutions (de- and remineralization) was measured with an inductively coupled plasma optical emission spectrometer (ICP-OES; Spectro Flame M 120, Spectro Analytical Instruments GmbH and Co. KG, Kleve, Germany). Before the elements were determined, calibration was performed with calcium and phosphorous standard solutions (Merck KGaA, Darmstadt, Germany). From each solution 300 μl samples were taken in triplicate and three measurements of calcium and three of phosphorous were performed to ensure the precision of the measurements. Both calcium and phosphorous contents were measured in μg/ml.

2.5. *Temperature measurements*

In addition to the mineral dissolution investigation, intrapulpal temperature measurements were taken. For this purpose, 20 freshly extracted human third molars were obtained. The pulpal tissue was removed and the root canals were enlarged up to an ISO 80 size with K-files. Measuring sensors (NiCr–Ni, Greisinger, Germany) were inserted into the pulp chamber through the root apex and the entire root canal system was filled with a nano thermo-conductive paste (thermal conductivity >4.5 W/m-K, Titan Technology, Taipei Hsien, Taiwan) to enable good contact between the sensor and the tooth. The end of the sensor was placed so that it touched the dentine wall at the closest distance to the irradiation area, and its location was controlled radiographically.[23] Temperature changes were recorded with a T202 thermometer (Digitron Instrumentation Ltd., Devon, UK) at mean rate of 1 per second and the accuracy of the measurement was ±0.2 °C. For the irradiation procedures the samples were fixed over a thermal bath with a controlled temperature of 37.3 °C. The buccal half of the teeth was left exposed to the air and the palatine portion was immersed in water. The irradiation was performed with the laser handpiece fixed over the samples and with the centre of the beam positioned 1 mm below the enamel–dentine junction. The beam diameter was 2.5 mm and the samples were irradiated for 1 s. The laser parameters used were the same as those described above (Table 1), except that the samples were not moved and therefore 10 pulses were overlapped. Temperature measurements started 1 s before the beginning and ended 120 s after the laser irradiation.

2.6. *Statistical analysis*

The data were submitted to analysis of variance (ANOVA) ($\alpha = 0.05$) and post hoc comparisons with un-paired *t*-test in order to detect statistically significant differences between the groups. The significance level for the *t*-test was corrected using the Bonferroni adjustment to 0.003.

3. Results

The mean calcium and phosphorous concentrations for each group and the differences between the groups are presented in Tables 2 and 3. In the demineralization solution both calcium and phosphorous losses of groups L11F and GF (fluoride) were statistically significant lower than those in the group receiving no treatment ($p < 0.01$ and $p < 0.01$, for both calcium and phosphorous). Moreover, group L11F showed statistically significant lower means than the fluoride group (GF) ($p < 0.01$ for both calcium and phosphorous). The highest percentage of reduction in calcium loss was 15% and was observed for the group irradiated with 11 J/cm^2 after the fluoride treatment (L11F). In the remineralization solutions, there was a statistically significant higher amount of phosphorous in groups L11F and GF than in control ($p < 0.01$ and $p < 0.01$ respectively). However for the calcium means only GF had statistically significant higher means than the control group ($p < 0.01$).

Table 2 – Means and standard deviation (SD) of calcium and phosphorous contents (μg/ml) in the demineralization solution.

	Calcium		Phosphorous	
	Mean	SD	Mean	SD*
GC (control)	63.74	(±2.2)a	103.49	(±2.2)a
GF (fluoride)	60.92	(±1.9)b	97.23	(±2.0)b
L8 (laser 8 J/cm^2)	63.7	(±1.9)a	101.7	(±2.2)a
L8F (laser 8 J/cm^2 + fluoride)	63.69	(±1.2)a	101.15	(±2.3)a
L11 (laser 11 J/cm^2)	64.27	(±2.2)a	101.67	(±4.2)a
L11F (laser 11 J/cm^2 + fluoride)	53.84	(±3.1)c	93.26	(±2.8)c

Different letters indicate statistically significant difference ($\alpha = 0.003$).

Table 3 – Means and standard deviation (SD) of calcium and phosphorous contents (μg/ml) in the remineralization solution.

	Calcium		Phosphorous	
	Mean	SD	Mean	SD
GC (control)	55.39	(±6.1)a	86.22	(±8.4)a,c
GF (fluoride)	77.34	(±17.7)b	108.73	(±15.1)b
L8 (laser 8 J/cm^2)	60.86	(±2.1)a	93.91	(±4.2)a
L8F (laser 8 J/cm^2 + fluoride)	54.59	(±2.3)a	89.40	(±2.1)a,c
L11 (laser 11 J/cm^2)	57.64	(±3.9)a	84.65	(±3.4)c
L11F (laser 11 J/cm^2 + fluoride)	60.56	(±1.5)a	105.8	(±4.24)b

Different letters indicate statistically significant difference ($\alpha = 0.003$).

The temperature measurements showed that both irradiation conditions caused intrapulpal temperature increase below 2 °C. The highest temperature increase and the time after which the temperature returned to its initial values were respectively 0.3 °C and 12 s for the irradiation with 8 J/cm^2 and 1.8 °C and 93 s for the irradiation with 11 J/cm^2 (Fig. 1).

4. Discussion

The results of the present study showed that the irradiation of dentine with a CO_2 laser ($\lambda = 10.6$ μm) at 11 J/cm^2 and 10 ms pulse duration, after fluoride application was indeed able to cause a decrease in the loss of calcium and phosphorous in the demineralization solution. The calcium loss in this group was even statistically significant lower than the observed in the fluoride-treated group. Thus, the possibility of enhancing the effects of fluoride through CO_2 laser irradiation has been demonstrated. Especially interesting to note is that these results were obtained with a clinical CO_2 laser and using parameters which did not cause any visible thermal damage to the tooth surfaces.

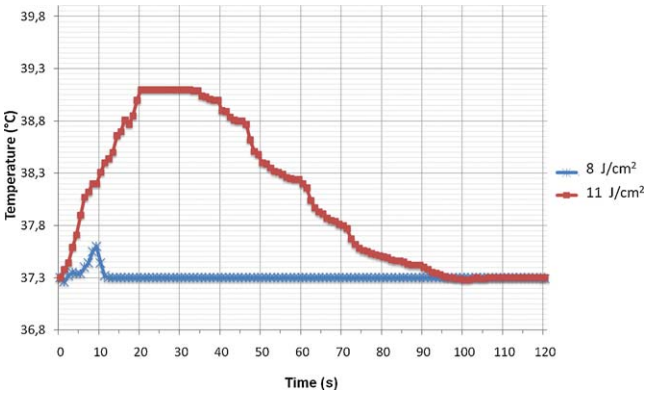

Fig. 1 – Temperature excursions inside the pulp chamber for both irradiation parameters. The maximum temperature increase after irradiation with 8 J/cm^2 occurred after approximately 10 s and was 0.3 °C. For irradiation with 11 J/cm^2 it occurred after approximately 20 s and was 1.8 °C.

Similar findings have been observed by other authors measuring calcium and phosphorous dissolution[14–16] and lesion depth[19] in CO_2 laser-irradiated dentine. Nonetheless decrease in calcium and phosphorous losses after irradiation with the set of parameters used in the present study has not been demonstrated before. Moreover, most of the previous studies were conducted with a CO_2 laser emitting in the continuous-wave mode, which is not the safest condition for irradiating vital teeth.[18]

The lowest energy density tested in this study (8 J/cm^2) did not cause any significant reduction in mineral loss either alone or in combination with fluoride. This was initially not expected, because according to the literature and the characteristics of the laser–tissue interaction for the 10.6 μm wavelength, this energy density could already be sufficient to promote the necessary changes in the tissue. For example, in a study conducted with the same pulse duration (10 ms) as used in the present study, but in enamel, a 67%-inhibition of demineralization was observed with 10 J/cm^2.[24] Thus, knowing that for similar irradiation intensities the temperatures produced in dentine are two times higher than they are in enamel, theoretically only half of the amount of an

energy density, successfully tested in enamel, would be necessary to cause the same effects in dentine.[18] Therefore, we expected to obtain a reduction in calcium loss already with the lowest energy density tested in the present study, but this was not confirmed. These results are probably explained by the fact that the energy applied to the tissue is not the only factor influencing the temperature excursions. The number of pulses applied to the same spot and the repetition rate also play an important role in the gradients of temperature formed. Thus, the observation that in the present study there were only 3 overlapped pulses, and the repetition rate was five times smaller than the one used by Steiner-Oliveira et al., may explain why the temperature increase after 8 J/cm^2 irradiation was not sufficient to make dentine more resistant to acid dissolution.

It is possible to reduce the energy density needed to cause an increase in acid resistance in dentine by decreasing the pulse duration. Shortening the laser pulses from 100 to 5–8 μs caused chemical changes in the dentine structure, which are supposed to render dentine more resistant to acid dissolution using only 0.5 J/cm^2.[18] The same effects using exactly the same energy density and irradiation conditions are probably not obtainable with a 10.6 μm CO_2 laser, because of its lower absorption (813 cm^{-1}) in dentine as compared with the 9.6 μm (6500 cm^{-1}). However a proportional reduction in the energy density with the reduction in the pulse duration may be expected. Therefore the idea of the present study was to find the lowest energy density capable of reducing the acid dissolution of dentine with the shortest pulse duration available for the clinical CO_2 laser used, in this case 10 ms.

The reduction in the pulse duration may also decrease the risk of excessive temperature increase in deeper tissue layers.[25] In the pulp for instance, the increase of more than 5.5 °C in temperature can cause irreversible damage in 15% of the cases and should therefore be avoided.[26] Such a high intrapulpal temperature was not observed in this study. Both conditions tested with 10 ms pulse duration caused a temperature increase below 2 °C in the pulp indicating safety of the treatment. Due to the technical difficulties in conducting intrapulpal temperature measurements with the teeth being moved, the temperature changes had to be measured in a static condition. Consequently the number of overlapped pulses applied to the samples had to be 3 times higher. Such an exaggerated situation certainly resulted in a higher heat generation and propagation into the tissue than a lower pulse overlap would have caused.[27,28] Therefore the observation of a relatively low temperature increase in spite of the irradiations being performed in a more heat-generating manner increases the safety margin of the results of this study.

Although the surface temperature during the irradiations could not be measured with the thermometers used in this study, the observed effects indicate an increase in the range between 100 and 300 °C.[18,29] Firstly, because the tissue was not ablated or melted, which indicates a temperature below 1200 °C.[30] Secondly, the only visible change at the surface was a whitish appearance, probably indicating water loss.[30] Besides, the typical colour changes indicating protein denaturation (350 °C) were not seen.[30,31] And finally the irradiation alone did not cause any significant changes in the dentine resistance to acid dissolution, which indicates that the temperature was not high enough to eliminate carbonate and cause crystal growth.[32–34]

The mechanism through which laser irradiation interacts with dental tissues, in the absence of fluoride, is mostly related to the temperature increase caused after absorption. In general, dentine irradiation with a CO_2 laser causes changes both to the mineral and to the organic matrix. Depending on the energy applied, carbonate can be reduced or eliminated and crystallinity can be increased.[18,30] Also reduction of collagen content, loss of water and formation of amorphous carbon bands have been observed.[35] It is, though, specially the reduction of carbonate and hydroxyapatite phase changes that happen between 600 and 900 °C that have shown to be related to decrease of tooth solubility after laser irradiation.[18,30,36] These tissue modifications are temperature-related and not all laser irradiation conditions are able to cause heating exactly in the range to positively modify the tissue and turn it more caries-resistant. This may be one of the reasons why laser irradiation alone was not able to decrease demineralization in the present study.

The decrease in dentine mineral dissolution observed with the combined use of laser and fluoride is probably related to the increase in the typical effects of fluoride by means of laser. Fluoride interacts with tooth mineral in two different ways. One is through incorporation into the hydroxyapatite crystal forming fluoridated hydroxyapatite, and the other is through the formation of a fluoride-rich layer containing calcium fluoride-like material (CaF_2-like) over the tooth surface.[37] The formation of a CaF_2-like rich layer has been said to be the main factor responsible for caries reduction through topic fluoride application. Nevertheless these globules are only loosely bound to the dental structure and are soluble at low pH. Furthermore, a drastic reduction in these deposits is observed approximately 5 days after application.[38,39] In the case of the combined use of laser and fluoride, it has been demonstrated that the formation of both loosely and firmly bound fluorides is enhanced by laser irradiation. However enhancement of calcium fluoride-like material (loosely bound) deposition through laser treatment seems to be more effective than the formation of fluorhydroxyapatite.[19] Therefore it is reasonable to speculate that the temperature increase caused by laser irradiation may increase the stability of the CaF_2-like deposits formed, and this may be one of the mechanisms through which laser-treated dentine is more resistant to acid dissolution than only fluoride-treated dentine.

The 15% reduction of calcium loss obtained in the present study is rather limited if a clinical application is concerned. This would probably result only in short-term caries prevention or would require constant re-treatment. Therefore, the present results should not be understood as a direct clinical indication but as an orientation to further development of the laser parameters. Nevertheless, since higher percentages of caries inhibition have already been obtained using laser prototypes to treat dental enamel[4,6] and quality of laser technology is advancing quickly; it might be soon possible to cause also for dentine high increase of caries resistance. Once this is achieved, preventive treatment of elderly patients presenting exposed root surface due to gingival retraction might become a reality. Especially the patients at high-risk or those who will start medical treatments causing decrease of

salivary flow (i.e. head and neck radiotherapy) could benefit from such kind of therapy.[40] Nonetheless, it should be kept in mind that the research with lasers is still very new and several improvements have to be made before it can be used in a clinical context.

Although the laser and fluoride treatment was not tested *in vivo* in the present experiment, the pH-cycling method is the model of choice for simulating caries *in vitro* and provides good predictability of clinical efficacy. Both the de- and remineralization periods are reproduced and are known to cause subsurface lesion formation with the characteristics of true white-spot lesions.[41]

Considering the fact that several recent studies have failed to find any increase in dentine acid resistance after CO_2 laser irradiation, the positive results observed for the combination of the laser irradiation with fluoride should be further studied.[12,13,42] Especially the mechanisms leading to increased dentine acid resistance after combined laser and fluoride treatment should be further studied, in order to allow optimization of the treatment conditions. The maximum reduction of 15% calcium loss in the demineralization solution was also significantly higher than in the fluoride treatment alone and shows that there could be a possibility of synergistically combining the two treatments.

5. Conclusion

CO_2 laser irradiation (10.6 μm) with 540 mJ, 10 Hz, 11 J/cm^2 of fluoride-treated dentine surfaces decreases the loss of calcium in the demineralization process, *in vitro*. This surface treatment was more effective in decreasing calcium loss than fluoride treatment only, and caused intrapulpal temperature increase below 2 °C. Laser irradiation alone did not influence dentine dissolution in the artificial caries model tested.

Contributors

M. Esteves-Oliveira is the principal investigator; D.M. Zezell is the physicist (professor) with whom the investigations were planed, elaborated and discussed; P.A. Ana is the PhD researcher who gave us assistance in conducting the measurements and in discussing the results; S.S. Yekta is the PhD researcher who was involved in the writing of the manuscript; F. Lampert is the senior author, full professor with expertise in field of lasers in dentistry and provided the conditions for the temperature measurements; C.P. Eduardo is the senior author, full professor with expertise in the field of laser applications in dentistry and responsible for the planning, discussion of results and elaboration of the manuscript.

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