UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Efeitos *in vitro* do fluoreto no potencial anticárie do laser de CO_2 no esmalte dentário desmineralizado.

Dissertação de Mestrado apresentada a Faculdade de Odontologia de Piracicaba da UNICAMP para obtenção do título de mestre em Odontologia, na área de Odontopediatria.

Orientadora: Marinês Nobre dos Santos Uchôa

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"*Nem olhos viram, nem ouvidos*
ouviram, nem jamais penetrou em
coração humano o que Deus tem
preparado para aqueles que o amam."
(1 Coríntios 2:9)

Resumo

O objetivo desse estudo foi avaliar se a irradiação com o laser de CO_2 acentua a incorporação de flúor no esmalte dentário desmineralizado e avaliar se a irradiação com o laser de CO_2 laser (λ=10.6 μm) com duas densidades de energia (11,3 J/cm^2 e 20,0 J/cm^2), combinado com aplicação de flúor fosfato acidulado (FFA) pode inibir a progressão da lesão em esmalte dentário. 315 espécimes de esmalte cariado (EEC) foram utilizados em dois experimentos. No experimento 1, 150 EEC foram divididos em 10 grupos, as quais foram irradiados e combinados com uma aplicação de FFA antes, durante ou após irradiação. Em seguida, 50 espécimes tiveram a superfície avaliada pela microscopia eletrônica de varredura (MEV). A concentração de fluoreto de cálcio (CaF_2) formada na superfície do esmalte foi determinada em 100 EEC (N=10, 10 grupos). Após analise de CaF_2, uma nova análise MEV foi realizada em 50 espécimes. No experimento 2, 165 EEC (11 grupos, n=15) foram submetidos aos mesmos tratamentos do experimento 1 + grupo com apenas lesão de cárie. Após ciclagem de pH, o flúor nas soluções desmineralizadoras (desmin) e remineralizadoras (remin), microdureza e profundidade de lesão foram determinadas. Os resultados foram analisados pelo teste ANOVA e Tukey e pelo teste de Kruskal-Wallis e Student-Newman-Keuls (α=0.05). Os resultados mostraram que a irradiação isolada ou combinada com FFA acentua a formação de CaF_2 na superfície do esmalte, a concentração de flúor nas soluções des-remin e inibe a progressão da lesão ($p<0.05$). As observações em MEV mostraram evidências de derretimento, fusão e trincas. Conclui-se que a irradiação com o laser isolada ou combinada com FFA acentua a incorporação de CaF_2 e inibi a progressão de lesão de cárie no esmalte dentário desmineralizado. No entanto nenhum efeito sinérgico foi encontrado.

Abstract

The aim of this study was to investigate if CO_2 laser irradiation enhances fluoride uptake by demineralized dental enamel and to evaluate whether enamel lesion progression can be inhibited by CO_2 laser (λ=10.6 μm) irradiation with two fluencies (11.3 J/cm^2 and 20.0 J/cm^2) combined with acidulated phosphate fluoride (APF) application. 315 specimens of carious enamel (SCE) were used in two experiments. In experiment 1, 150 SCE were allocated to 10 groups, which were irradiated combined with one APF application performed either before, during or after irradiation. Following, 50 specimens had their surface examined for morphological changes by scanning electron microscopy (SEM). Fluoride as CaF_2 formed on enamel surface was determined in 100 SCE (n=10, 10 groups). After CaF_2 analysis, a new SEM analysis was performed in 50 specimens. In experiment 2, 165 SCE (11 groups, n=15) were submitted to the same treatments as in experiment 1 + a caries lesion only group. After pH cycling, fluoride in demineralizing (Demin) and remineralizing (Remin) solutions, microhardness and lesion depth were determined. The results were analyzed by ANOVA and Tukey test and by Kruskal-Wallis rank test and Student-Newman-Keuls test (α=0.05). The results showed that irradiation alone or combined with APF enhanced CaF_2 formation on enamel surface, fluoride concentration in de-remin solutions and inhibited lesion progression ($p<0.05$). SEM observations showed evidences of melting, fusion and cracks. In conclusion, laser irradiation alone or combined with APF enhances CaF_2 uptake and inhibits lesion progression on demineralized dental enamel. However no synergic effect was found.

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INTRODUÇÃO GERAL

A cárie dentária é uma doença crônica caracterizada pela destruição progressiva dos tecidos duros da estrutura dentária. O uso de compostos fluoretados tem sido utilizado com o objetivo de deter a progressão desta doença. No entanto, o fluoreto não tem sido capaz de controlar completamente o desenvolvimento das lesões de cárie, o que facilitou o aparecimento de um grupo polarizado com essa doença, ou seja, grupos de indivíduos que continuam apresentando alta atividade de cárie (Seppä, 2001). Tais fatos enfatizam a necessidade do aperfeiçoamento de métodos preventivos já existentes, com a introdução de técnicas inovadoras que possam agir como coadjuvantes na prevenção e controle da cárie dentária.

Diferentes tipos de lasers, tais como Nd:YAG, Argônio, Er:YAG e dióxido de carbono (CO_2) têm sido estudados para uso em Odontologia. O emprego dos lasers de alta potência consiste no tratamento do esmalte dentário para a obtenção de superfícies mais resistentes aos ácidos produzidos pelas bactérias cariogênicas (Stern et al.,1972; Kantola, 1972; Kantola et al., 1973; Nelson et al., 1986, 1987; Featherstone et al., 1991, 1998; Kantorowitz et al., 1998; Hsu et al., 2000).

O laser de CO_2 atua na desmineralização do esmalte reduzindo sua solubilidade aos ácidos. Os comprimentos de onda obtidos com os lasers de CO_2 (λ= 9,3, 9,6, 10,3 e 10,6 μm) são mais apropriados para a utilização em esmalte dentário, pois produzem radiação na região do infravermelho que coincide com algumas bandas de absorção da hidroxiapatita, principalmente os grupamentos fosfato e carbonato (Rodrigues et al., 2006). Quando a luz é absorvida nos poucos micrometros externos da superfície do esmalte dentário e convertida em calor, ocorre perda de carbonato do mineral e fusão dos cristais de hidroxiapatita, tendo como consequência uma diminuição na reatividade ácida desta estrutura (Fried et al., 1997). O nível de interação que ocorre entre o tecido dentário e o laser é dependente das propriedades estruturais do tecido irradiado, bem como do

comprimento de onda, densidade de energia, duração de pulso e taxa de repetição do laser (Gimbel, 2000).

Segundo Featherstone (2000), os comprimentos de onda mais indicados para uso na prevenção de cárie dentária são 9,3 μm e 9,6 μm com duração de pulso de 100 μs ou menos. No entanto, até o momento, não existem aparelhos de laser comercialmente disponíveis que possam produzir tais condições, de modo que as pesquisas realizadas com estes parâmetros utilizaram protótipos. Consequentemente, em busca de simplificação, e aproveitamento da tecnologia já existente, muitas pesquisas tem empregado o comprimento de onda 10,6 μm (Kantorowitz et al., 1998; Hsu et al., 2000; Klein et al., 2005; Steiner-Oliveira et al., 2006; Tagliaferro et al., 2007).

Quando o laser de CO_2 é associado a agentes fluoretados, o seu efeito na redução da desmineralização pode ser potencializado (Fox et al., 1992; Hsu et al., 1998; Hsu et al., 2001; Nobre dos Santos et al., 2001; Rodrigues et al., 2006). No entanto, os estudos que mostraram a potencialização deste efeito utilizaram laser de CO_2 com comprimentos de onda de 9.3 a 9.6, que só existe disponível em forma de protótipo.

Apesar dos lasers de CO_2 serem objeto de estudo desde 1960, os trabalhos científicos ainda apresentam discrepâncias no modo de atuação destes lasers e consequentemente, para que esta tecnologia possa ser empregada clinicamente com segurança, torna-se necessária a realização de novos estudos. Além disso, não esta estabelecido se a irradiação com o laser aumentaria a incorporação de flúor na superfície e consequentemente o seu efeito preventivo.

Dessa forma, os objetivos do presente estudo foram:

- Avaliar se a irradiação com o laser de CO_2 (λ=10.6 μm) acentua a incorporação de flúor no esmalte dentário desmineralizado.

- Avaliar se a irradiação com o laser de CO_2 laser com duas densidades de energia (11,3 J/cm^2 e 20,0 J/cm^2), combinado com aplicação de flúor fosfato acidulado (FFA) pode inibir a progressão da lesão em esmalte dentário desmineralizado.

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CAPÍTULO 1

CO_2 laser irradiation enhances CaF_2 formation and inhibits lesion progression on demineralized dental enamel – In vitro study.

Abstract

The aim of this study was to investigate if CO_2 laser irradiation enhances fluoride uptake by demineralized dental enamel and to evaluate whether enamel lesion progression can be inhibited by CO_2 laser (λ=10.6 μm) irradiation with two fluencies (11.3 J/cm^2 and 20.0 J/cm^2) combined with acidulated phosphate fluoride (APF) application. 315 specimens of carious enamel (SCE) were used in two experiments. In experiment 1, 150 SCE were allocated to 10 groups, which were irradiated combined with one APF application performed either before, during or after irradiation. Following, 50 specimens had their surface examined for morphological changes by scanning electron microscopy (SEM). Fluoride as CaF_2 formed on enamel surface was determined in 100 SCE (n=10, 10 groups). After CaF_2 analysis, a new SEM analysis was performed in 50 specimens. In experiment 2, 165 SCE (11 groups, n=15) were submitted to the same treatments as in experiment 1 + a caries lesion only group. After pH cycling, fluoride in demineralizing (Demin) and remineralizing (Remin) solutions, microhardness and lesion depth were determined. The results were analyzed by ANOVA and Tukey test and by Kruskal-Wallis rank test and Student-Newman-Keuls test (α=0.05). The results showed that irradiation alone or combined with APF enhanced CaF_2 formation on enamel surface, fluoride concentration in de-remin solutions and inhibited lesion progression ($p<0.05$). SEM observations showed evidences of melting, fusion and cracks. In conclusion, laser irradiation alone or combined with APF enhances CaF_2 uptake and inhibits lesion progression on demineralized dental enamel. However no synergic effect was found.

Introduction

Dental caries continues to be an oral health problem in most industrialized countries [Petersen et al., 2005]. Thus, the development of more effective methods to prevent dental caries is extremely important to control the disease [Rodrigues et al., 2004].

Studies over the past 30 years have indicated that lasers such as the carbon dioxide (CO_2), Nd: Yag and Argon can be used to thermally modify the chemical composition of dental enamel to render it more resistant to acid dissolution and potentially more resistant to dental caries [Stern et al., 1966; Gerard et al., 2005].

Carbon dioxide lasers were developed in 1964 by Patel et al. and they seem to be the most appropriate lasers for preventing dental caries. Studies carried out to evaluate the effect of a CO_2 laser on enamel and dentin structures showed its absorption by dental tissues to be high [Fried et al., 2002]. This is a result of the fact that the CO_2 laser produces radiation in the infrared region, which coincides closely with some of the apatite absorption bands, mainly phosphate and carbonate group absorption bands [Nelson et al., 1986]. During irradiation, chemical and morphological changes [Chiang et al., 2008; Fox et al., 1992; Moshonov et al., 2005., McCormack et al., 1995; Nomelini et al., 2009] can be induced in the irradiated dental enamel, thereby changing the susceptibility of its modified mineral content to organic acids in the oral environment [Rodrigues et al., 2006; Tepper et al., 2004].

The efficacy of CO_2 laser irradiation combined with fluoride in caries inhibition has been demonstrated by previous studies [Hsu et al., 2001; Nobre dos Santos et al., 2001; Tagliaferro et al., 2007; Steiner-Oliveira et al., 2008; Rodrigues et al., 2006; Esteves-Oliveira et al., 2011]. The irradiation combined with fluoride may produce numerous spherical or globular precipitates that morphologically resemble calcium fluoride-like deposits on enamel surfaces[Chin-Ying et al.,2004; González-Rodríguez et al., 2011]. These 'loosely-bound' fluoride precipitates may serve as a reservoir to replenish fluoride used up during periodic episodes of enamel demineralization. However, there are still very few studies that investigated the ability of CO_2 laser application to increase fluoride uptake by demineralized enamel and inhibit the progression of enamel mineral loss. Moreover,

most studies that evaluated the effects of CO_2 laser irradiation on enamel demineralization were performed with a prototype laser equipment at 9.6 µm wavelength that has 10 times higher absorption in enamel (8,000 cm^{-1}) than the 10.6 µm wavelength (825 cm^{-1}) [Zuerlein et al., 1999]. Thus, the purpose of this *in vitro* study was firstly, to investigate if CO_2 laser irradiation enhances CaF_2 uptake by demineralized enamel. Secondly, to evaluate after a cariogenic challenge, whether enamel lesion progression can be inhibited by CO_2 laser (λ=10.6 µm) irradiation with two fluences (11.3 J/cm^2 and 20.0 J/cm^2) alone or combined with acidulated phosphate fluoride (APF) application either before, during or after laser irradiation.

Materials and Methods

Experimental Design

This study was approved by the Research and Ethics Committee of the Piracicaba Dental School at University of Campinas in Piracicaba, SP, Brazil (Protocol No. 160/2009). Three hundred and fifteen dental enamel specimens were previously submitted to caries-like lesion formation. The demineralized enamel specimens (DES) were used in two experiments in the following way: One hundred and fifty specimens were employed in experiment 1 and 165 DES were used in experiment 2. In experiment 1, 150 enamel specimens were irradiated with a CO_2 laser at 10.6 µm with two energy densities (11.3 J/cm^2 and 20.0 J/cm^2) and received acidulated phosphate fluoride (APF) application alone and either before, during or after laser irradiation. Following, 50 specimens (10 groups, n=5) had their surface examined for morphological changes by scanning electron microscopy (SEM). Fluoride as CaF_2 formed on enamel surface by APF gel application alone and either before, during or after laser irradiation not subjected to the cariogenic challenge, was determined in 100 DES (10 groups, n=10,). After CaF_2 analysis, a new SEM analysis was performed in 50 specimens (10 groups, n=5). In experiment 2, 165 DES were submitted to the same treatments as in experiment 1 with the inclusion of a caries lesion only group (11 groups, n=15). After pH cycling, fluoride in Demin and Remin solutions were determined with an ion selective electrode. The inhibition of lesion progression was

determined by microhardness and lesion depth analyses. The factors under study were laser irradiation, topical fluoride application and moment of the fluoride application on demineralized dental enamel. The response variables in this study were calcium fluoride concentration on enamel surface, enamel mineral loss and lesion depth as well as fluoride concentration in the de/remineralizing solutions measured after pH cycling.

Tooth selection and Sample Preparation

To perform this *in vitro* study, 160 sound impacted human third molars used in this investigation were collected from adults living in Piracicaba, SP, Brazil. The teeth had more than two thirds of formed root, and were free of apparent caries, macroscopic cracks, and abrasions as well as staining, as assessed by visual examination. The teeth, stored in 0.1% thymol solution, were sterilized using gamma radiation (Rodrigues et al., 2004) and sectioned mesiodistally using a water-cooled diamond saw as well as a cutting machine (Isomet, Buehler, Lake Bluff, Ill., USA). The tooth halves were polished for 30 sec using a 5μm alumina/water suspension micropolish (Instrumental, Jabaquara, SP, Brazil) to expose fresh enamel. The slabs were coated with an acid-resistant varnish leaving a window of 4 mm^2 of exposed enamel in the middle third of both buccal and lingual surfaces.

Caries-Like Lesion Formation

Early caries lesions was performed in 315 enamel specimens according to Paes Leme et al. [2003]. Early caries lesions were produced in these specimens by individual immersion in an acetate buffer (6.25mL of solution/mm^2 of exposed enamel): 0.05 mol/L, pH 5.0, 50% saturated with hydroxyapatite (Gen-phos HA Hospitália Cirúrgica Catarinense Ltda., Florianópolis, SC, Brazil) for 48 h at 378C. The specimens were then randomly assigned to 10 groups in experiment 1 and to 11 groups in experiment 2.

Experiment 1

Determination of CaF_2- like Formed on Enamel

To perform experiment 1, 150 enamel specimens were irradiated with a CO_2 laser at 10.6 µm with two energy densities (11.3 J/cm^2 and 20.0 J/cm^2) and received acidulated phosphate fluoride (APF) application alone and either before, during or after laser irradiation. Initially, after being subjected to the treatments, 50 specimens (10 groups, n=5) had their surface examined for morphological changes by scanning electron microscopy (SEM) to investigate the effect of APF gel and laser applications before CaF_2 extraction. Fluoride as CaF_2 formed by APF gel application alone or either before, during or after laser irradiation was determined on 100 DES (10 groups, n=10) not submitted to the cariogenic challenge. After CaF_2 analysis, a new SEM analysis was performed in 50 specimens (10 groups, n=5) to evaluate the enamel physical changes after CaF_2 extraction. For CaF_2 analysis the treated enamel specimens, were individually immersed in plastic tubes containing 0.08 mL of 1.0 M KOH solution and gently agitated at room temperature for 24h, according to Calavaska, et al [1975]. After this period, the extracts were neutralized with 0.08 mL of TISAB II containing 1.0 M HCL and analyzed with standard solutions containing from 0.031 to 0,500 µg F/mL were used. The amount of "CaF_2" formed on the surface of each enamel specimen, was calculated and expressed as µg F/cm^2.

Scanning Electron Microscopy Analysis

After irradiation and fluoride application, morphological investigations were performed before and after CaF_2 extraction in order to verify the physical changes promoted by irradiation and fluoride application on the enamel surface. In both analyses, five specimens of each group were evaluated. Specimens were mounted on aluminum stubs and sputter coated with gold (~10-12nm thickness) by the BAL-TEC SCD 050 (Wetzlar, Liechtenstein/Vienna, Austria). SEM observations were made with a JEOL JSM-5600 LV Scanning Electron Microscope (Jeol, Peaboy, MA, USA) at 15 kV with magnifications up to X2000.

Experiment 2

In experiment 2, 165 demineralized enamel specimens were randomly assigned to 11 groups (n =15) as follows:

1. carious enamel only (control);
2. carious enamel + pH cycling (control);
3. carious enamel + APF + pH cycling;
4. carious enamel + laser (11.3 J/cm^2)+ pH cycling;
5. carious enamel + APF + laser (11.3 J/cm^2) + pH cycling;
6. carious enamel + laser (11.3 J/cm^2) APF + pH cycling;
7. carious enamel + APF + laser (11.3 J/cm^2 – during irradiation) + pH cycling;
8. carious enamel + laser (20.0 J/cm^2)+ pH cycling;
9. carious enamel + APF + laser (20.0 J/cm^2) + pH cycling;
10. carious enamel + laser (20.0 J/cm^2) + APF + pH cycling;
11. carious enamel + APF + laser (20.0 J/cm^2 – during irradiation) + pH cycling.

Fluoride Treatment

A single application of APF gel (Odahcam, Dentsply, Herpo, Petrópolis, RJ, Brazil) containing 1.23% F (NaF) at pH 3.5 was performed on the carious enamel specimens from groups 3, 5, 6, 7, 8 9, 10 and 11. Fluoride was applied to the slabs for 4 min alone and either before, after or during the laser treatment. The gel on the slab surfaces was removed with a cotton roll.

Laser Irradiation Parameters

Laser irradiation was carried out by scanning the exposed enamel of each specimen for approximately 30 s by manually moving the laser tip at a distance of five mm from the tip of the hand piece to the tooth. To perform enamel surface irradiation, a pulsed CO_2 laser at 10.6 μm wavelength (Union Medical Engineering Co. Model UM-L30, Yangju-si, Gyeonggi-Do, Korea) was used for irradiation with the following parameters: 10 ms pulse duration, 10 ms of time off, 50 Hz repetition rate, beam diameter of 0.3 mm.

Using a power meter (Scientech 373 Model-37-3002, Scientech Inc., Boulder, CO, USA) the average power outputs were measured and found to be 0.4 W and 0.7 W for the correspondent lased groups. Thus, the laser fluencies applied on enamel were 11.3 and 20.0 J/cm^2 respectively. A 10 mm distance from the tip of the hand piece to specimen was maintained during irradiation.

pH Cycling

The pH cycling model used in this study was based on the one described by Featherstone et al. [1986] and modified by Argenta et al. [2003]. Specimens from groups 2 to 11 were submitted to the pH-cycling regimen. Each slab was kept in a demineralizing (Demin) solution (6.25 mL/mm^2 exposed enamel) containing 2.0 mmol/L calcium, 2.0 mmol/L phosphate in 75 mmol/L acetate buffer pH 4.6 for three hours. They were then placed in a remineralizing (REmin) solution (3.12 mL/mm^2 exposed enamel) containing 1.5 mmol/L calcium, 0.9 mmol/L phosphate, and 150 mmol/L KCl in 20 mmol/L cacodylic buffer pH 7.0 for an average of 21 hours each day. Remin solutions were changed at 4th day of the pH cycling. The cycle was repeated for eight days. Between the demineralizing and remineralizing stages and at the end of the pH-cycling, the slabs were washed with deionized water for ten seconds and wiped with tissue paper. Both solutions contained thymol to prevent microbial growth. To provide a reference for initial mineral content loss, specimens from Group 1 (carious enamel only) were not submitted to the pH cycling regimen.

Determination of fluoride concentration in De-Remineralizing solutions

After pH cycling, fluoride concentrations in the Demin and Remin solutions (Remin 1- until 4th day and Remin 2- after 4th day) were analyzed on days three through six and seven through twelve. For this analysis, duplicate aliquots of the solutions were mixed with TISAB III at a ratio of 1:0.1. Fluoride concentrations measured immediately after preparation of the de- and remin solutions and before pH-cycling were 0.046 and 0.031 µg/mL, respectively. Fluoride determination was performed using an Orion 96-09 ion-selective electrode (Orion Research Inc., Boston, MA, USA) and an Orion EA-940 digital

ion-analyzer previously calibrated with standardized solutions (0,031 to 0,500 µg F/mL) and expressed as µg F/mL .

Cross-Sectional Microhardness Testing

After pH cycling, the specimens were longitudinally sectioned through the border of the exposed enamel. Each cut section was embedded in acrylic resin and serially flattened and polished. The hardness profile was determined using a Future Tech FM-ARS microhardness testing device (Future-Tech Corp., Tokyo, Japan) with a Knoop diamond under a 25-g load for five seconds. Thirty-six indentations (three rows of 12 indentations each) were made with the long axis of the Knoop diamond parallel to the outer enamel surface, maintaining a 10 µm interval between 10 and 60 µm and then a 20 µm interval from 60 to 180 µm across the lesion and into the underlying enamel. The mean hardness values of the three rows at each distance from the surface were then averaged and expressed as Knoop hardness number The mean values (kg/mm^2) at all measuring points of each distance from the surface were then averaged and the area of microhardness 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 [Cury et al., 2010].

Polarized Light Microscopy Analysis

After microhardness analysis, fifteen slabs from each group were cut from the middle of the exposed enamel window and 200 µm thick sections were obtained. For that, a water-cooled diamond saw as well as a cutting machine (Isomet, Buehler, Lake Bluff, Ill., USA) were used. Sections were then polished with 600 and 1200 grit-abrasive-paper to obtain a thickness of 100 ± 20 µm and then imbibed in water and standard X10 magnification photomicrographs were taken. The measurements of the enamel demineralization depth were determined by a previously calibrated examiner who was blinded to the experimental groups using a Leica DMLP polarized light microscope (Leica Microsystems Wetzlar, Germany) coupled to a Leica DFC 280 digital system and the

software Leica Application Suite (LAS). To determine lesion depth, 03 measurements were performed in the central area of each sample with a 100 µm of distance among them.

Statistical Analysis

The Kolmogorov-Smirnov test was performed to assess whether the distributions of the studied variables (tooth's mineral loss and fluoride concentration in the de-remineralizing solutions) deviated from normality. The differences among groups regarding mineral loss effects of the laser irradiations, combined or not with APF gel, expressed by enamel mineral loss (ΔS) and lesion depth as well as fluoride concentration as CaF_2 were evaluated by ANOVA followed by Tukey test and fluoride concentration in the de-remineralizing solutions by Kruskal-Wallis rank test followed by Student-Newman-Keuls test. Statistical analysis was performed using SPSS .13.0 (Statistical Package for Social Science Inc., Chicago, IL, USA) and BioEstat 5.0 (Mamirauá, Belém, PA, Brazil) with a 5% significance level.

Results

Experiment 1

The results obtained by CaF_2 determination in previously demineralized human dental enamel are shown in table 2 (experiment 1). From this table, it can be seen that the highest CaF_2 concentration was formed on enamel surface of specimens treated with CO_2 laser with 11.3 J/cm^2 + APF (during irradiation) ($p<0.05$). This table also demonstrates that in comparison with control group, except for laser 11.3 J/cm^2 and laser 20.0 J/cm^2 groups, all treatments significantly favored the CaF_2 formation on enamel surface ($p<0.05$).

The representative SEM observations of the enamel surfaces receiving fluoride treatment alone, combined fluoride-laser treatments, and control are shown in Figs. 1–8, respectively. Figure 1a shows normal untreated enamel surface (control). Figure 1b shows enamel surface treated with CO2 laser (11.3 J/cm^2). The enamel surface is thermally altered

and evidences melting and fusion. Figure 1c depicts a 20.0 J/cm^2 laser-treated surface, evidencing wider thermal alteration. Roughness and some craters are observed, compatible with heat fusion of the surface. Figure 2a depicts the enamel surface treated with APF alone, showing that before KOH treatment, spherical deposits which morphologically resemble calcium fluoride can be seen. After KOH treatment, no evidence of CaF_2 is visualized (Fig. 2b). From figures 3a – 8a it is clear that laser irradiation improved CaF_2 formation on the enamel surface weather fluoride was applied before, during or after laser irradiation. However, after extraction of CaF_2, no fluoride was observed in the SEM images. (Fig. 3b-8b)

Table 2. Fluoride concentration (mean ± SD; n = 10 – Experiment 1) and Enamel lesion depth and Mineral los, according to the groups (mean ± SD; n = 15 – Experiment 2).

Treatment groups	Experiment 1	Experiment 2	
	CaF_2,µg/cm^2	Lesion Depth, µm	ΔS, kg/mm^2 x µm
Carious lesion	———	55.3±03.9^a	7,211.3±1,026.3^a
Carious lesion + pH cycling	0.26±0.01^a	139.9±10.7^b	12,242.9±2,605.0^b
APF	1.99±0.10^b	58.8±06.9^a	7,398.5±1,593.0^a
Laser 11.3 J/cm^2	0.24±0.01^a	58.8±05.4^a	7,520.6±0,879.6^a
APF + Laser 11.3 J/cm^2	3.94±0.19^{bc}	59.8±11.3^a	7,799.8±1,552.9^a
Laser 11.3 J/cm^2 + APF	2.26±0.12^{bd}	59.8±09.0^a	7,869.6±1,001.1^a
Laser 11.3 J/cm^2+ APF (during irradiation)	5.87±0.27^c	59.2±12.2^a	7,703.8±1,924.8^a
Laser 20.0 J/cm^2	0.15±0.01^{ad}	57.4±11.1^a	7,717.7±2,211.4^a
APF + Laser 20.0 J/cm^2	2.54±0.16^b	57.6±09.4^a	8,085.7±1,267.4^a
Laser 20.0 J/cm^2+ APF	3.93±0.19^{bc}	55.7±06.1^a	7,570.0±1,831.9^a
Laser 20.0 J/cm^2 +APF (during irradiation)	3.04±0.13^{bc}	57.4±05.1^a	8,355.8±0,736.4^a

Means followed by distint letters are statistically different by ANOVA followed by Tukey test ($p<0.05$).
Note: Specimens from experiment 1 were not submitted to the pH-cycling regimen.

Experiment 2

Table 1 shows the fluoride concentrations in the de- and remineralizing solutions used during the pH-cycling used to simulate the dynamic of caries development. From this table it can be seen that except for groups laser 11.3 and 20.0 J/cm^2, all groups showed fluoride concentrations in demin solution significantly higher than that of control group ($p<0.05$). In the remin solution 1, the same behavior of fluoride increase was noticed. Table 1 also reveals that in respect to fluoride concentration in remin solution 2, no difference was observed among APF, laser 11.3 J/cm^2 + APF (during irradiation) as well as APF + laser 20.0 J/cm^2, laser 20 J/cm^2 + APF and laser 20.0 J/cm^2 + APF (during irradiation) groups and the control group. Moreover, a statistically significant decrease in fluoride concentration in remin solution 2 was observed in group laser 11.3 J/cm^2 while significantly higher concentration were found in groups APF + laser 11.3 J/cm^2 and laser 11.3 J/cm^2 + APF ($p<0.05$).

The severity of enamel lesion progression (table 2) determined by the area of microhardness loss (ΔS) showed that the groups treated with APF gel alone or either before, during or after laser irradiation had a statistically lower demineralization than the negative control group ($p < 0.05$). However the differences among them were not significant ($p > 0.05$)

Considering the quantitative polarized light microscopy analysis, Table 2 reveals that lesion depth of dental enamel ranged from 138.35 µm (control group – caries lesion + pH cycling) to 54.40 µm (laser 20.0 J/cm^2 + APF group). The results also shows that lesion depth was significantly shallower in all groups when compared with control group ($p<0.05$). Moreover, no difference was found between the caries lesion only group and all treatments ($p > 0.05$).

Table 1. Fluoride concentration (µg/mL) in the demin- and remineralizing solutions according to the treatments (mean ± SD).

Groups	Demineralizing solutions (µg F/mL)	Remineralizing solutions 1 (µg F/mL)	Remineralizing solutions 2 (µg F/mL)
Carious lesion + pH cycling	0.051±0.002^{a}	0.021±0.000^{a}	0.046±0.008^{ab}
APF	0.274±0.085^{b}	0.065±0.043^{ab}	0.043±0.009^{a}
Laser 11.3 J/cm^2	0.048±0.003^{a}	0.020±0.000^{a}	0.027±0.000^{c}
APF + Laser 11.3 J/cm^2	0.091±0.019^{c}	0.421±0.211^{c}	0.068±0.020^{d}
Laser 11.3 J/cm^2+ APF	0.088±0.017^{cd}	0.330±0.303^{cd}	0.079±0.046^{d}
Laser 11.3 J/cm^2 + APF (during irradiation)	0.087±0.022^{cd}	0.486±0.441^{cd}	0.065±0.030^{bde}
Laser 20.0 J/cm^2	0.004±0.002^{a}	0.019±0.000^{a}	0.016±0.000^{c}
APF + Laser 20.0 J/cm^2	0.069±0.011^{d}	0.127±0.077^{bd}	0.053±0.027^{ab}
Laser 20.0 J/cm^2 + APF	0.100±0.215^{c}	0.264±0.153^{cd}	0.048±0.020^{ae}
Laser 20.0 J/cm^2+APF (during irradiation)	0.090±0.021^{c}	0.291±0.180^{cd}	0.069±0.031^{bd}

Means followed by distint letters are statistically different by the Kruskal Wallis test followed by Student-Newman-Keuls test($p<0.05$).

Discussion and Conclusion

This study evaluated the ability of CO_2 application to increase fluoride uptake by enamel and inhibit enamel mineral loss. Fluoride has been proven to be an effective anticaries agent and CaF_2-like material is the major reaction product formed during F topical application on dental hard tissues. The formation of CaF_2 is important because it is well known that it interferes with de-and remineralizing phases of the carious process [Ten Cate, 1997], acting as a pH-controlled reservoir of fluoride ion on enamel or in dental plaque to be released during cariogenic challenges [Rolla et al., 1990; Ogaard., 2001]. In respect to CaF_2-like material formed on enamel surface (Table 2), the results of the present

study showed that combined therapies as well as fluoride application alone significantly favored the enhancement of CaF_2-like formation material on enamel surface ($p<0.05$). Moreover, the highest CaF_2-like material formation was found in laser 11.3 J/cm^2 + APF during irradiation group. A further analysis of these results reveals that with this laser condition, the CaF_2-like material formation was 22.6 times higher than in control group. This result is in line with Goodman and Kaufman [1977] who found a 14-fold increase in fluoride uptake on enamel powders after laser irradiation. However it should be noted that these authors employed an argon laser and a very distinct methodology. Additionally, with fluoride alone a 7.6 times higher CaF_2-like material formation was observed in relation to control group. Thus, compared to APF alone the combined therapy enhanced the CaF_2-like material formation in approximately 3 times. This high CaF_2-like material formation was also observed on SEM images of fluoride-laser treated enamel surfaces (Fig 3a-8a). On the surfaces treated by fluoride only, the deposits were considerably less than those on the fluoride laser treated enamel surface (Fig. 2a). Our results are in line with previous studies performed by González-Rodríguez et al. [2011], Chin-Ying et al. [2004] and Tepper et al. [2004]. In respect to the effect of laser irradiation in enhancing fluoride uptake two possible mechanisms are proposed. Since heat was found to enhance the uptake of fluoride, the thermal effect of the laser was speculated to be the main factor in promoting fluoride uptake [Goodman and Kaufman, 1977; Putt et al., 1978]. In line with this assumption, in the present study high concentrations of CaF_2-like material was found on enamel treated with fluoride before or during irradiation (Table 2). Secondly, laser-induced surface alteration, such as an increase in cracks and roughness, may also play a role in increasing fluoride uptake when fluoride is applied after irradiation [Zhang et al., 1996]. In this study, part of the CaF_2-like material formed on enamel surface could have been released from the tooth structure during an acid attack, and in turn facilitate inhibition of demineralization during the subsequent cariogenic challenge (pH cycling). This phenomenon probably happened in the present study since a statistically significant increase in fluoride concentrations in the de-mineralizing solutions when fluoride alone or the combined therapies were used (Table 1). It is interesting to note that the increase in CaF_2-like material formation is coincident with fluoride enhancement occurring in the demin solution. This

increase is probably a consequence of CaF_2 solubilization during the cariogenic challenge used in the present study [Ogaard, 2001]. This result partially explains the enamel demineralization reduction effect found in the present study since previous work by Fox et al. [1992] demonstrated that only 0.526 µmol/L of fluoride (0,01 ppm) in the demineralizing solution produced a 6 times higher reduction in enamel solubility after irradiation with CO_2 laser. According to these authors [Fox et al., 1992b], this is related to the fact that the pH threshold is lowered by 0.4 unit by this fluoride concentration. Regarding remin solutions 1, the combined therapies as well as fluoride alone significantly increased fluoride concentration (Table 1). Similar results were also found by Steiner-Oliveira et al. [2008]. However, a different pattern of fluoride concentration was noted in the remin solution 2. In this solution, only the APF applied before or after the lower energy density produced a significant increase in fluoride concentration.

After the *in vitro* simulation of a high cariogenic challenge the present study showed that carious enamel treated by CO_2 laser irradiation combined or not with APF application, was effective in inhibiting mineral loss in carious enamel (Table 2). This finding is in line with previously reported results [Esteves-Oliveira et al., 2009; Tepper et al., 2004; Fox et al., 1992; Hsu et al., 1998; Hsu et al., 2001; Steiner-Oliveira et al., 2006; Tagliaferro et al., 2007]. In addition, the finding that no difference in enamel mineral loss and lesion depth was observed between the caries lesion only group and all treatments indicates that isolated treatments as well as the combined laser and APF treatment inhibited further lesion progression promoted by the pH-cycling regime.

As for the mechanism of laser action, it remains unclear although reports of several related studies have been published. The most frequently mentioned hypothesis for laser effects states that caries inhibition is due to the melting and fusion of hydroxyapatite crystals [Ferreira et al., 1989; Tagomori et al., 1995]. In the present investigation the SEM images (Fig.1.b-c; 2.a-8.b) suggested that fusion and melting phenomena may be related to the inhibition of demineralization found in the irradiated groups. These findings are in agreement with the reports by Kantorowitz et al. [1998], Klein et al. [2005], McCormack et al. [1995] and Steiner-Oliveira et al [2006] whereby the energy densities were similar or higher than those used in the present study. These results may suggest that the enamel

surface was sealed by the laser irradiation together with the CaF_2 formed on enamel surface (Fig.3.a; 5.a; 6.a; 8.a).

In our study, the absence of statistical difference among groups 3,4,5,6,7,8,9,10 and 11 (Table 2) showed that there was no synergism between CO_2 laser and fluoride. This finding also observed for enamel lesion depth, is in accordance with Phan et al. [1999] and Tepper et al. [2004] who were also unable to show a synergistic effect between the association of CO_2 laser and amine fluoride and APF respectively. However, a synergistic effect between CO_2 laser and fluoride was demonstrated in other investigations, using different irradiation parameters and mainly employing a 9.6 µm CO_2, before or after fluoride application [Fox et al., 1992; Nobre-dos-Santos et al., 2001; Rodrigues et al., 2006]. The reason we found no synergism between laser irradiation and APF application is probably related to the fact that he 9.6 µm wavelength has an absorption in enamel that is 10 times higher (8,000 cm^{-1}) than the absorption of 10.6 µm (825 cm^{-1}) and has therefore been considered the most promising for use in caries prevention. However, the lower absorption of the 10.6 µm wavelength results in a higher penetration depth and can therefore affect a thicker enamel layer. Consequently, it has been suggested that most of the caries-preventive effect obtained with the 10.6 µm wavelength could be longer lasting. Moreover, the 10.6 µm laser line is the commercially available medical CO_2 laser.

As would be expected, in the present study the APF application inhibited enamel demineralization. This confirms previous evidence of the caries-inhibiting effect of fluoride gels [Marinho et al., 2011b]. However, no statistically significant difference was found between the fluoride and laser groups, showing that laser treatment may be a good alternative to prevent enamel demineralization in tooth surfaces where fluoride is apparently not as effective as it is on smooth surfaces, such as in surfaces adjacent to restorations, in the pit and fissures of occlusal surfaces regions and around orthodontic brackets.

In conclusion, our results showed that CO_2 laser irradiation, at λ 10.6 µm, alone or in combination with a single application of APF enhanced CaF_2-like material formation on enamel surface and inhibited demineralization progression in carious dental enamel.

However, there was no evidence for an additional effect when the enamel surface was treated with the combination of CO_2 laser and APF.

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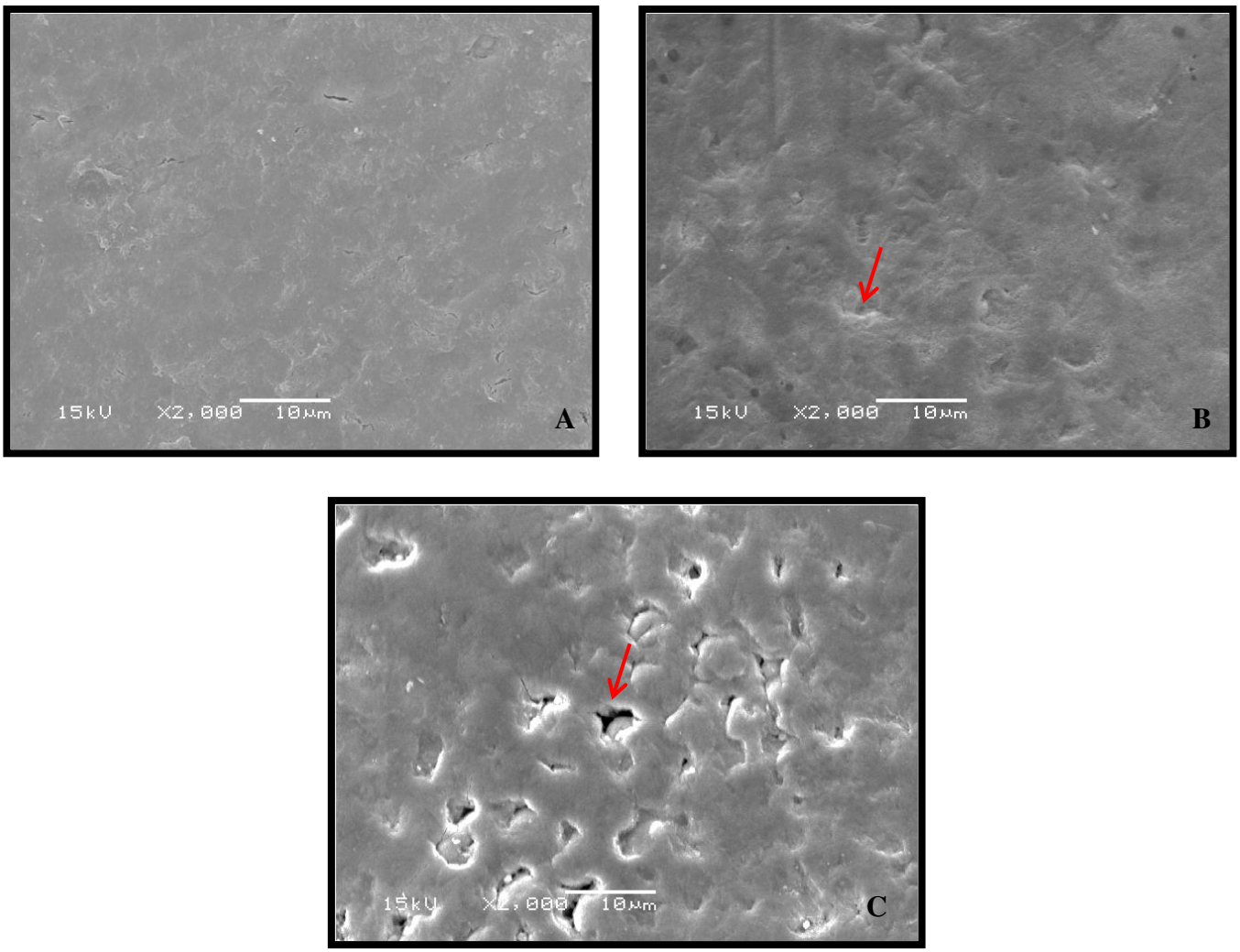

Fig. 1.a. Control Group. A representative SEM micrograph on enamel surface of the control sample (without any treatment).
Fig. 1.b. Laser 11.3 J/cm^2 Group. SEM micrograph after irradiation with 11.3 J/cm^2 on enamel. Surface alterations (melting and fusion) are evident.
Fig. 1.c. Laser 20.0 J/cm^2 Group. SEM micrograph after irradiation with 20.0 J/cm^2 on enamel. Roughness, some craters, melting and fusion are observed.

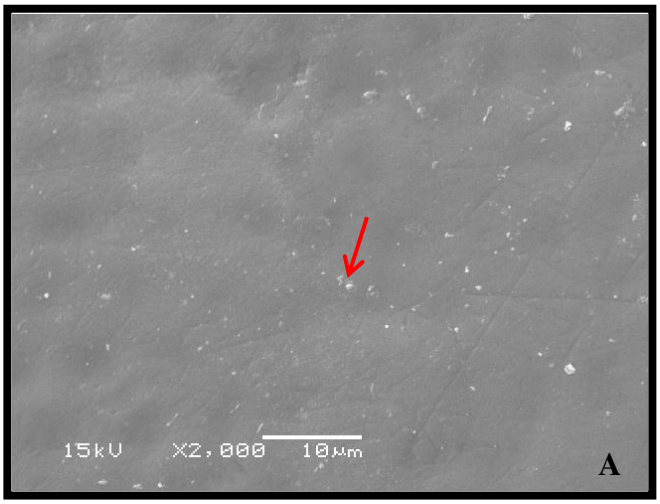

Fig. 2.a. APF Group. SEM micrograph after application of fluoride on enamel. Note the calcium fluoride-like deposits. The surface deposits are much less than that on fluoride-laser treated surfaces.

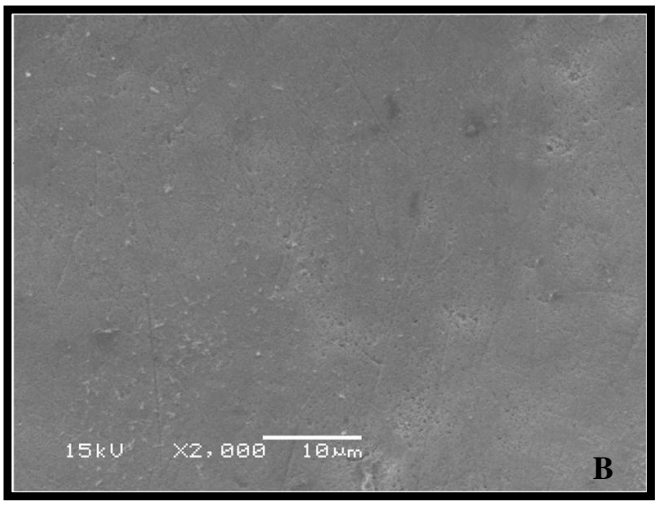

Fig. 2.b. APF Group. SEM micrograph after application of fluoride on enamel and subsequent KOH treatment. No deposits are seen.

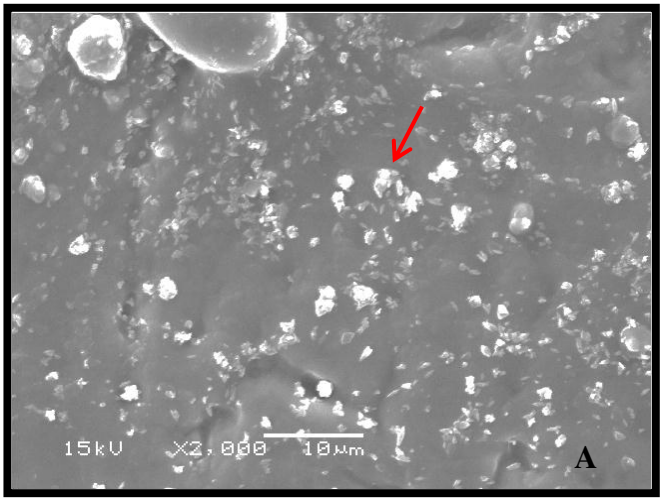

Fig. 3.a. APF+Laser 11.3 J/cm^2 Group. SEM micrograph after application of fluoride on enamel and irradiation with 11.3 J/cm^2. Note the calcium fluoride-like deposits.

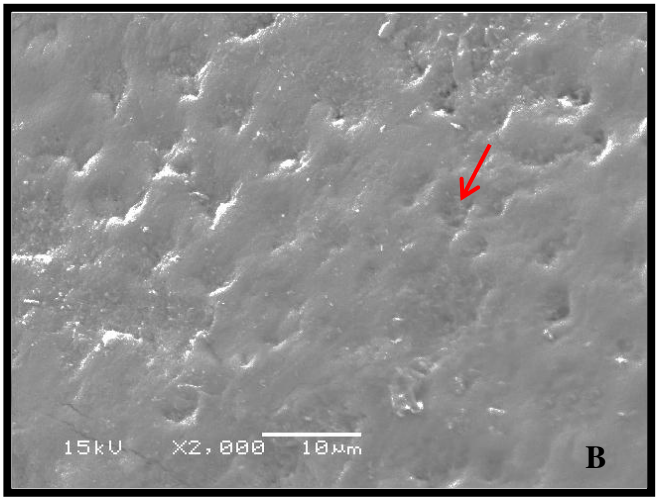

Fig. 3.b. APF+Laser 11.3 J/cm^2 Group. SEM micrograph after application of fluoride on enamel, irradiation with 11.3 J/cm^2 and subsequent KOH treatment. No deposits are seen only surface alterations (melting and fusion) are evident.

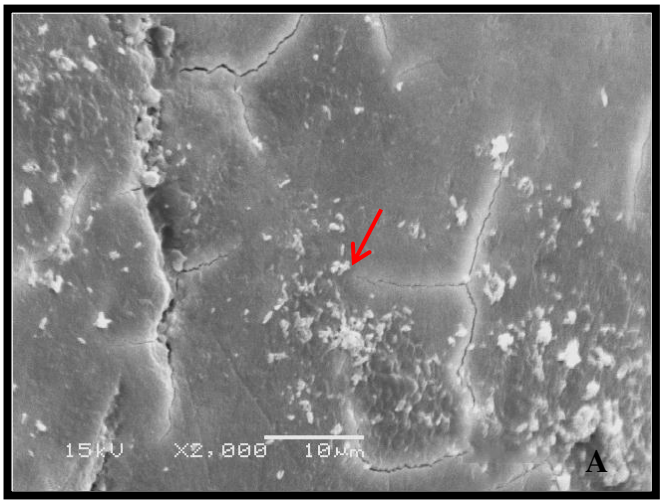

Fig. 4.a. Laser 11.3 J/cm^2 + APF Group. SEM micrograph after irradiation with 11.3 J/cm^2 on enamel and application of fluoride. Calcium fluoride-like deposits and cracks are observed.

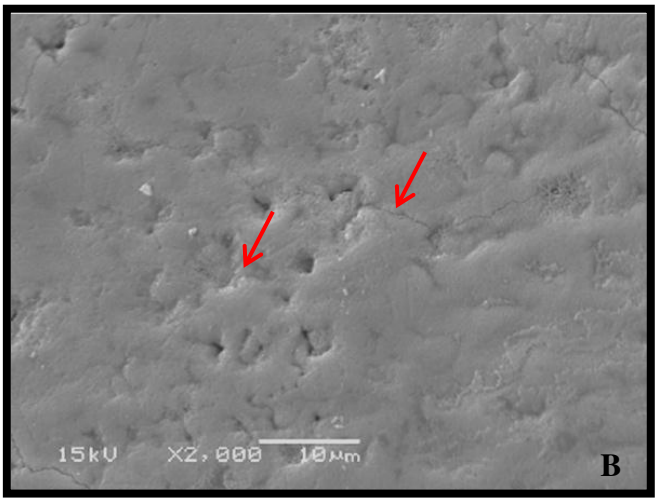

Fig. 4.b. Laser 11.3 J/cm^2 + APF Group. SEM micrograph after irradiation with 11.3 J/cm^2 on enamel, application of fluoride and subsequent KOH treatment. No deposits are seen. Melting, fusion and cracks are evident.

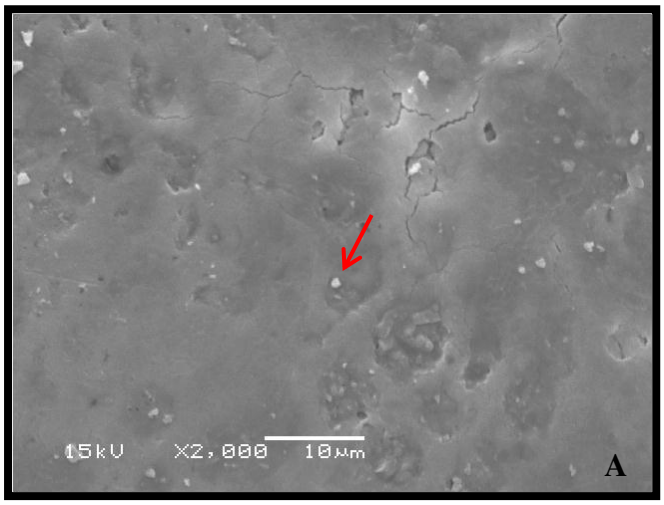

Fig. 5.a. Laser 11.3 J/cm^2 + APF Group. SEM micrograph after fluoride application during the irradiation with 11.3 J/cm^2. Note the calcium fluoride-like deposits and surface alterations (melting and fusion).

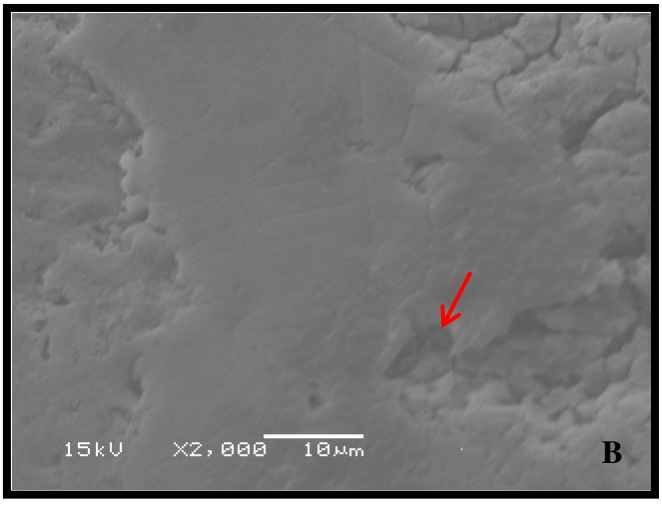

Fig. 5.b. Laser 11.3 J/cm^2 + APF Group. SEM micrograph after application of fluoride during the irradiation with 11.3 J/cm^2 on enamel and subsequent KOH treatment. No deposits are seen only surface alterations (melting and fusion) are evident.

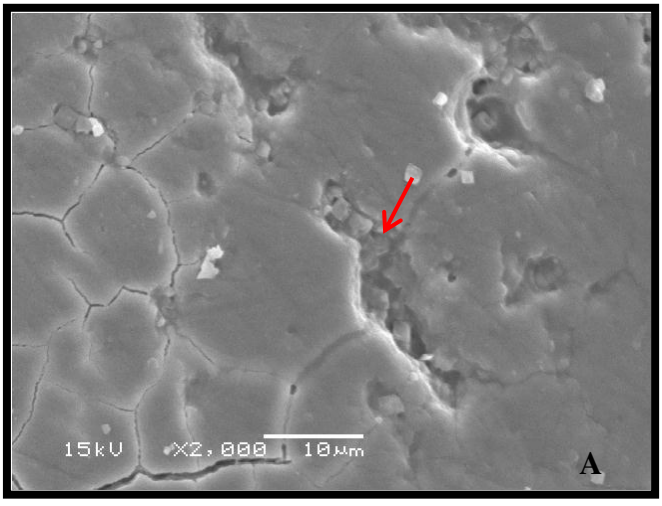

Fig. 6.a. APF+Laser 20.0 J/cm^2 Group. SEM micrograph after fluoride application and irradiation with 20.0 J/cm^2. Craters, cracks, melting, fusion and calcium fluoride-like deposits are observed.

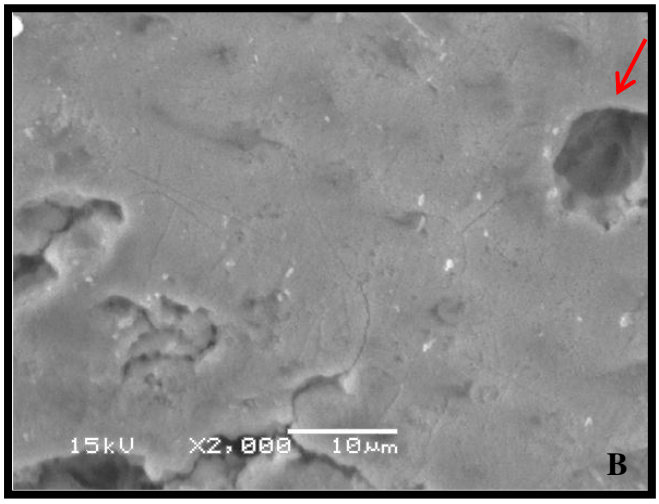

Fig. 6.b. APF+Laser 20.0 J/cm^2 Group. SEM micrograph after application of fluoride on enamel, irradiation with 20.0 J/cm^2 and subsequent KOH treatment. No deposits are seen, only melting and fusion on surface are evident.

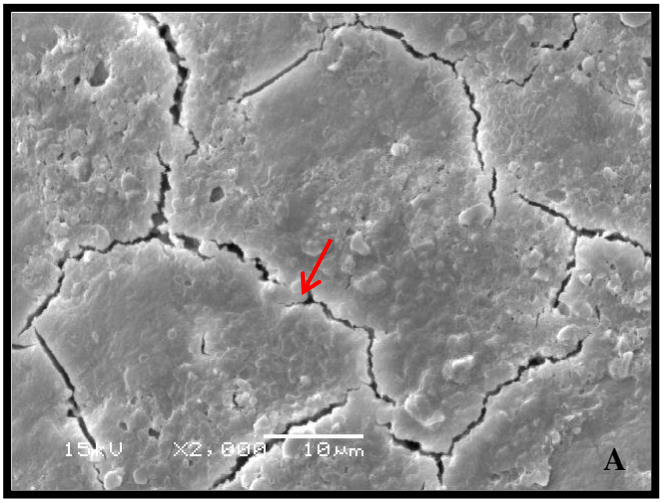

Fig. 7.a. Laser 20.0 J/cm^2 + APF Group. SEM micrograph after irradiation with 20.0 J/cm^2 on enamel and application of fluoride. Cracks, melting, fusion and calcium fluoride-like deposits are observed.

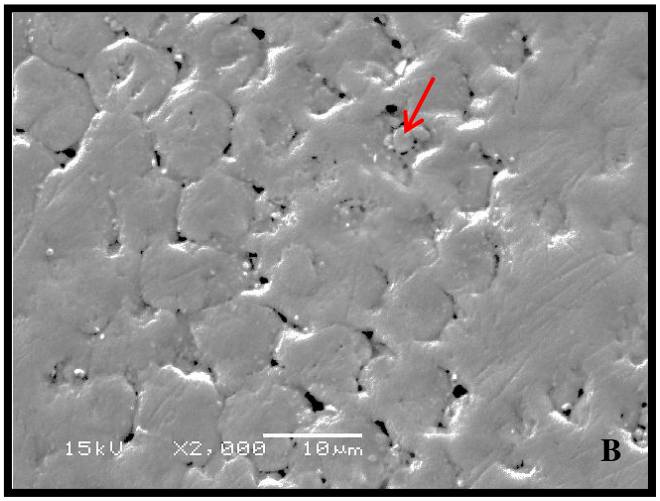

Fig. 7.b. Laser 20.0 J/cm^2 + APF Group. SEM micrograph after irradiation with 20.0 J/cm^2 on enamel, fluoride application and subsequent KOH treatment. No deposits are seen. Melting and fusion on surface are evident.

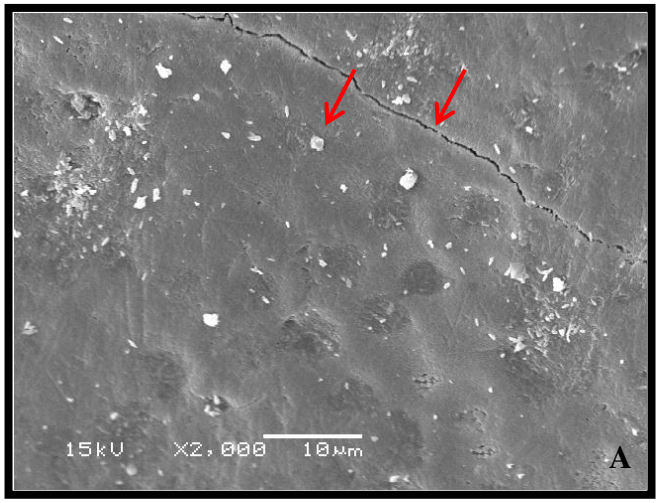

Fig. 8.a. Laser 20.0 J/cm^2 + APF Group. SEM micrograph after fluoride application during the irradiation with 20.0 J/cm^2. Cracks, melting, fusion and calcium fluoride-like deposits are observed.

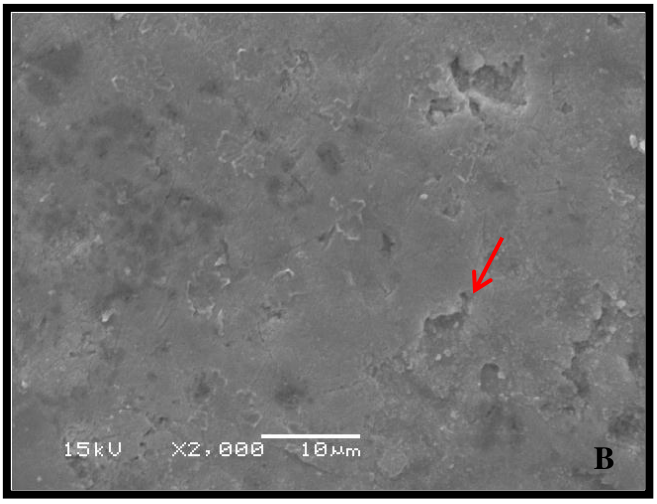

Fig. 8.b. Laser 20.0 J/cm^2 + APF Group. SEM micrograph after fluoride application during the irradiation with 20.0 J/cm^2 and subsequent KOH treatment. No deposits are seen, only melting and fusion on surface are evident.

CONCLUSÕES GERAIS

Os resultados encontrados no estudo realizado permitem-nos concluir que:

- A irradiação com o laser de CO_2 (λ10.6 μm) isolada ou combinada com o flúor fosfato acidulado acentua a incorporação de CaF_2 no esmalte dentário desmineralizado.

- A irradiação com o laser de CO_2 laser com duas densidades de energia (11,3 J/cm^2 e 20,0 J/cm^2), combinado com aplicação de flúor fosfato acidulado (FFA) inibe a progressão de lesão de cárie no esmalte dentário desmineralizado. No entanto nenhum efeito sinérgico foi encontrado.

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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 **"Avaliação dos efeitos do fluoreto fortemente e fracamente ligados na efetividade do laser de CO2 em reduzir a desmineralização e aumentar a remineralização do esmalte dentário - Estudos in vitro"**, protocolo nº 160/2009, dos pesquisadores Marinês Nobre dos Santos Uchôa e Bruna Raquel Zancopé, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 09/12/2009.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Evaluation of the strong and weakly on effect of the fluorid in the effectiveness of the CO2 laser in reducing the demineralization and increasing the remineralization of the dental enamel - Studies in vitro"**, register number 160/2009, of Marinês Nobre dos Santos Uchôa and Bruna Raquel Zancopé, 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 12/09/2009.

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.

Anexo 2

Comprovante de submissão do artigo

Marines Nobre

De: <david.beighton@kcl.ac.uk>
Data: sexta-feira, 24 de fevereiro de 2012 14:39
Para: <nobre@fop.unicamp.br>
Assunto: Caries Research - Manuscript ID CRE-2012-Feb-00041
24-Feb-2012

Dear Prof. Nobre dos Santos:

Your manuscript entitled "CO2 laser irradiation enhances CaF2 formation and inhibits lesion progression on demineralized dental enamel – In vitro study." has been successfully submitted online and is presently being given full consideration for publication in Caries Research.

Your manuscript ID is CRE-2012-Feb-00041.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your street address or e-mail address, please log in to Manuscript Central at http://mc.manuscriptcentral.com/cre and edit your user information as appropriate.

You can also view the status of your manuscript at any time by checking your Author Center after logging in to http://mc.manuscriptcentral.com/cre .

Thank you for submitting your manuscript to Caries Research.

Yours sincerely,

Prof. David Beighton
Caries Research

david.beighton@kcl.ac.uk

Apêndice 1

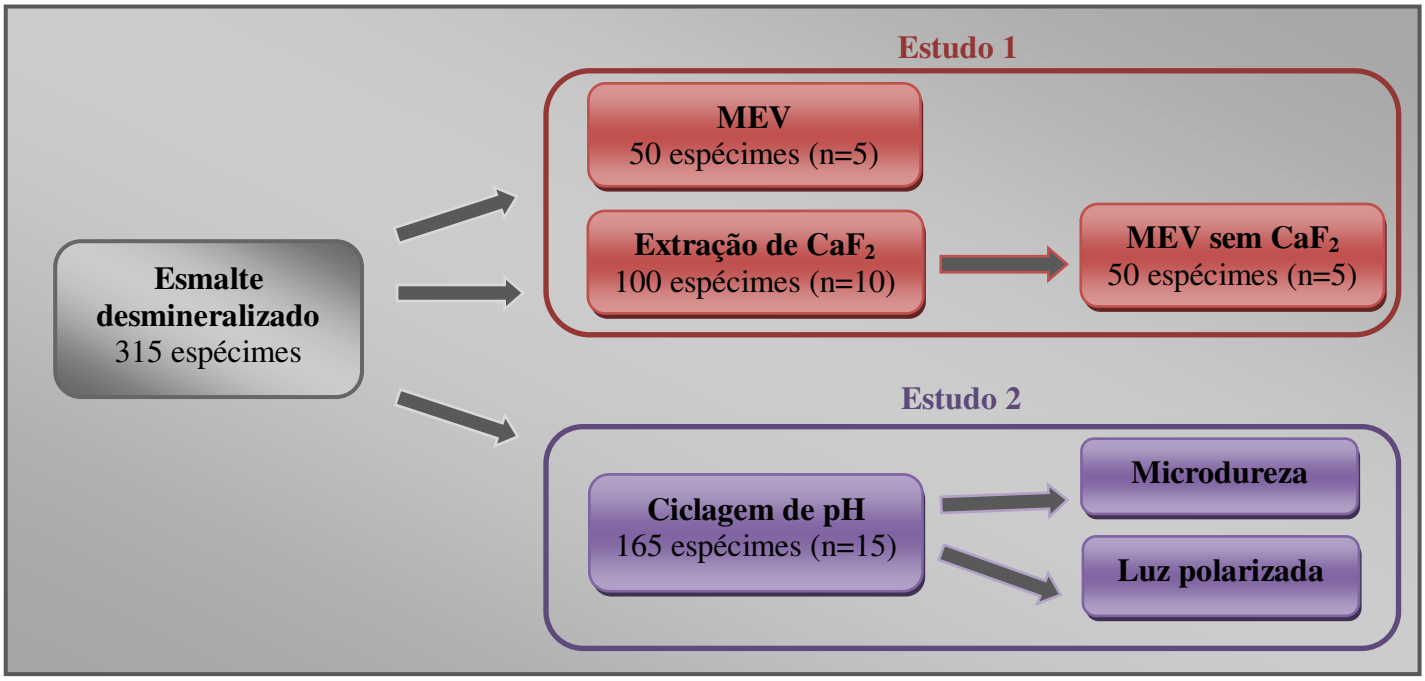

Figura 1. Esquema ilustrativo do delineamento experimental

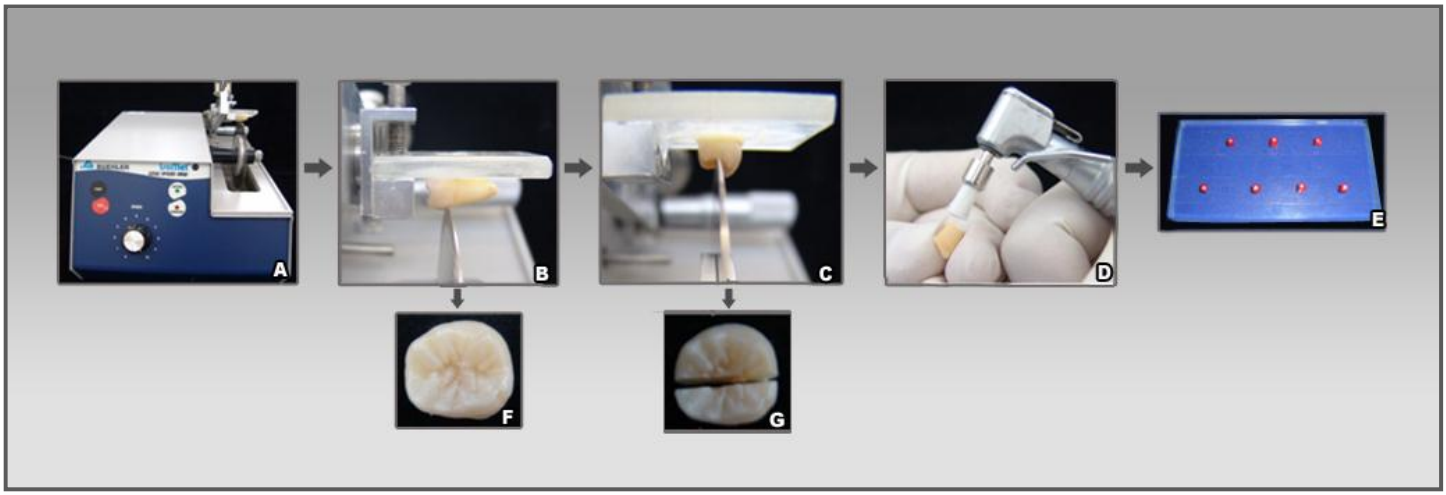

Figura 2. Preparo dos espécimes; **A.** Cortadeira Isomet. Buehler. Lake Bluff. Ill.. USA; **B** e **F.** Corte e remoção da porção radicular; **C e G**. Secção longitudinal da coroa no sentido mésio distal; **D.** Profilaxia do espécime; **E.** Delimitação da área exposta para ser submetida aos testes.

Apêndice 2

Figura 1. A. Esquema ilustrativo da produção de lesão de cárie; **B.** Estufa para acondicionamento dos tubos.

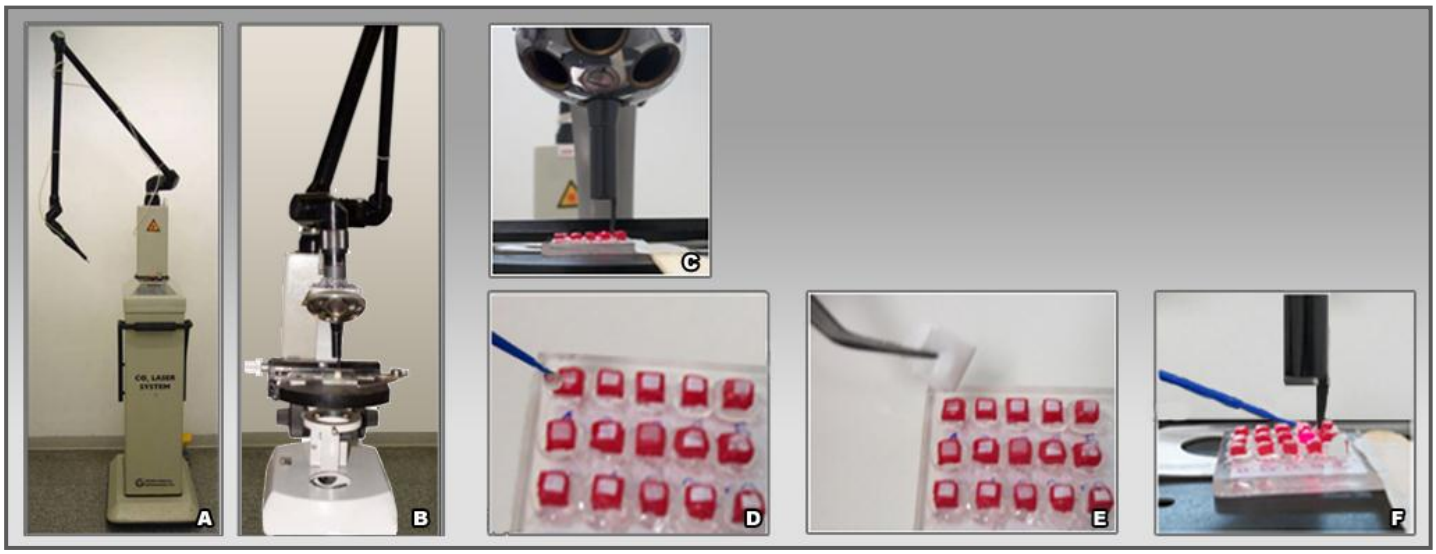

Figura 2.A. Laser de CO_2; **B.** Laser de CO_2 acoplado ao microscópio para varredura da área de esmalte; **C.** Irradiação dos espécimes com laser de CO_2; **D.** Aplicação de flúor; **E.** Remoção dos excessos de flúor**; F.** Aplicação de flúor durante a irradiação do espécime com laser de CO_2.

Apêndice 3

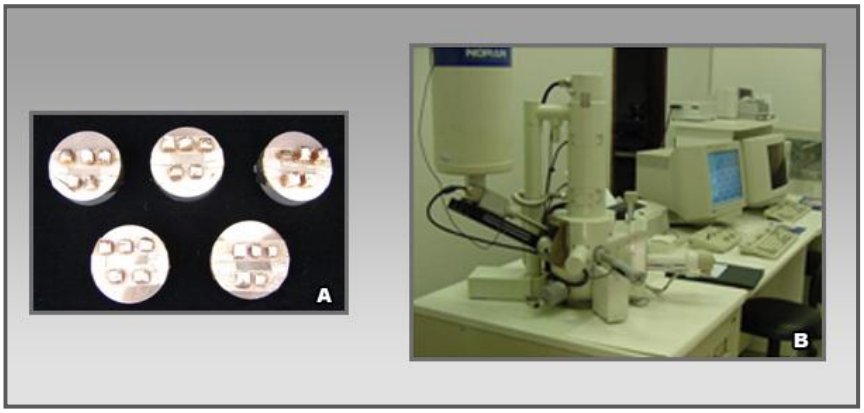

Figura 1. A. Espécimes após serem metalizados para a microscopia eletrônica de varredura; **B.** Microscópio Eletrônico de varredura

Figura 2.A Analisador de ions acoplado com Eletrodo íon-seletivo Orion 96-09.

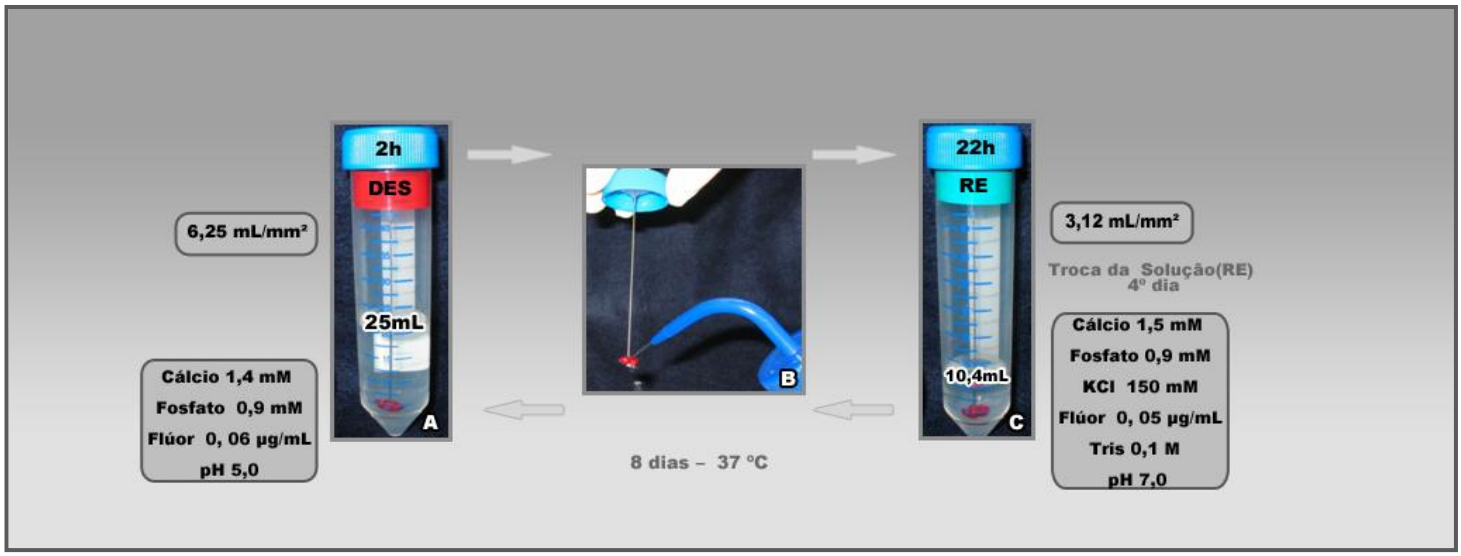

Figura 2. Esquema ilustrativo de ciclagem de pH; **A.** Tubo de ciclagem com solução desmineralizadora; **B.** Lavagem com água deionizada na troca de soluções na ciclagem; **C.** Tubo de ciclagem com solução remineralizadora.

Apêndice 4

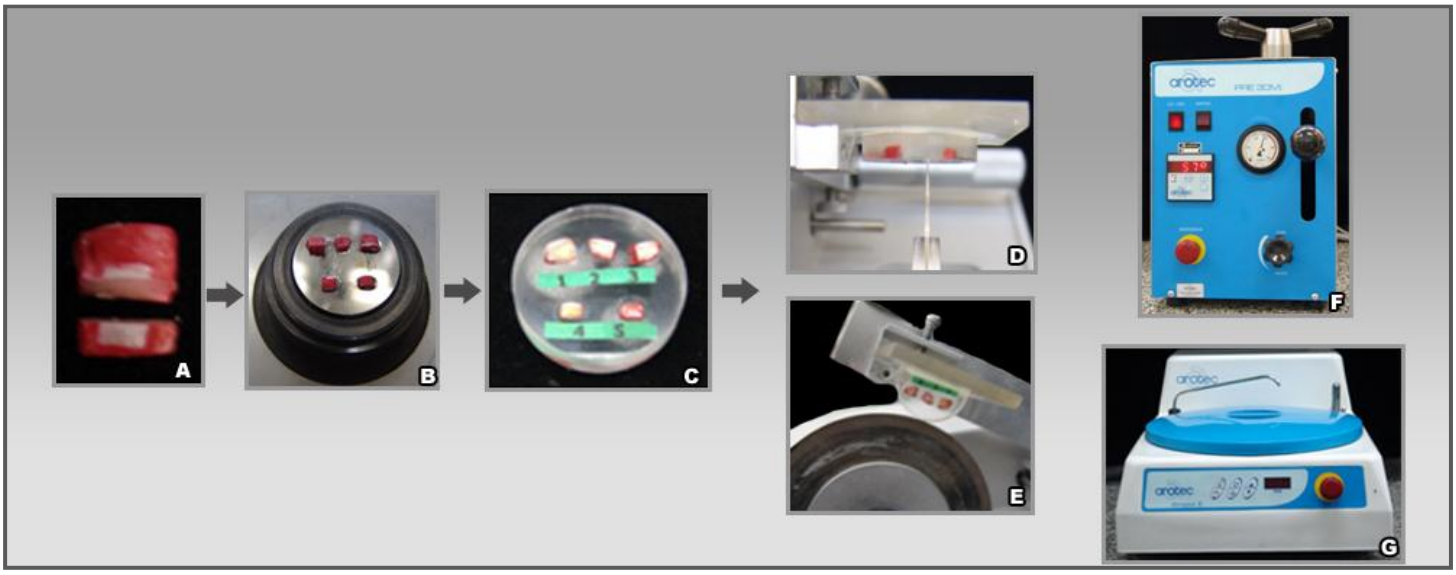

Figura 1.A. Espécime após o corte da área delimitada; **B.** Espécimes posicionados na embutidora; **C.** Aspecto final após os espécimes serem embutidos em resina acrílica; **D.** e **E.** Corte dos espécimes embutidos em fatias para análise de luz polarizada; **F.** Embutidora Arotec; **G.**Politriz Arotec.

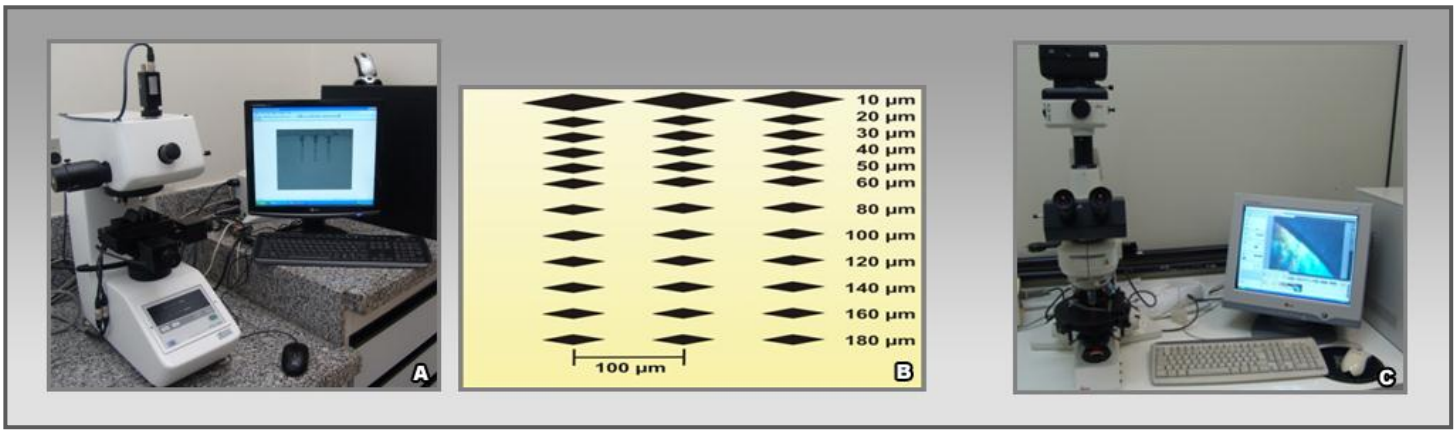

Figura 2.A. Microdurômetro HMV-2 Shimadzu hardness tester (Shimadzu Corporation. Kyoto. Japan); **B.** Ilustração representativa das impressões realizadas nos espécimes durante a análise de microdureza; **C.** Microscópio de luz polarizada Leica Microsystems Wetzlar. Germa