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ARIANY BORGES CARVALHO

“EFFECTS OF REPEATED CO_2 LASER APPLICATIONS ON PRIMARY ENAMEL DEMINERALIZATION – AN *IN VITRO* STUDY”

“EFEITO DE APLICAÇÕES REPETIDAS DO LASER DE CO_2 SOBRE A DESMINERALIZAÇÃO NO ESMALTE DE DENTES DECÍDUOS – ESTUDO IN VITRO”

PIRACICABA
2013

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Orientador: Profa. Dra. Marinês Nobre dos Santos Uchôa

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Dissertação de Mestrado apresentada ao programa de Pós-Graduação em Odontologia da Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do título de Mestra em Odontologia, na área de Odontopediatria.

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defendida pela aluna ARIANY BORGES CARVALHO
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Juliano Pelim Pessan

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"O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis."

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RESUMO

Estudos prévios mostraram que uma irradiação do esmalte dentário com uma aplicação do laser de dióxido de carbono (CO_2) modifica a superfície desse substrato e traz benefícios no que concerne ao aumento na resistência aos ácidos, diante à desafios cariogênicos. No entanto, ainda não se sabe se a irradiação repetida do esmalte decíduo com o laser de CO_2 promoveria efeitos adicionais na resistência à desmineralização do esmalte. Assim sendo, este estudo teve como objetivo verificar, *in vitro*, se o acúmulo de irradiações do esmalte decíduo com laser de CO_2, com comprimento de onda 10,6 µm e densidade de energia de 20,0 J/cm^2 seria capaz de aumentar a inibição da perda mineral do esmalte decíduo, quando submetido à situações de alto desafio cariogênico. Para isto, 80 espécimes de esmalte hígido de molares decíduos foram selecionados após análise pontual prévia de Ca pela Espectrometria de Fluorescência de Raio-X (µ-EDXRF) e aleatoriamente divididos em 4 grupos: controle (C), 1 aplicação do laser de CO_2 (L1), 2 aplicações do laser de CO_2 (L2) e 3 aplicações do laser de CO_2 (L3). Os espécimes foram submetidos à análise da área de Ca por meio do µ-EDXRF antes da aplicação de laser e após a ciclagem de pH. Depois, foram divididos para serem analisados por Microdureza e Microscopia Eletrônica de Varredura (MEV). Os resultados foram analisados por ANOVA seguida de teste t, teste de Kruskal-Wallis, test t pareado e regressão linear simples ($p<0,05$). Em relação à microdureza, todas as condições de irradiação foram significativamente efetivas em reduzir a perda mineral quando comparadas ao grupo controle ($p<0,01$), não havendo diferenças entre os grupos L1 e L2. Entre os grupos irradiados, o grupo L3 apresentou os maiores resultados de redução de perda mineral ($p<0,01$). Os resultados da análise final do µ-EDXRF mostraram que somente o grupo L1 apresentou uma porcentagem significativamente menor de Ca quando comparado com outros grupos e o test t pareado mostrou que houve um aumento de Ca após a ciclagem de pH no grupo L2 ($p=0,046$). Os resultados da MEV mostraram fusão e derretimento na superfície de esmalte irradiada. Assim, três aplicações repetidas sobre o esmalte de dentes decíduos com o laser de CO_2, nas condições empregadas no estudo, aumentou significativamente a inibição de desmineralização do esmalte.

Palavras-chave: Desmineralização, laser de CO_2, dentes decíduos, ciclagem de pH, cárie

Abstract

Previous studies have shown that one single irradiation of primary dental enamel with carbon dioxide (CO_2) laser promotes ultrastructural crystallographic effects on enamel surface and turns it more acid-resistance under a cariogenic challenge. However, the effects of repeated applications of CO_2 laser irradiation on the inhibition of demineralization in primary enamel have not been investigated. Thus, the aim of this study was to investigate, *in vitro*, if the repeated application of CO_2 laser irradiation on the primary enamel with a wavelength of 10.6 µm and density of 20.0 J/cm^2 could enhance the inhibition of enamel demineralization, under a high cariogenic challenge. For this study, 80 specimens of sound primary enamel were selected after timely analysis by Energy Dispersive X-ray Fluorescence Spectroscopy (µ-EDXRF) and randomly divided into 4 groups: control (C), 1 application of CO_2 laser (L1), 2 applications of CO_2 laser (L2) and 3 applications of CO_2 laser (L3). The specimens were evaluated by µ-EDXRF before laser applications and after pH cycling. Right after the specimens were submitted to Cross-Sectional Enamel Micro-hardness (CSEM) and Scanning Electron Microscopy (SEM) analyses. The results were analyzed by ANOVA followed by t- test, Kruskal-Wallis test, a paired t test and simple logistic regression analysis ($p<0.05$). CSEM data showed a statistically significant difference between control and all irradiated groups ($p<0.01$), but no difference was found between the irradiated L1 and L2 groups ($p>0.05$). The lowest enamel mineral loss occurred in L3 group ($p<0.01$). The µ-EDXRF results showed a significant decrease of Ca only on the L1 group. The results of paired t test showed that calcium content was higher only in primary enamel submitted to two laser aplications ($p=0.046$). SEM results showed that with repeated applications of the CO_2 laser, a progressive melting and recrystallization of the enamel surface occurred. In conclusion, our results showed that three repeated irradiations of primary dental enamel with a CO_2 laser significantly enhanced the inhibition of primary enamel demineralization.

Key words: Demineralization, CO_2 laser, primary teeth, pH cycling, caries

SUMÁRIO

INTRODUÇÃO

Embora tenha ocorrido o declínio da prevalência da doença cárie na última década, ainda representa a doença crônica bucal mais prevalente na infância (Oral Health in America, 2000). No Brasil, ocorreu um declínio da prevalência da doença cárie de 17% entre os anos de 2003 e 2010, porém, para os dentes decíduos, a essa prevalência ainda é alta, de modo que aos 5 anos de idade 53,4% das crianças apresentam um índice ceo-d de 2,3 (SB-Brasil - Ministério da Saúde, 2010).

Tendo em vista que em pacientes de alto risco à cárie deve-se preferencialmente optar por terapias que não dependam de cooperação dos mesmos para que se obtenha êxito, prevenir a formação da lesão de cárie, tem sido uma preocupação constante dos dentistas. A irradiação do esmalte dental com o laser de CO_2 pode representar uma alternativa a ser empregada nestes pacientes. O efeito do laser na inibição da desmineralização do esmalte dental tem sido evidenciado por vários estudos, *in vitro*, que têm mostrado que os lasers de CO_2 podem ser usados para modificar a composição química e/ou a morfologia da superfície do esmalte dental e inibir o desenvolvimento e a progressão de lesões de cárie (Fried *et al*., 1996, 1997; Kantorowitz *et al*., 1998; Rodrigues *et al*., 2004; Steiner-Oliveira e*t al*., 2006; Tagliaferro *et al*., 2009).

Alguns comprimentos de onda obtidos com os lasers de CO_2 (λ= 9.3 e 9.6 µm) são mais apropriados à prevenção da cárie dental, pois produzem radiação na região do infravermelho, que coincide com algumas bandas de absorção da hidroxiapatita, principalmente os grupamentos fosfato e carbonato (Featherstone *et al*., 1998; Kantorowitz *et al*., 1998). No entanto, até o momento, não existem aparelhos de laser disponíveis comercialmente que produzam tais condições, assim as pesquisas realizadas com estes parâmetros utilizaram protótipos (Featherstone *et al*., 1998; Featherstone *et al*., 2001; Nobre dos Santos *et al*., 2001). Desta forma, em busca de simplificação, e aproveitamento da tecnologia já existente, muitas pesquisas têm empregado o comprimento de onda 10,6 µm demonstrando resultados que apresentaram mudanças na superfície do esmalte (Nelson *et al*., 1987; Kantola *et al*., 1973; Stern *et al*., 1972).

O coeficiente de absorção do laser pelo tecido dental deve ser alto o suficiente para modificar a superfície sem danificar o tecido circunjacente e o tecido pulpar

(Shoji *et al.,* 1985; Malmstrom *et al.,* 2001; Steiner-Oliveira *et al.,* 2006). Com o uso do laser de CO_2, a maior parte da luz é absorvida nos poucos micrometros externos da superfície do esmalte e convertida em calor, causando perda de carbonato do mineral, fusão/derretimento dos cristais de hidroxiapatita; tendo como consequência a diminuição na dissolução ácida do esmalte (Featherstone & Nelson, 1987).

Diversas explicações têm sido sugeridas para esse aumento na resistência do esmalte irradiado aos ácidos, como diminuição da permeabilidade do esmalte a agentes químicos, causada pelo derretimento e fusão da superfície (Stern *et al*., 1966), redução da solubilidade do esmalte resultante da alteração na fase mineral (Kuroda & Fowler, 1984), redução do conteúdo de água, proteína e carbonato e formação de compostos fosfatados (Nelson *et al*., 1986, 1987); ou seja, alterações químicas (Stern *et al.,* 1972; Kuroda & Fowler, 1984) ou morfológicas; ou a combinação de ambas. O exato mecanismo ou associação de mecanismos pelos quais se dá essa diminuição da susceptibilidade do esmalte à desmineralização, ainda não está totalmente elucidado.

A análise da literatura evidencia que enquanto inúmeras pesquisas investigaram os efeitos do laser de CO_2 no esmalte de dentes permanentes, apenas o estudo realizado por Tagliaferro *et al.* (2007) evidenciou a efetividade do laser de CO_2 em reduzir a progressão da desmineralização do esmalte de dentes decíduos. Posteriormente, estes autores observaram que este efeito no aumento da resistência do esmalte de dentes decíduos à desmineralização decorria da redução de carbonato do esmalte (Tagliaferro *et al*., 2009). Dessa forma, considerando-se que o esmalte de dentes decíduos apresenta maior concentração de carbonato do que o esmalte de dentes permanentes (Sonju Clasen & Ruyter, 1997), torna-se relevante investigar se aplicações repetidas de laser de CO_2 seriam mais efetivas em aumentar a resistência do esmalte dentário decíduo à desmineralização frente a um alto desafio cariogênico.

Assim, o objetivo do presente estudo foi avaliar, *in vitro*, os efeitos do acúmulo de aplicações repetidas de laser de CO_2 na resistência à perda mineral do esmalte de dentes decíduos.

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CAPÍTULO

Effects of repeated CO_2 laser applications on primary enamel demineralization – An *in vitro* study

Carvalho, AB[1]; Rodrigues, LP[1]; Zancopé, BR[1]; Parisoto, TM[1]; Soares, LES[2]; Steiner-Oliveira, C[3]; Rodrigues , LKA[4]; Nobre-dos-Santos, M[1]*

[1]Department of Pediatric Dentistry, Piracicaba Dental School, University of Campinas (UNICAMP), Piracicaba, 13414-900, Brazil.

[2]Faculty of Dentistry and Laboratory of Biomedical Vibrational Spectroscopy, Research and Development Institute, IP&D, UNIVAP, São José dos Campos, 12244-390, Brazil.

[3]Department of Restorative Dentistry/Special Laboratory of Lasers in Dentistry (LELO), School of Dentistry, University of São Paulo, São Paulo, 05508-070, Brazil.

[4]Department of Restorative Dentistry, Faculty of Dentistry of Federal University of Ceará, Fortaleza, Ceará, 60020-181, Brazil.

*Correspondence to: Marinês Nobre dos Santos.
e-mail: nobre@fop.unicamp.br

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Abstract

Previous studies have shown that the one single irradiation of primary dental enamel with carbon dioxide (CO_2) laser promotes ultrastructural crystallographic effects on enamel surface and turns it more acid-resistance under a cariogenic challenge. However, the effects of repeated applications of CO_2 laser irradiation on the inhibition of demineralization of primary enamel have not been investigated. Thus, the aim of this study was to investigate, *in vitro*, if the repeated application of CO_2 laser irradiation of the primary enamel with a wavelength of 10.6 µm and density of 20.0 J/cm^2 could enhance the inhibition of enamel demineralization, under a high cariogenic challenge. For this study, 80 specimens of sound primary enamel were selected after timely analysis by Energy Dispersive X-ray Fluorescence Spectroscopy (µ-EDXRF) and randomly divided into 4 groups: control (C), 1 application of CO_2 laser (L1), 2 applications of CO_2 laser (L2) and 3 applications of CO_2 laser (L3). The specimens were evaluated by µ-EDXRF before laser applications and after pH cycling. Following, the specimens were submitted to Cross-Sectional Enamel Micro-hardness (CSEM) and Scanning Electron Microscopy (SEM) analyses. The results were analyzed by ANOVA followed by t- test, Kruskal-Wallis test, a paired t test and simple logistic regression analysis ($p<0.05$). The results of CSEM showed that all irradiation conditions were significantly effective in inhibiting enamel demineralization when compared to control group ($p<0.01$) and no difference was found between L1 and L2 groups. Among irradiated groups, the L3 group provided the highest protection against enamel mineral loss ($p<0.01$). The µ-EDXRF results showed a significant decrease of Ca only on the L1 group. The results of paired t test showed that calcium content was higher only in primary enamel submitted to two laser applications ($p=0.046$). The SEM analysis showed melting and fusion on irradiated enamel surface. In conclusion, the results showed that three repeated irradiations of primary dental enamel with a CO_2 laser significantly enhanced the inhibition of enamel demineralization.

INTRODUCTION

In spite of the decline in dental caries prevalence that has been observed over the last few decades, not only in industrialized countries, but also in developing ones, it still remains a major public health problem in some strata of the population [1,2]. In Brazil, a national research [3] showed that 53.4% of the five-years-old children were affected by caries and the average caries in the primary dentition was 2.3 teeth. Tayanim *et al.* (2005) reported that there was a bias of the disease, or groups of individuals that continue to exhibit high caries activity [4]. The use of fluoridated vehicles, which deliver fluoride to the oral cavity, has contributed substantially to the widespread decline in caries incidence in some western countries [5,6]. However, there is evidence that the anticaries effect of fluoride is related to its sustained presence in the oral environment, making the effect dependent on the patient's oral hygiene habits [7]. For effective prevention, therapies not dependent on the patient's compliance would be more advantageous for young children specially those at high caries risk. Thus, use of a pulsed CO_2 laser at 10.6 µm might be a good alternative for these patients. Moreover, when caries prevention is the target, laser treatment may be performed in a unique section being more comfortable for using in children.

Since the development of the rubi laser by Maiman in 1960 [8], several studies have demonstrated that laser technology can be employed in many areas of interest in Dentistry such as caries diagnosis [9-24] and others. Despite the uncertainly on how CO_2 laser irradiation inhibits enamel demineralization, several studies have demonstrated its efficacy in reducing enamel demineralization under a high cariogenic challenger [25-29].

This demineralization reduction could be explained by the presence of hydroxyapatite, which has absorption bands in the infrared region (9.0-11.0 µm) due the presence of phosphate, carbonate and hydroxyl groups in its crystal [18,30]. These absorption bands coincide closely with the radiation produced by the CO_2 laser [31-33]. Besides the works performed in permanent and primary dental enamel, studies were also conducted in dentin, showing that laser energy density in the range of 4.0 to 6.0 J/cm^2 can be safely applied to dental root to reduce demineralization [21] and that irradiation of root dentine with the same laser at fluency of 12.0 J/cm^2 was able to inhibit root surface demineralization when associated with fluoride [22].

There is still controversy regarding the exact action mechanism of CO_2 laser in the inhibition of enamel demineralization. Most theories focus on enamel mineral phase changes, such as surface melting and hydroxyapatite crystal recrystallization. It is well known that irradiation of dental hard tissue with lasers of sufficient power leads to a variety of structural and ultrastructural changes in the tissue near the surface, and several studies have shown that irradiation by CO_2 laser at 10.6 μm can produce surface changes in enamel [34,35]. However, these enamel changes were investigated in permanent teeth. In this respect, only the work from Tagliaferro *et al*. (2009) [36], showed that carbonate content was reduced in irradiated primary enamel specimens but no physical change on enamel surface was noted [25]. In light of the uncertain mechanism of interaction between CO_2 laser and enamel, a more sensitive analysis should be performed to clarify the modification on enamel induced by laser irradiation, especially in the chemical aspect.

Indeed, most of above-mentioned laser studies were conducted in permanent teeth and one might consider that there are differences in the pattern of caries development and prevention when permanent and primary teeth are compared [37]. Moreover in all previous studies, the inhibition of enamel demineralization effect was obtained when CO_2 laser irradiation was performed over the enamel surface only one time. However, one might consider that the higher carbonate content of primary enamel can require more than one laser application for a better effect on the inhibition of deciduous enamel demineralization. Thus, this *in vitro* study aimed to evaluate the effects of cumulative irradiation of CO_2 laser (λ=10.6 μm) on the inhibition of primary enamel demineralization.

MATERIALS AND METHODS

Experimental design

This study was approved by the Research and Ethics Committee of Piracicaba Dental School - University of Campinas (Protocol No. 157/2009) according to the Brazilian Resolution of the National Commission for Ethics in Research. In order to standardize the enamel mineral content, 143 specimens of primary enamel were initially submitted to a nondestructive punctual semiquantitative elemental analysis of calcium (Ca) using Energy-Dispersive X-ray Fluorescence Spectrometry (μ-EDXRF). After selection of 80 enamel specimens, they were randomly assigned according to a computer generated list (software R) to one of the following 4 groups (n= 20): no laser application - Control (C), 1 application of CO_2 laser (L1), 2 applications of CO_2 laser (L2) and 3 applications of CO_2 laser (L3). Before laser application, specimens from all groups were submitted to initial area μ-EDXRF analysis. Then, all specimens were submitted to a pH-cycling process to simulate a high cariogenic challenge. Following, all specimens were submitted to a final area μ-EDXRF analysis. After that, 15 samples of each group were submitted to Cross-Sectional Enamel Micro-hardness (CSEM) analysis while 05 specimens were analyzed by Scanning Electron Microscopy (SEM) in order to evaluate the morphological changes on the primary enamel surface after repeated applications of CO_2 laser. Calcium percentages obtained by μ-EDXRF performed before laser application and after pH cycling as well as the area of CSEM loss were statistically analyzed by ANOVA followed by t-test and Kruskal-Wallis test ($p<0.05$). The area of CSEM loss was also evaluated by simple linear regression ($p<0.05$).

Tooth selection and sample preparation

To perform this *in vitro* study, 143 carious free human primary molars, stored in a supersatured 0.1% thymol solution, were selected, cleaned and sterilized using gamma radiation. These teeth were sectioned mesiodistally using a water-coated diamond saw cutting machine (Isomet, Buehler, Lake Bluff, IL, USA) to obtain 143 unabraded enamel slabs (4 x 4 mm). All slabs were obtained from the medium third of buccal or lingual coronal surfaces of each tooth. Then, enamel surface was screened for cracks and white spots under a stereoscopic microscope. The slabs were coated with an acid-resistant varnish leaving a window (2 x 2 mm) of exposed enamel surface and were kept moist at 100% relative humidity with thymol 0.1% before experimentation begun.

Energy-Dispersive X-ray Fluorescence Spectrometry (μ-EDXRF) measurements

In order to standardize and select 80 enamel samples, 143 enamel specimens were submitted to a punctual analysis of the calcium content. Thus, enamel specimens were set side by side on a glass plate with modeling clay. Semiquantitative elemental analysis of calcium (Ca) was carried out by an energy-dispersive micro X-ray fluorescence spectrometer (model μ-EDX 1300, Shimadzu, Kyoto, Japan), equipped with a rhodium X-ray tube and Si (Li) detector cooled by liquid nitrogen (N_2) and coupled to a computer system for data acquisition and processing. The voltage in the tube was set at 15 kV, with automatic adjustment of the current and incident beam diameter of 50 μm. Three points in the center of the irradiated enamel slab was chosen for measurement. The measurement were performed with a count rate of 100 seconds per point (live time) and dead time of 25%. The energy range scans was 0.0 – 40.0 eV. The equipment was calibrated and adjusted using certified commercial stoichiometric hydroxyapatite (Aldrich, synthetic $Ca_{10}(PO_4)_6(OH)_2$, grade 99.999%, lot 10818HA) as a reference. The measurements were collected using classic parameters for Ca X-ray emission. Energy calibration was performed using equipment-specific internal standards. The equipment calibration and chemical balance were performed as previously reported [38]. In order to select the primary enamel samples we considered the mean calcium obtained by the average of punctual analysis (32.5%). In the same way, we selected enamel samples having a 5% variation for more and to less (28-37%) of calcium.

After selection of 80 samples, they were subjected to an area analysis and then randomly assigned according to a computer generated (software R) to one of the following 4 groups (n= 20): control (C), 1 application of CO_2 laser (L1), 2 applications of CO_2 laser (L2) and 3 applications of CO_2 laser (L3). After laser treatment and pH cycling all the samples were subjected to a final area analysis.

Laser Irradiation

The enamel surface irradiation was carried out using an X-Y position platform. They were scanned at a distance of 10 mm from the tip of the hand piece for approximately

30 seconds. The scanning speed was approximately 1 mm/s. A commercially available model UM-L30 pulsed CO_2 laser (Union Medical Engineering Co., Yangju-si, Gyeonggi-Do, Korea) at a wavelength of 10.6 µm was used for irradiating the groups L1, L2 and L3 with the following parameters: 10 ms pulse duration, 10 ms of time off (total time of 20 ms), 50 Hz repetition rate, 0.3 mm beam diameter. Using a power meter (Scientech 373 Model-37-3002, Scientech Inc., Boulder, CO, USA), the average power output was measured and found to be 0.7 W. Thus, the laser fluency applied on enamel was approximately 20.0 J/cm^2 per pulse. These parameters have been previously tested and show no possibility of pulp damage [39].

pH-Cycling Process

The pH-cycling model used in this study was based on the on described by Nobre-dos-Santos *et al*. (2001) [40] with modifications by Steiner-Oliveira *et al*. (2008) [41]. The enamel specimens were kept individually in a demineralizing solution (2.0 mM calcium, 2.0 mM phosphate, in 75 mM acetate buffer, pH 4.6) for 3 hours (20 mL per slab – 5mL/mm^2), and in a remineralizing solution (1.5 mM calcium, 0.9 mM phosphate, 150 mM of KCl, in 20 mM cacodylic buffer, pH 7.0) for approximately 21 hours (10 mL per slab – 2.5 mL/mm^2) each day. This cycle was repeated daily for 5 days and then the enamel slabs remained in the remineralization solution for 2 days. This pH-cycling regimen was carried out at 37°C. Between immersions in demineralizing and remineralizing solutions and at the end of the pH-cycling regimen, enamel slabs were rinsed with deionized water for 10 seconds and wiped with tissue paper. The demineralizing and remineralizing solutions contained thymol to avoid bacterial and fungal growth. The enamel slabs were kept moist at 100% relative humidity with thymol 0.1% until the analysis.

Cross-Sectional Enamel Micro-hardness (CSEM) analysis

For cross-sectional enamel micro-hardness analysis, 15 samples of each group were longitudinally sectioned after pH-cycling with a cut through the center of the exposed enamel area. One of the halves was embedded in a thermo-acrylic resin Vipicril Plus (VIPI Ind. e Com., Pirassununga, SP, Brazil) and heated in the PRE 30MI (Arotec SA Ind. E

Com., Cotia, SP, Brazil). Then they were serially polished using an AROTEC APL-4 (Arotec SA Ind. E Com., Cotia, SP, Brazil) polishing machine and sandpapers, followed by diamond abrasive paste on polishing cloths. The slabs were assessed for microhardness test with a Knoop diamond under a 25 g load for 5 seconds mounted on a FM-100 micro-hardness tester (Future-Tech Corp., Tokyo, Japan) and read at 50X magnification. Thirty-six identations (three rows of 12 identations each) were made with the long axis of the Knoop diamond parallel to the outer enamel surface, maintaining a 10 µm interval from edge up to 60 µm and then a 20 µm interval from 60 µm to 180 µm across the lesion and into the underlying enamel. The distance between the rows was 100 µm. The mean hardness values of the three rows at each distance from the surface were then averaged and expressed as Knoop hardness number (KHN). The means values (kg/mm^2) at all measuring points of each distance from the surface were then averaged and the area of hardness loss versus lesion depth (ΔS) was calculated by numerical integration using the trapezoidal rule by the difference between the area under the curve (kg/mm^2 x µm) of the sound enamel minus the area of the demineralized one [42].

Scanning Electron Microscopy (SEM) analysis

Morphological investigation was performed in order to verify the effects of repeated applications of CO_2 laser on the enamel surface. Thus, 5 specimens from each group were examined under scanning electron microscopy. They were dried and mounted on a holder using double-sided adhesive carbon tape. The samples were sputter-coated with gold (~10-12 nm thickness) by the BAL-TEC SCD 050 Sputter Coater (Wetzlar, Liechtenstein/Vienna, Austria). Observations were made with a JEOL JSM 5600 LV Scanning Microscope (Jeol, Peaboy, MA, USA) at 15 kV, operating at 2500x magnification.

Statistical analysis

The normal distribution of the sample data were assessed by The D'Agostino test and the Lilliefors test ($p<0.05$).

μ-EDXRF– The calcium percentages obtained by X-ray analysis performed before laser application and after pH-cycling were statistically compared among the control and lasers groups using ANOVA followed by t- test and Kruskal-Wallis test, according to data distribution (α=0.05). In addition, a paired t test was preformed to compared means and standard deviations (n=20) of calcium and percentages obtained by μ-EDXRF analysis before and after laser application and pH cycling.

CSEM– The area of CSEM loss was compared among the control and lasers groups using ANOVA followed by t- test ($p<0.05$). In addition, simple linear regression was performed to verify if the laser applications were able to reduce the area of CSEM loss (dependent variable) compared with the control group (α=0.05).

All analysis were performed using BioEstat 5.3® software and Stata 11.0. software.

RESULTS

The enamel lesion progression (Table 1 and Figure 1) determined by the area of micro-hardness loss (ΔS) showed that all irradiation conditions were significantly effective in inhibiting enamel demineralization when compared to control group ($p<0.01$). A further analysis of our results demonstrated that among irradiated groups, the L3 group provided the highest protection against enamel mineral loss (< 0.01) and no difference was found between L1 and L2 groups.

Table 2 shows means and standard deviations of calcium areas before and after laser application (initial and final μ-EDXRF measurements). From this table, it can be seen that no statistical difference between the control and irradiated groups was found for calcium percentages (p=0.0926) before pH-clycing. The μ-EDXRF measurements performed after pH cycling (final analysis) showed that calcium percentage was significantly lower for L1 group when compared with control group (p=0.05) and irradiated groups (p=0.001). Moreover, L2 and L3 groups did not differ from control group. Table 3 shows the results of paried t test of initial and final means and standard deviations of calcium. This table evidenced that calcium content was higher only in primary enamel submitted to two laser applications (p=0.046).

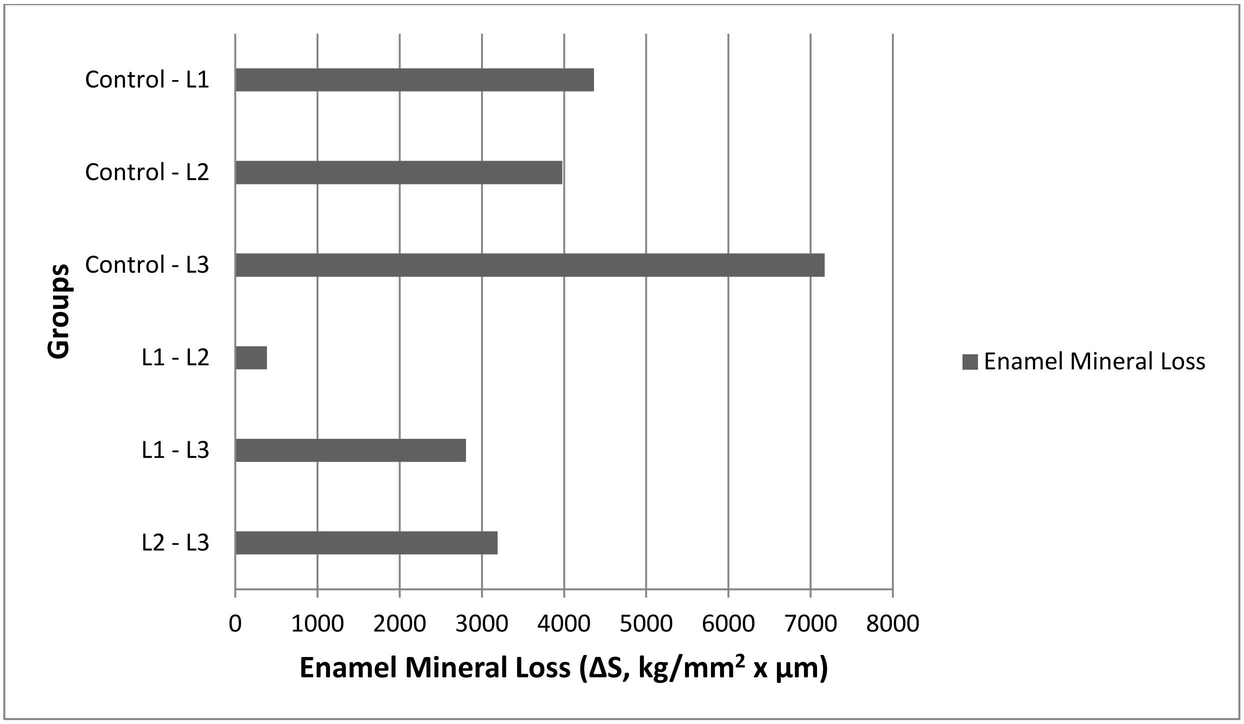
The SEM observations showed evidence of melting and fusion in the specimens treated with the CO_2 laser in all the laser applications (Fig. 2. b, c and d). These morphological changes were more common in specimens irradiated with more than one laser application (Fig. 2.c and d). Surface alterations have progressed to the point that crystal definition was no longer visible (Fig. 2.c). Recrystallization was observed more frequently on enamel surface after L3 irradiation (Fig. 2.d). Crystal coalescence and alignment of crystals along enamel rods as well as droplets of recondensed enamel mineral (Fig. 2.d).

Table 1. Means and standard deviations of enamel mineral loss according to the groups (n= 15)

Groups	ΔS, kg/mm^2 x µm	p_value$_{anova}$
Control	9146.97± 2717.05a	
L1	4782.73± 1514.40b	<0.01
L2	5167.04± 1729.94b	
L3	1975.05± 1436.10c	

Means followed by distinct letters are statistically different by Anova followed by t- test (p <0.05).

Figure 1. Difference of means and standard deviations of enamel mineral loss according to the following comparisons (n=15):



Simple linear regression, $p<0.01$, r^2 adjusted= 0.64.

Table 2. Means and standard deviations (n=20) of calcium and percentages obtained by µ-EDXRF analysis before and after laser applications and pH-cycling

Component	Groups	Mean ±SD Initial	Mean ±SD Final	p_value	CI – 95%
Ca	Control	31.2 ± 3.3a	33.5± 3.7bc	0.0570	-4.7565 to 0.0781
	L1	30.9 ± 4.3a	30.1± 6.6a	0.6152	-2.4369 to 4.0113
	L2	33.4 ± 4.1a	36.6± 4.3b	0.0467	-6.2656 to 0.0461
	L3	33.7 ± 3.7a	32.8± 4.3ac	0.4789	-3.2828 to 1.5984

Data evaluated by Kruskal Wallis test ($p <0.05$); Calcium before treatments (initial): p = 0.0926, without significance. Calcium after treatments (final): p = 0.001, where different small letters on columns shows statistical significance among groups and paired t test $\alpha = 0.05$.

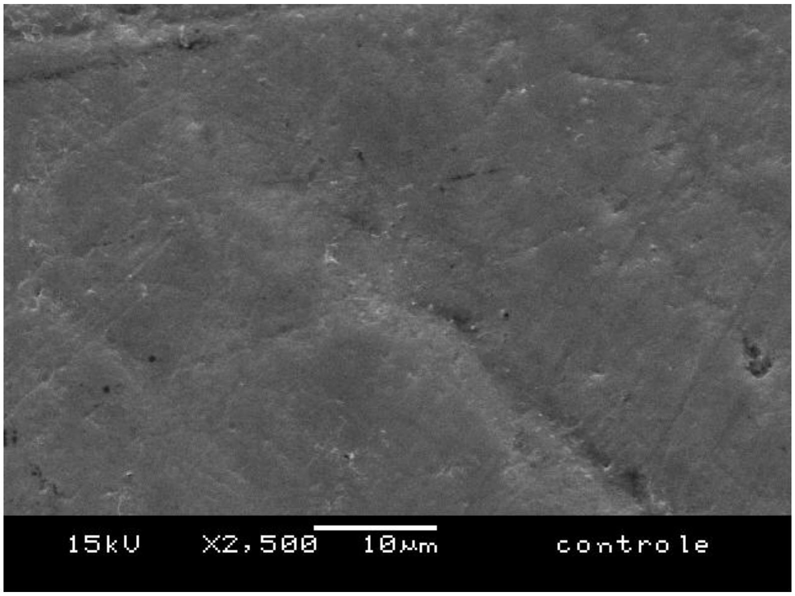


Figure. 2.a. Control group. SEM micrograph of non-irradiated enamel surface after pH cycling.

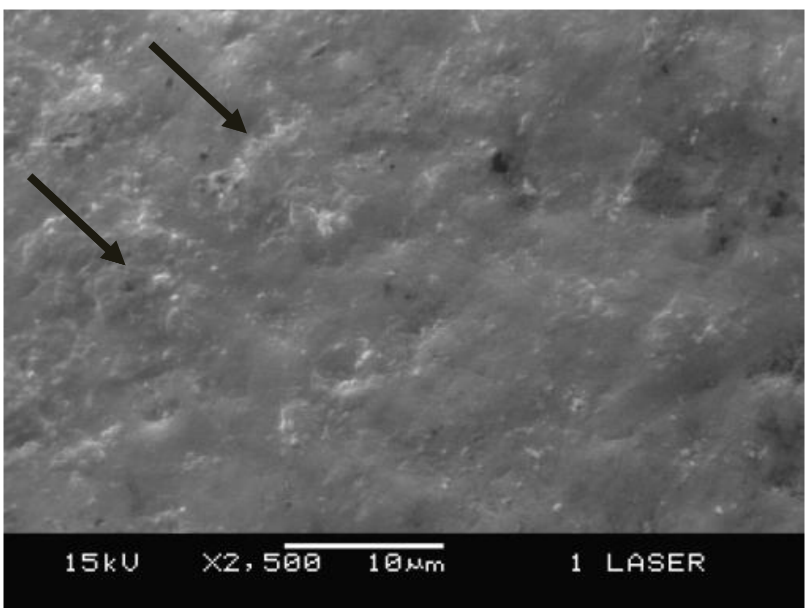


Figure. 2.b. L1 Group. Representative scanning electron micrograph of enamel surface after 01 CO_2 laser application and pH cycling. Enamel crystals melting and fusion are evident.

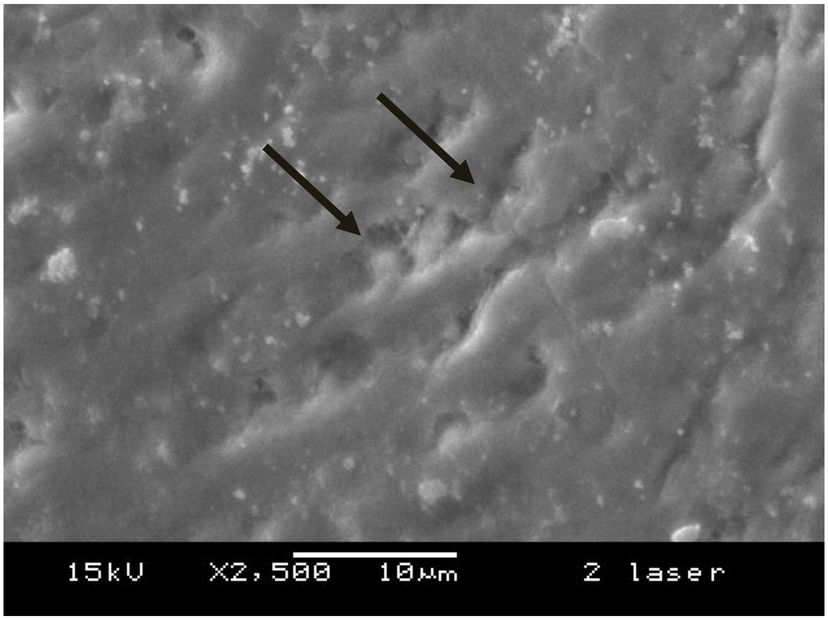


Figure. 2.c. L2 Group. Representative scanning electron micrograph of enamel surface after 02 CO_2 laser applications and pH cycling. Surface alterations (melting and fusion) has progressed to the point that crystal definition was no longer visible.

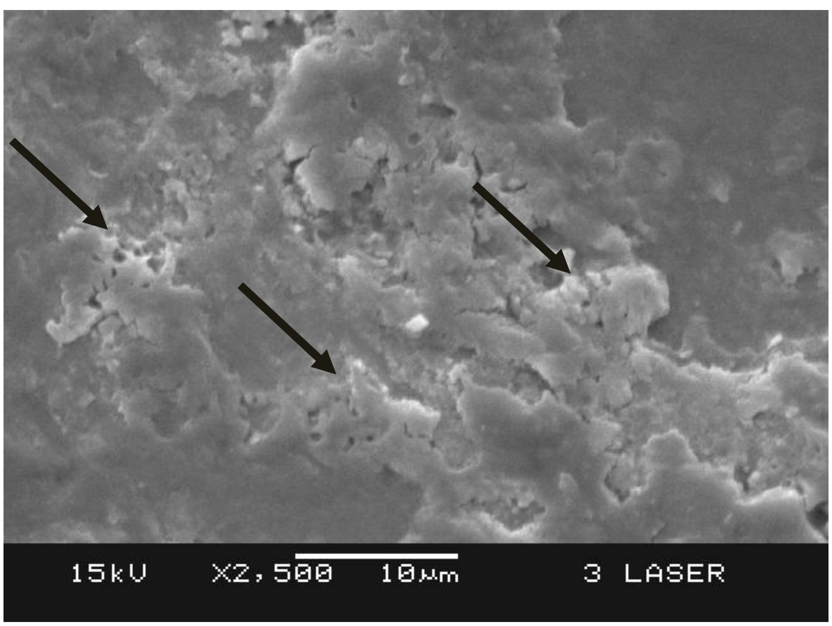


Figure. 2.d. L3 Group. Representative scanning electron micrograph of enamel surface after 03 CO_2 laser applications and pH cycling. Crystal coalescence and alignment of crystals along enamel rods as well as droplets of recondensed enamel mineral.

DISCUSSION AND CONCLUSION

The successful use of lasers for caries prevention depends on the promotion of demineralization inhibitory effect on dental enamel. The results of this study demonstrated for the first time that repeated irradiation of sound primary dental enamel with a CO_2 laser significantly enhanced the inhibition of enamel demineralization (Table 1). After one laser irradiation the enamel mineral loss was lower than the one noticed for control group ($p<0.01$). This result is in line with previous investigation performed on carious primary enamel by Tagliaferro *et al*. (2007) [36]. However, these authors employed a four times lower fluency (5 J/cm^2). With two laser irradiations no further improvement in the inhibition of enamel demineralization was obtained although the final calcium analysis showed a higher calcium content in L2 group when compared with all groups ($p=0.01$). Moreover, the results of this study showed that the highest demineralization inhibitory effect was obtained when sound primary enamel was submitted to three CO_2 laser applications ($p<0.01$) (Table 1- group L3). In this respect, when analyzing the results of the simple linear regression analysis, our study showed that when three CO_2 laser applications were performed the mineral loss was reduced in average, 7171.93, when compared with no laser application ($p<0.01$) (Figure 1). This result may be partially explained by the higher carbonate content in deciduous teeth when compared to the permanent enamel. Furthermore, the deciduous enamel contains significantly more type A carbonate (carbonate in the hydroxide positions) than permanent enamel (Sonju Clasen & Ruyter, 1997) [43]. In line with this assumption, research performed by Tagliaferro *et al.* (2009) [25] showed a decrease in intensity of the organic bands in the range of 1200-3100 cm^{-1} in carious and irradiated primary enamel. However, since FT-Raman spectroscopic analysis was not performed in the present study, we can only speculate that the hole of CO_2 laser in reducing type A carbonate might have made the caries-preventive effects of repeated CO_2 laser applications more evident in primary enamel.

Regarding morphological changes occurring on enamel surface after laser application, the SEM data of the present study suggest that the fusion and melting phenomena (Figure 2.b.c.d) are related to the inhibition of enamel demineralization found in the irradiated groups. These features are in agreement with data reported by Klein *et al.* (2005) [44] and Steiner-Oliveira *et al.* (2006) [39] and suggest that enamel surface was sealed by the laser irradiation and became less permeable to the subsequent diffusion of ions into and out of the enamel (Ferreira *et al.,* 1989) [30]. On the other side, SEM observations by Tagliaferro *et al.* (2009) [25] evidenced little or no morphologic change, and no melting or formation of craters on primary enamel surface. However, using these laser parameters these authors concluded that although no morphologic change occurred on enamel surface, CO_2 laser irradiation was highly effective in inhibiting lesion progression in primary carious enamel (Tagliaferro *et al.,* 2007) [36]. The SEM results of this study also showed crystal coalescence as well as droplets of recondensed enamel mineral (Fig. 2.d). This result is in agreement with Stern *et al*. (1972) [16] and Malmstrom *et al.* (2001) [45] and partially explains the higher inhibition of enamel demineralization noticed when three laser application were performed (Table 1, group L3). In summary, our findings showed that morfological changes occuring on irradiated enamel surface, give support to the most frequently mentioned hypothesis for laser effect stating that inhibition of enamel demineralization is due to melting and fusion of hydroxyapatite crystals [30,46].

The µ-EDXRF analysis is based on bombarding the specimen with a beam of high voltage electrons that are refracted at different energy levels from individual minerals. The change in the energy returned from the specimen reflects the change in its mineral content [47]. This method allows for accurate and non-invasive analysis of specimens. In this study, comparison of mineral content performed before and after laser irradiation and pH cycling showed that calcium content was higher only in primary enamel submitted to two laser aplications ($p=0.046$ - paired t test). These findings only partially explains the demineralization inhibitory effect observed after two laser irradiations (Table 2) since no calcium change occurred in L3 group that presented the lowest mineral loss. In this way, we believe the enamel demineralization inhibitory effect found for L1 and L2 groups was most due to morphological changes occurring on enamel surface mainly melting and fusion.

In conclusion, our results showed that all irradiation conditions were significantly effective in inhibiting demineralization of sound primary enamel (operated at

10.6 μm and at an energy density of 20.0 J/cm^2) and that three repeated irradiations of primary dental enamel with a CO_2 laser significantly enhanced the inhibition of enamel demineralization.

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CONCLUSÃO

O estudo do laser de CO2 (10.6 µm) como um dos métodos alternativos na redução da desmineralização do esmalte dentário tem crescido ao longo de 40 anos. No entanto, ainda não há protocolos definidos que padronizem os parâmetros do laser, nem tão pouco, um número significante de estudos clínicos/ *in situ* randomizados que viabilizem seu uso na prática clínica.

Uma aplicação do laser de CO_2 (10.6 µm – 20 J/cm^2) sobre a superfície do esmalte dentário foi capaz de modificar a superfície do esmalte aumentando sua resistência à desmineralização. No entanto, 3 aplicações de laser de CO_2 sobre o esmalte de dentes decíduos diminuiu significativamente a perda mineral.

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APÊNCICE 1

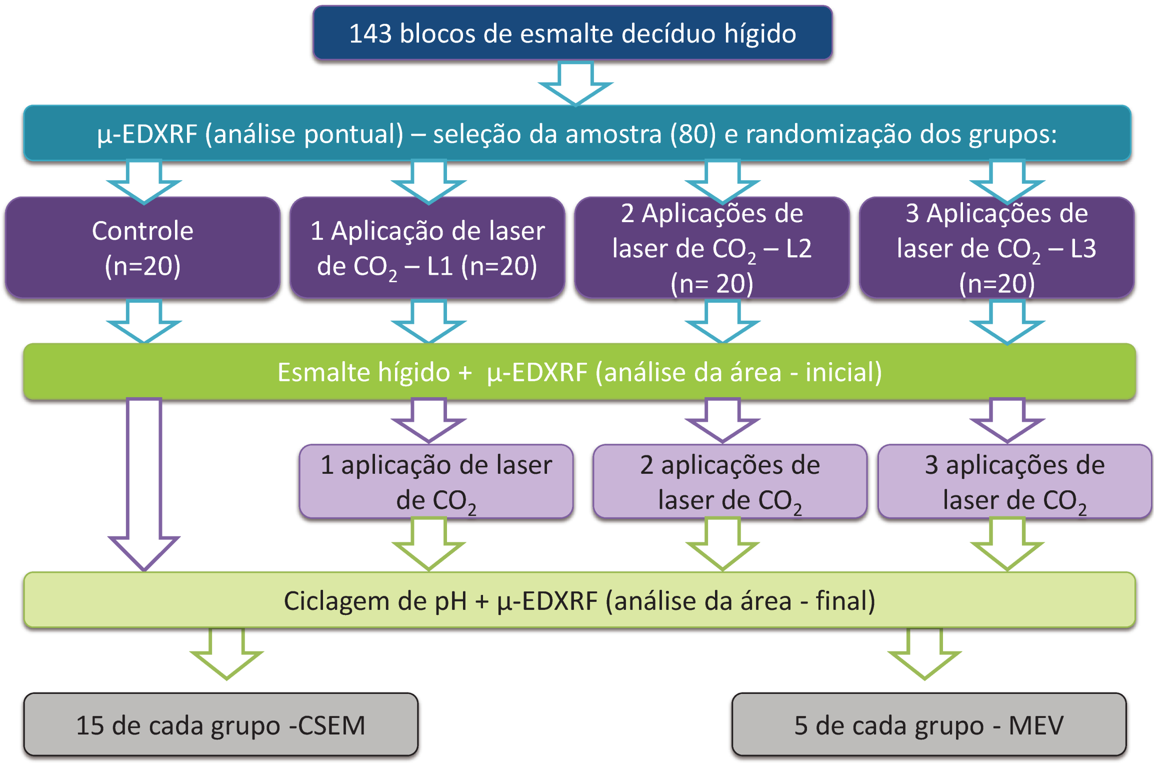

Fig. Delineamento experimental

APÊNDICE 2

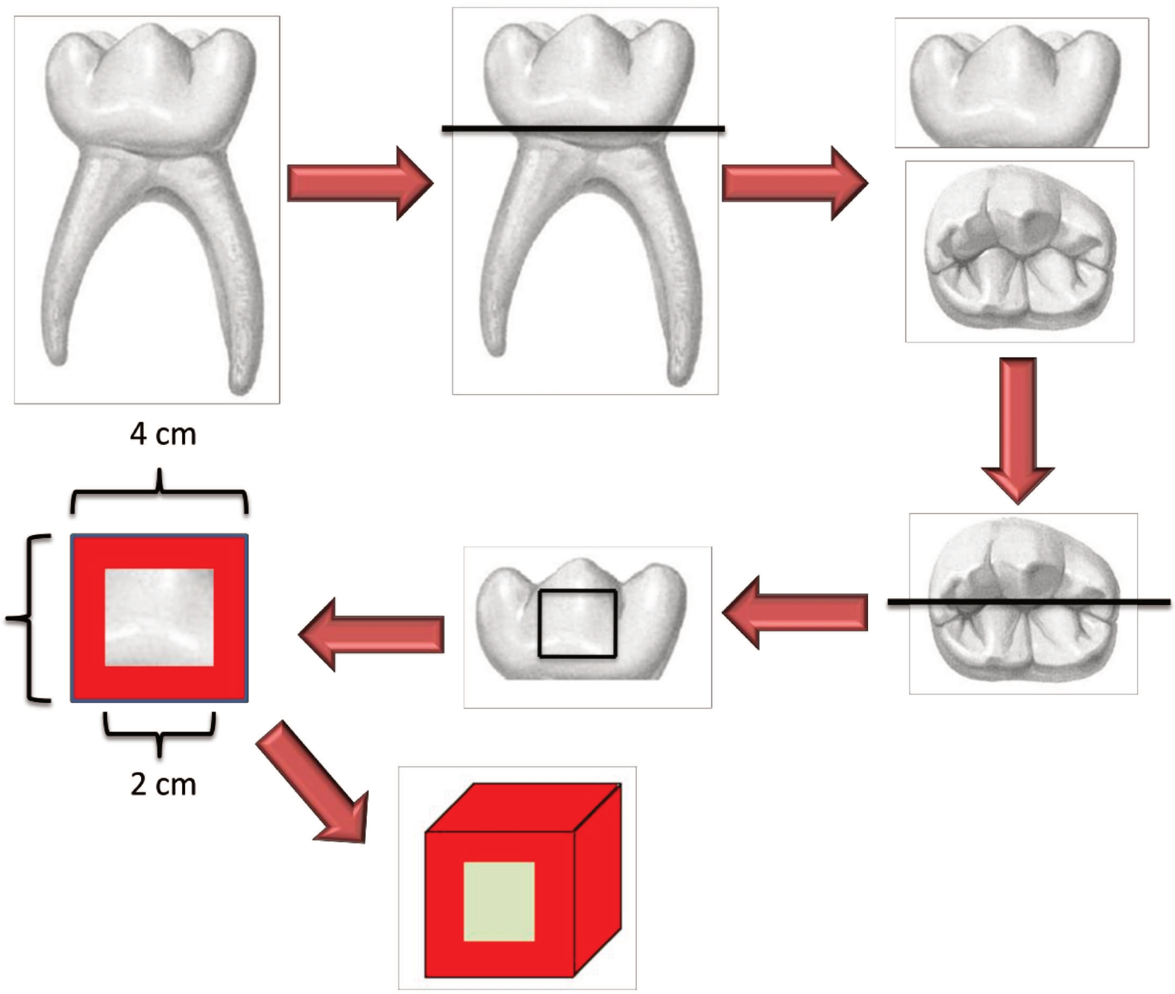

Fig. Esquema ilustrativo do preparo dos blocos de esmalte

APÊNDICE 3

Fig. µ-EDXRF - modelo µ-EDX 1300, Shimadzu, Kyoto, Japão

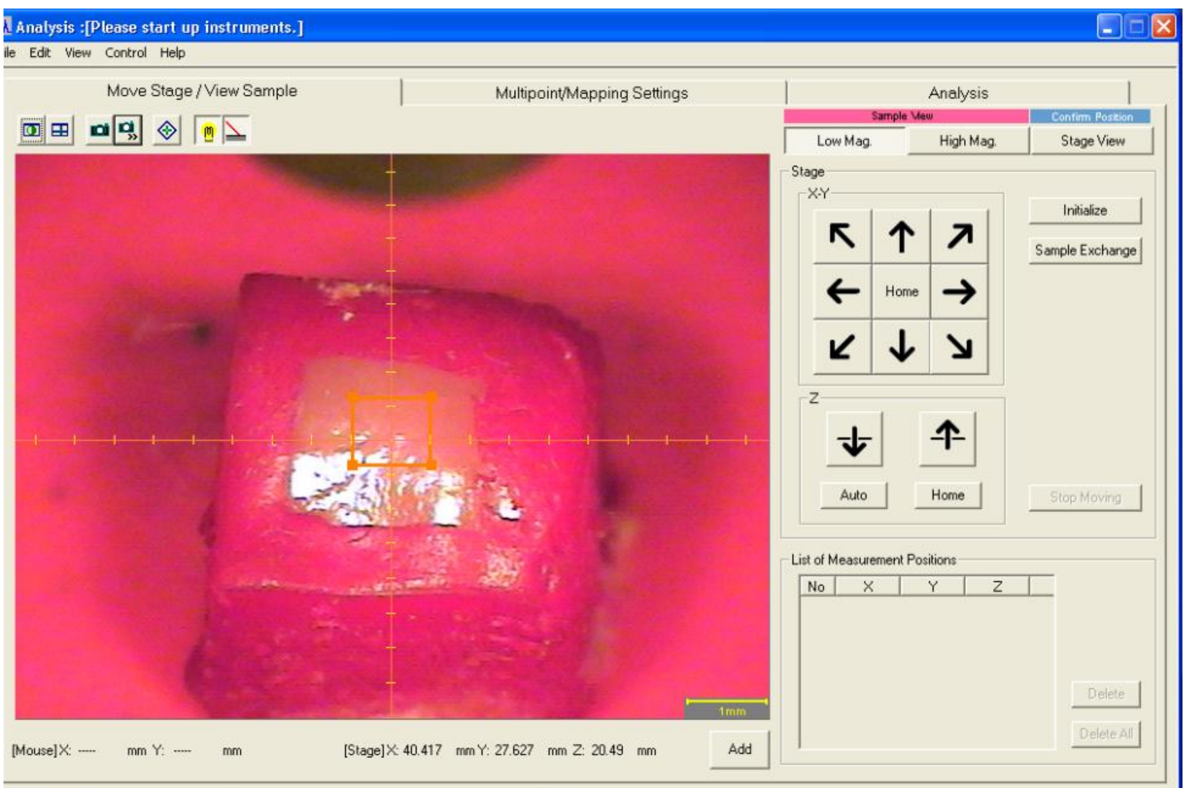

Fig. Análise da área – µ-EDXRF

APÊNDICE 4

Fig. Laser de CO_2

Fig. Laser de CO_2 acoplado à base adaptada de microscópio para varredura da área de esmalte

APÊNDICE 5

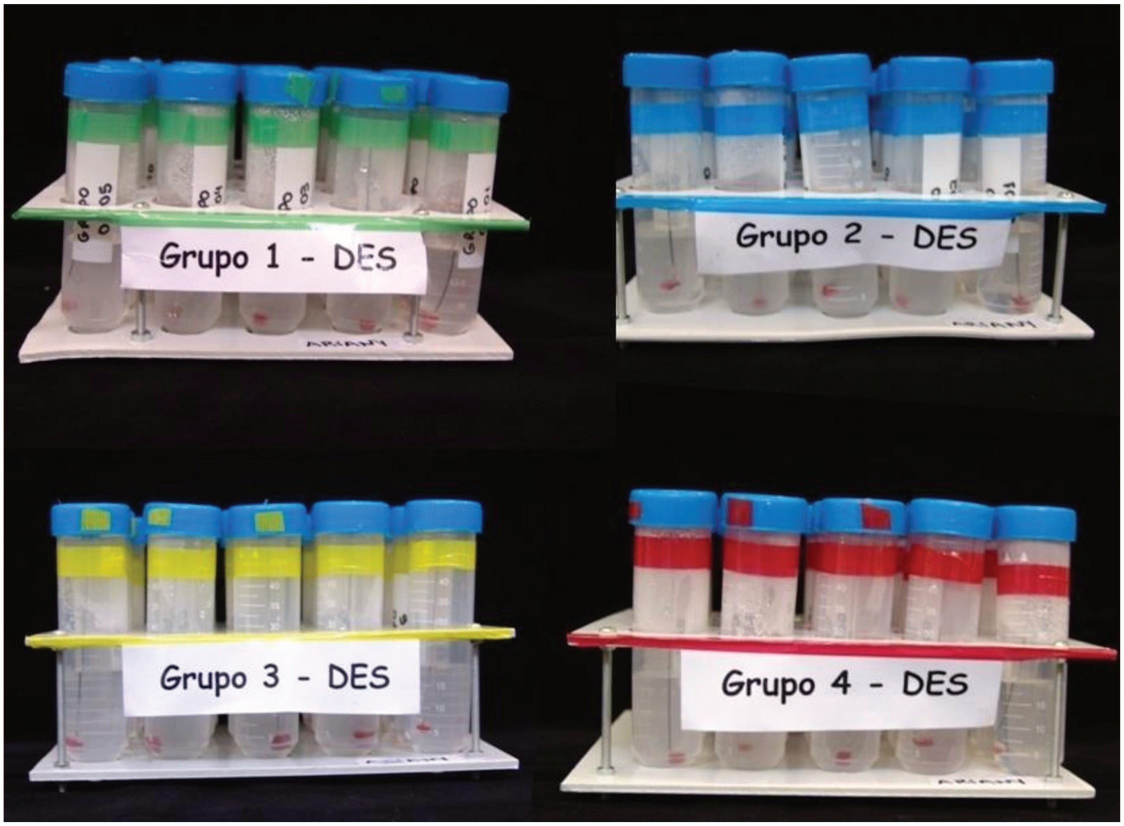

Fig. Ciclagem de pH – Espécimes separados por grupos imersos em solução DES

APÊNDICE 6

Fig. Microdurômetro

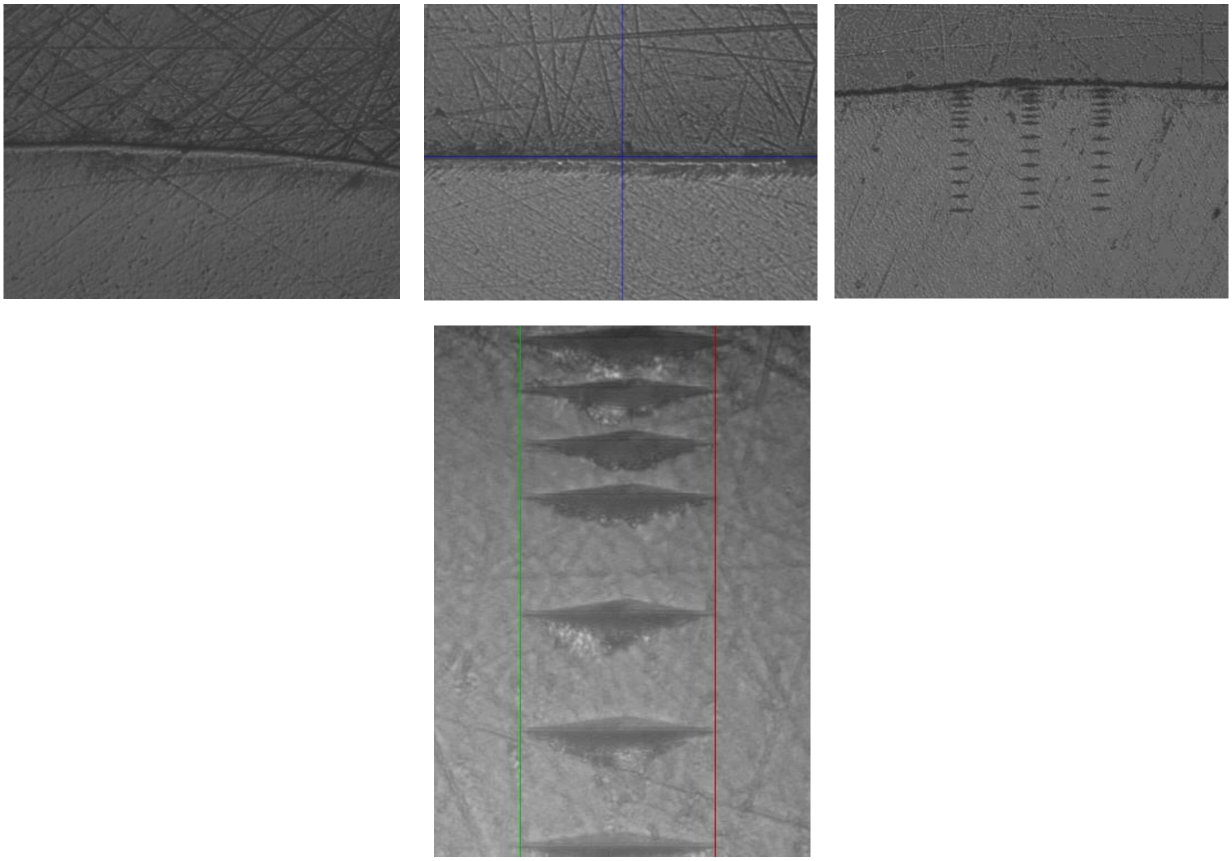

Fig. Imagens de identações realizadas para Dureza Knoop

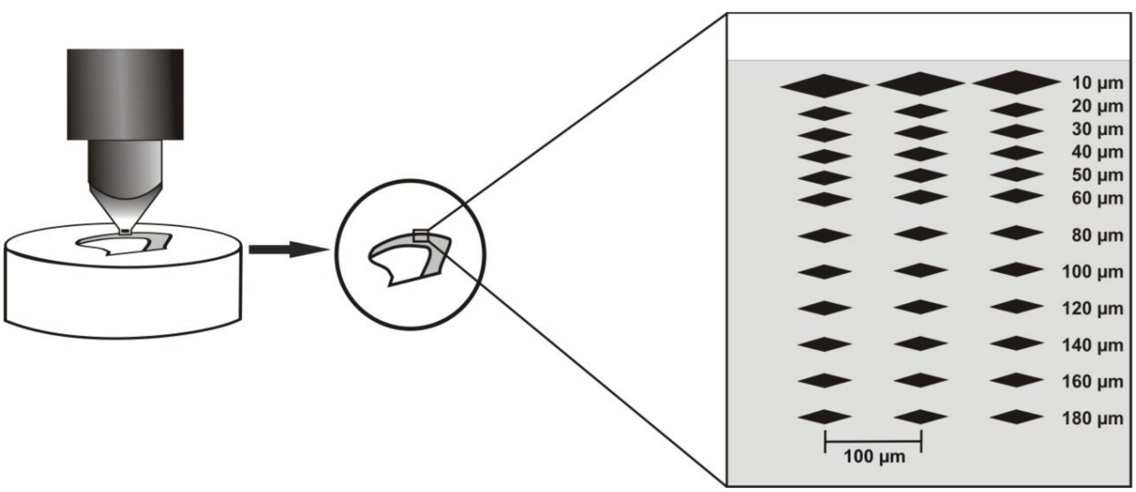

Fig. Ilustração representativa das impressões realizadas nos espécimes durante análise de microdureza

ANEXO 1

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"**Efeitos do tratamento repetido do esmalte com laser de CO2 na desmineralização do esmalte de dentes decíduos e permanentes - Estudos in vitro **"**, protocolo nº 157/2009, dos pesquisadores Marinês Nobre dos Santos Uchôa e Ariany Borges Carvalho, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 21/12/2009.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "Effects of the repeated treatment of the enamel with laser of CO2 in the desmineralization of the enamel in deciduous and permanent teeth – Studies in vitro", register number 157/2009, of MarinêsNobre dos Santos Uchôa and Ariany Borges Carvalho, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 12/21/2009.

Prof. Dr. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.