Universidade Estadual de Campinas

Faculdade de Odontologia de Piracicaba

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"EFEITOS DO LASER DE CO_2 NA DESMINERALIZAÇÃO DO ESMALTE AO REDOR DE BRAQUETES ORTODÔNTICOS"

Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, para obtenção do título de Mestre em Odontologia, Área de Odontopediatria.

Orientadora: Profa. Dra. Marinês Nobre dos Santos Uchôa

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"A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original".

[Albert Einstein]

RESUMO

A aplicação do laser de dióxido de carbono (CO_2) à estrutura do esmalte modifica a composição química e/ou morfológica dessa superfície e inibe o desenvolvimento e a progressão de lesões cariosas. Porém, não foram realizadas pesquisas que tenham verificado se a irradiação do esmalte dental ao redor de braquetes ortodônticos com esse laser é efetiva em reduzir a desmineralização nessa região numa situação de alto desafio cariogênico. Assim, esta dissertação objetivou verificar, *in vitro,* se a irradiação do esmalte dental com laser de CO_2 (λ =10.6 μm e 10.0 J/cm^2), associada ou não a liberação de fluoreto pelo material de colagem, seria capaz de reduzir a perda mineral do esmalte, ao redor de braquetes ortodônticos, quando submetido a uma situação de alto desafio cariogênico. Nesse estudo, 24 blocos de esmalte bovino foram divididos em 4 grupos, em triplicata: 1 – resina composta não liberadora de fluoreto Transbond (T); 2 – cimento de ionômero de vidro modificado por resina Fuji (F); 3 – laser de CO_2 + resina composta não liberadora de fluoreto (TL); 4 – laser de CO_2 + cimento de ionômero de vidro modificado por resina (FL). Um grupo contendo blocos de esmalte foi incluído apenas para análise de microdureza. Após a colagem dos braquetes, os espécimes foram suspensos em água destilada deionizada esterilizada e esterilizados com radiação gama. A seguir, foram transferidos para o meio de cultura esterilizado de caldo de cérebro-coração (BHI) contendo sacarose a 5% e os 4 grupos experimentais foram inoculados com uma cultura overnight de *Streptococcus mutans*. Diariamente, o meio BHI foi trocado e analisado quanto à contaminação microbiológica. Após 6 dias de incubação (37^{o}C - 10% CO_2), o biofilme foi coletado e submetido as análises microbiológica (UFC/mg) e bioquímica. Além disso, microdureza do esmalte seccionado longitudinalmente foi determinada. Os dados foram analisados pelos testes ANOVA e Tukey, com alfa a 5%. As concentrações de polissacarídeo insolúvel em água (μg/mg) no biofilme foram: T – 213,206(±421,746)^a, F – 111,208(±43,501)^a, TL – 124,626(±37,488)^a e FL – 138,83(±118,893)^a. As concentrações de cálcio (μg/mg) foram: T – 340,5(±27,01)^a, F – 329,5(±143,97)^a, TL – 412,3(±228,80)^a e FL – 411,8(±252,59)^a. As concentrações de fluoreto (μg/mg) no biofilme foram: T – 0,001(±0,005)^a, F – 0,010(±0,021)^a, TL – 0,0009(±0,002)^a e FL – 0,002(±0,007)^a. As

concentrações de fósforo (µg/mg) foram: T – 0,162(±0,134)[a], F – 0,149(±0,066)[a], TL – 0,170(±0,104)[a] e FL – 0,148(±0,029)[a]. Os resultados (expressos 10^7 UFC/mg) obtidos da análise microbiológica foram: T – 2,54(±2,58)[a], F – 2,90(±3,08)[a], TL – 2,59(±3,13)[a] e FL – 2,30(±4,04)[a]. A média numérica da microdureza knoop (kg/mm^2) variou de 195,5(±87,3)[c], 209,8(±75,0)[bc], 218,2(±113,6)[ab] e 229,1(±82,7)[a] para os grupos T, F, TL e FL, respectivamente. Em conclusão, esse estudo demonstrou que o uso do laser de CO_2 (λ =10.6 µm e 10.0 J/cm^2) sozinho ou combinado com o material de colagem liberador de fluoreto foi capaz de reduzir a perda mineral do esmalte ao redor de braquetes ortodônticos, quando submetidos a uma situação de alto desafio cariogênico com biofilme de *Streptococcus mutans*.

Palavras-chave: Laser de CO_2, esmalte bovino desmineralizado, braquetes ortodônticos, fluoreto, desafio microbiológico.

ABSTRACT

The application of carbon dioxide laser (CO_2) on dental enamel structure modifies the chemical and/or morphologic composition of this surface and inhibits the development and progression of caries lesion. However, no research verified the if the irradiation of dental enamel around orthodontic brackets was able to reduce the enamel mineral loss in this region in a high cariogenic challenge. Thus, this dissertation aimed to verify *in vitro*, if the irradiation of the dental enamel with a laser of CO_2 (λ =10.6 µm and 10.0 J/cm^2), associated or not with fluoride released from a bonding material, will be able to reduce the enamel mineral loss around orthodontic brackets, when submitted to a high cariogenic challenge situation. In this study, twenty four enamel slabs were divided into 4 groups in triplicate: 1. nonfluoride-releasing composite resin Transbond (T); 2. resin-modified glass ionomer cement Fuji (F); 3. CO_2 laser + nonfluoride-releasing composite resin (TL); 4. CO_2 laser + resin-modified glass ionomer cement (FL). One group with 6 specimens of sound enamel were used to determine the sound enamel microhardness. After brackets bonding, the specimens were immersed in sterile deionized distilled water and sterilized with gamma radiation. Following, the specimens were transferred to a sterile brain-heart infusion broth (BHI) with a 5% sucrose solution and the 4 experimental groups were inoculated with an overnight culture of *Streptococcus mutans*. The BHI medium was daily changed and analyzed to check for microbiological contamination. After 6 days of incubation (37^{o}C - 10% CO_2), the biofilm was collected and submitted to microbiological (CFU/mg) and biochemical analyses. Additionally, microhardness assay of the enamel longitudinally sectioned was determine. The data were statistically analyzed by the ANOVA and Tukey' tests, with an alpha of 0.05. The concentrations of water-insoluvel polysaccharide (µg/mg) in biofilm were: T – 213.206(±421.746)^a, F – 111.208(±43.501)^a, TL – 124.626(±37.488)^a e FL – 138.83(±118.893)^a. The concentrations of calcium (µg/mg) were: T – 340.5(±27.01)^a, F – 329.5(±143.97)^a, TL – 412.3(±228.80)^a e FL – 411.8(±252.59)^a. The concentrations of fluoride (µg/mg) in biofilm were: T – 0.001(±0.005) ^{a}, F – 0.010(±0.021)^a, TL – 0.0009(±0.002)^a e FL – 0.002(±0.007)^a. The concentrations of phosphorus (µg/mg) were: T – 0.162(±0.134)^a, F – 0.149(±0.066)^a, TL –

0.170(±0.104)[a] e FL – 0.148(±0.029)[a]. The results (expressed 10^7 CFU/mg) obtained of microbiological analysis were: T – 2.54(±2.58)[a], F – 2.90(±3.08)[a], TL – 2.59(±3.13)[a] e FL – 2.30(±4.04)[a]. The mean knoop microhardness number (kg/mm^2) varied from 195.5(±87.3)[c], 209.8(±75.0)[bc], 218.2(±113.6)[ab] and 229.1(±82.7)[a] for T, F, TL and FL respectively. In conclusion, the present study demonstrated that the use CO_2 laser (λ =10.6 μm and 10.0 J/cm^2) alone or combined with the release of fluoride by bonding material was capable of reducing the enamel mineral loss around the orthodontic brackets, when submitted to a high microbiological cariogenic challenge with a *Streptococcus mutans* biofilm.

Key Words: laser/CO_2; demineralized bovine enamel; orthodontic brackets; fluoride, microbiological challenge.

SUMÁRIO

INTRODUÇÃO GERAL

Pacientes que fazem uso de aparelhos ortodônticos fixos apresentam alto risco de desenvolvimento de áreas de desmineralizacão do esmalte comumente chamadas de lesões de mancha branca (Gorelick *et al*., 1982; Mizrahi, 1982; Ogaard *et al*., 1988; Ogaard, 1989). Este achado clínico tem sido apontado como um dos riscos do tratamento ortodôntico. A lesão de mancha branca é considerada a precursora da lesão de cárie cavitada e tem sido atribuída ao acúmulo e retenção prolongada de biofilme na superfície do esmalte adjacente ao aparelho ortodôntico fixo (Darling, 1956; Darling, 1956; Mizrahi, 1982). A cárie é conceituada atualmente como uma doença biofilme-açúcar dependente. O biofilme formado na presença da sacarose tem uma matriz rica em polissacarídeos extracelulares (PECs)(Cury *et al*., 2000) e apresenta uma diminuição da concentração de cálcio, fósforo e fluoreto (Cury *et al*., 1997; Paes Leme *et al*., 2004; Pecharki *et al*., 2005). Os PECs insolúveis conferem maior porosidade ao biofilme dental (Dibdin e Shellis, 1988), o que permite a penetração de substratos acidogênicos para as camadas mais internas do biofilme (Zero *et al*.,1986). Diante desse fato, o controle da dieta, um controle mecânico efetivo do biofilme e a fluorterapia devem ser utilizados para prevenir a desmineralização do esmalte dentário ao redor de braquetes ortodônticos (Zachrisson, 1975; Shannon, 1981; O´Reilly *et al*., 1987). No entanto, estas medidas preventivas dependem da cooperação dos pacientes, o que compromete sua efetividade (Shannon, 1981; Geiger *et al*., 1992). Assim, devido à ação dos cimentos ionoméricos liberadores de fluoreto como carregadores passivos para a liberação do fluoreto na interface braquete-esmalte sem a necessidade da cooperação do paciente, estes cimentos, juntamente com o tratamento tópico de fluoreto, tem sido utilizado pelos ortodontistas para prevenir a lesão de mancha branca (Cohen *et al.,* 2003).

Vários tipos de cimentos liberadores de fluoreto têm sido introduzidos no mercado com a finalidade de colagem ortodôntica, porém, o mais utilizado é o cimento de ionômero de vidro modificado por resina (Komori *et al.,* 2003). Entretanto, o fluoreto tem um efeito parcial já que o mesmo não consegue impedir completamente o desenvolvimento da lesão de cárie. Nesse sentido, a modificação da estrutura do esmalte dental (hidroxiapatita) da superfície ao redor do braquete ortodôntico pela redução de seu

conteúdo de carbonato e fosfato decorrente da aplicação do laser de CO_2, poderia representar uma estratégia mais efetiva na prevenção da cárie nesta região.

Os estudos realizados nos últimos 10 anos demonstraram que a irradiação do esmalte dental com o laser de CO_2 torna o mesmo mais resistente ao desenvolvimento da cárie (Featherstone *et al.*, 1998; Kantorowitz *et al.,* 1998; HSU *et al.,* 2000; Klein *et al.*, 2005; Rodrigues *et al.,* 2006*;* Steiner-Oliveira *et al.*, 2006; Steiner-Oliveira *et al.*, 2008; Tagliaferro *et al.,* 2007). O laser de CO_2 é o mais apropriado para a aplicação no esmalte dentário, pois a radiação emitida pelo mesmo encontra-se na região do espectro do infravermelho e coincide com as bandas de absorção da hidroxiapatita, principalmente os grupamentos fosfato e carbonato (Nelson & Featherstone, 1982; Featherstone & Nelson, 1987).

Segundo Featherstone (2000), os comprimentos de onda mais indicados para uso na prevenção de cárie 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 comercial disponível que possa produzir tais condições, de modo que as pesquisas realizadas com estes parâmetros utilizaram protótipos (Featherstone *et al*., 1998; Young *et al*., 2000; Featherstone *et al*., 2001; Nobre dos Santos *et al*., 2001, 2002). Conseqüentemente, em busca de simplificação, e aproveitamento da tecnologia já existente, muitas pesquisas têm empregado o comprimento de onda 10,6 µm (Kantorowitz *et al*., 1998; Hsu *et al*., 2000, 2001; Klein *et al*., 2005).

Além da obtenção de um tecido mais resistente à dissolução ácida ao redor do braquete, outra possível vantagem do emprego do laser para prevenção de cárie quando do uso de aparelho ortodôntico fixo é o aumento da retenção do material de colagem ao esmalte irradiado. Este procedimento tem sido empregado com o objetivo de evitar os efeitos adversos do condicionamento ácido e aumentar a adesão da resina (Walsh *et al*., 1994; Shahabi & Walsh, 1996). Adicionalmente, existe a possibilidade de combinar-se o tratamento com laser com a liberação de fluoreto dos materiais de cimentação e colagem, o que poderia promover um sinergismo de efeitos na inibição da desmineralizacão ao redor dos braquetes.

Sendo assim, a utilização da tecnologia laser associada à colagem dos braquetes com cimento de ionômero de vidro em indivíduos portadores de aparelhos ortodônticos fixos poderia ser um recurso preventivo efetivo, com a vantagem de não depender somente da cooperação do paciente, além de ser um método indolor e não invasivo.

Porém, a análise da literatura evidencia que não foram realizadas pesquisas que tenham verificado se a irradiação do esmalte dental ao redor dos braquetes ortodônticos com o laser de CO_2 é efetiva em reduzir a perda mineral do esmalte nessa região numa situação de alto desafio cariogênico.

Desta forma, o objetivo do presente trabalho foi verificar se a irradiação do esmalte dental com laser de CO_2 (λ =10.6 μm e 10.0 J/cm^2) , associada ou não ao material de colagem liberador de fluoreto, seria capaz de reduzir a perda mineral do esmalte, ao redor de braquetes ortodônticos, quando submetido a uma situação de alto desafio cariogênico.

CAPÍTULO

Esta tese está baseada na Resolução CCPG/001/98/UNICAMP que regulamenta o formato alternativo para teses de Mestrado e Doutorado e permite a inserção de artigos científicos de autoria ou co-autoria do candidato (Anexo 1). Assim sendo, esta tese é composta de um capítulo contendo o artigo, conforme descrito abaixo:

✓ Capítulo

"CO_2 laser and bonding materials reduce enamel demineralization around orthodontic brackets." Souza-e-Silva CM, Steiner-Oliveira C, Parisotto TM, Rodrigues LKA , Kamiya RU, Nobre-Dos-Santos M. Este artigo foi submetido à publicação no periódico *Journal of Biomedical Materials Research. Part B, Applied Biomaterials.*

CO_2 laser and bonding materials reduce enamel demineralization around orthodontic brackets

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Abstract

Aim: To determine whether a CO_2 laser in association with fluoride released from a bonding material could reduce enamel demineralization around orthodontic brackets subjected to cariogenic challenge. *Material and Methods*: 24 bovine enamel slabs were randomly divided into four groups in triplicate: non-fluoride releasing Transbond composite resin (T – Control group), resin-modified Fuji glass ionomer cement (F), CO_2 laser + Transbond (TL), and CO_2 laser + Fuji (FL). Slabs were submitted to a 5-day microbiological caries model. The *Streptococcus mutans* biofilm that formed on the slabs was biochemically and microbiologically analyzed, and the enamel Knoop hardness number (KHN) around the brackets was determined. The data were analyzed by ANOVA and Tukey tests ($\alpha = 0.05$). *Results*: Biochemical and microbiological analyses revealed no statistically significant differences among the groups. Groups T, F, TL and FL showed KHN means (±SD) of $195.5(\pm87.3)^{c}$, $209.8(\pm75.0)^{bc}$, $218.2(\pm113.6)^{ab}$ and $229.1(\pm82.7)^{a}$, respectively. *Conclusion*: The use of a CO_2 laser (λ=10.6 µm; 10.0 J/cm^2) with or without F-bonding materials was effective for inhibition of demineralization around orthodontic brackets subjected to a cariogenic challenge. However, there was no evidence to suggest an additional effect when the enamel was treated with the combination of CO_2 laser and F-releasing material.

Keywords: laser/CO_2; demineralized bovine enamel; orthodontic brackets; fluoride, microbiological challenge.

Running Heads: CO_2 laser/fluoride effect in demineralization around brackets.

INTRODUCTION

Control of enamel demineralization around brackets is a major problem in orthodontic therapy with fixed intraoral appliances; such demineralization arises as a consequence of biofilm accumulation on the enamel and increased cariogenic challenge due to increased difficulty in maintaining oral hygiene.[1-6] White spot lesions occur in 50% of orthodontic patients[2,5] and can be considered a preliminary clinical sign of cavitated carious lesions.[7] Fluoride therapy via fluoridated dentifrices, fluoridated oral rinses, or topical fluoride application can be a valuable tool for preventing or reducing white spot lesion development in patients with orthodontic appliances.[8-12] However, this type of treatment requires patient compliance.[9,13] White chalky spot lesions to a depth of 75 μm can develop in 4 weeks. This is a shorter period than the typical interval between orthodontic appointments (6 to 10 weeks). Thus, although topical fluoride application has been recommended during this period to inhibit lesioning, treatment is not always maintained throughout the orthodontic intervention.[5] Manufacturers have therefore incorporated fluoride into orthodontic bonding cement to prevent or reduce enamel demineralization around the brackets.[6,14-16]

The material of choice for orthodontic fluoride release is conventional glass ionomer cement. However, its low adhesive strength and difficulty of manipulation present limitations to clinical use.[17,18] Orthodontists therefore use resin-modified glass ionomer cement for orthodontic bonding.[15] *In vitro* and *in vivo* studies have shown that resin-modified glass ionomer cement was more effective than composite resin for reducing enamel demineralization around the brackets,[6,16,19] indicating that therapies that do not depend on patient compliance could be effective for high caries risk individuals.[20] In this regard, application of carbon dioxide (CO_2) lasers with or without resin-modified glass ionomer cement can be used to prevent white chalky spot lesions around brackets in orthodontic treatment.

Several studies have demonstrated both *in vitro* and *in situ* that CO_2 lasers may be used to change the chemical composition and the enamel surface morphology to inhibit the rate of subsurface demineralization in enamel.[21-33] However, no studies have

investigated the effects of CO_2 lasers associated with fluoride-releasing orthodontic bonding materials on enamel demineralization around orthodontic brackets.

Thus, the aim of this study was to determine whether irradiation of enamel with a CO_2 laser in association with the application of fluoride-releasing bonding materials could decrease demineralization around orthodontic brackets subjected to cariogenic challenge.

MATERIALS AND METHODS

Experimental Design

The *in vitro* study used a randomized design in triplicate; enamel slabs were randomly allocated by a lottery method.[34] The factors under evaluation were bonding materials [resin-modified glass ionomer cement (F) and resin composite (T)] and CO_2 laser irradiation [Lased (L) and Unlased], resulting in four experimental groups: T, F, TL, and FL. All groups were inoculated with a cell suspension of *Streptococcus mutans* prepared from overnight growth of a pure culture. A biofilm subsequently formed over a five day period. Each group comprised 6 enamel slabs tested in triplicate, resulting in a total of 18 experimental units (n = 18). To verify the cariogenicity of the microbiological model used in this study, one control group without *Streptococcus mutans* inoculation was included for each triplicate but the results were only used for cross-sectional microhardness analysis.

After microbiological cariogenic challenge, enamel demineralization was assessed by cross-sectional microhardness analysis. The number of viable microorganisms and the concentration of water-insoluble polysaccharides, calcium, inorganic phosphorus and fluoride in the biofilm formed over the enamel slabs were analyzed.

Enamel Slab Preparation

Ninety bovine incisors free from structural defects were selected for this study.[35] After selection, the teeth were stored in a supersaturated 0.1% thymol solution at 4°C for 30 days.[36,37] Ninety enamel slabs (6 x 6 x 2 mm) were obtained from the buccal surface of each tooth using a water-cooled diamond saw and a cutting machine (Isomet 1000; Buehler, Lake, Bluff, IL, USA). The enamel slabs surfaces were polished for 30 seconds with 1 µm alumina paste and water. The slabs were then coated with an acid-resistant varnish, leaving

a 14.54 mm^2 window of exposed enamel for laser irradiation and the microbiological cariogenic challenge.

Laser Irradiation of Enamel Slabs

Irradiation of groups FL and TL was accomplished using a pulsed CO_2 laser at a wavelength of 10.6 μm (Model UM-L30, Union Medical Engineering Co., Yangju-si, Gyeonggi-Do, Korea), pulse duration 10 ms, rest cycle 10 ms, repetition rate 50 Hz, beam diameter 0.3 mm, and laser power of 0.7 W. The average power output was measured using a power meter (Scientech 373 Model-37-3002, Scientech Inc., Boulder, CO, USA) and found to be 0.7 W. Thus, the laser fluency applied to the enamel was approximately 10.0 J/cm^2. [32]

Each enamel slab was irradiated for approximately 10 seconds by manual scanning movement of the laser tip at a distance of 10 mm from the tip of the handpiece to the slab. The movement covered the boundary of the enamel area where the bracket would be placed.

Bracket Bonding Procedure

The brackets (Ref. 10.15.208, Dental Morelli, Sorocaba, SP, Brazil) were bonded to the enamel slabs with the direct bonding technique. A bracket clamp (Ref. 75.01.022, Dental Morelli, Sorocaba, SP, Brazil) was used to hold and keep the brackets in position on the center of the enamel surface. The bonding materials were applied to the bracket base and pushed against the enamel surface. The bonding materials employed were a composite resin (Transbond XT™; 3M Unitek, Monrovia, CA, USA) and a resin-modified glass ionomer cement (Fuji Ortho LC™; GC América Inc, Chicago, IL, USA). The manufacturers' recommendations were strictly followed for each material used. Excess adhesive around the bracket bases was removed with a clinical probe and the material was then light cured.

Sterilization Procedure

After being fixed with orthodontic wires previously tied to lids from a glass recipient, six slabs from each group were immersed in 50 mL of sterile deionized distilled water. In order to avoid contamination with non-experimental bacteria, samples were

irradiated at the Agricultural Nuclear Energy Center-University of São Paulo with gamma radiation (GAMMACELL 220 EXCEL (GC-220E)) at a sterilization dose of 14.5 kGy.[38]

Microbiological Caries Model

After sterilization, the enamel slabs of all groups were submitted to a bacterial caries model. These groups were transferred to glass recipients containing 35 mL of sterile brain-heart infusion broth (Difco™, 237500, Becton, Dickinson and Company Sparks, MD 21152 USA, Lot No. 6213021) and 5% sucrose (w/v).[37] The glass recipients of all groups except the control group were inoculated with a cell suspension of *Streptococcus mutans* (TCC 3440) at 1-2 X 10^8 CFU/mL that was prepared from an overnight growth pure culture. Groups were then incubated for 24 h at 37°C and a partial 10%-pressure CO_2. This inoculation was performed only once on the first day; on every subsequent day, the enamel slabs were washed twice with 0.9% sterile saline solution to remove loosely bound material of the enamel structure. They were also transferred to new culture medium each 24 h.[39] Every day, the contamination and turbidity of the media were determined. The biofilms formed on the slabs were analyzed on the sixth day. Slabs were washed twice in 0.9% sterile saline solution[40] and only biofilm located on the enamel surface was collected with a sterile plastic curette. The collected material was placed in two pre-weighed microcentrifuge tubes (one for chemical analyses and the other for microbiological counting), and the biofilm was then weighed using an analytical balance.

Biofilm Analysis

Microbiological Analysis

A 0.9% (w/v) NaCl (0.1 mL mg^{-1}) solution was added to biofilms stored in one of the microcentrifuge tubes. Microcentrifuge tubes were then sonicated (Ultrasonic Processor UP 400S, Hielscher Technology, Teltow, Germany) for 15 s at amplitude of 20% and 0.5 cycle in order to obtain suspensions of single cells.[41] These suspensions were serially diluted, and 1:1000000, 1:10000000 and 1:100000000 cells were plated with 25 µl drops in triplicate [42] onto BHI agar. Cultures were incubated at 37°C for 48 h in a partial 10%-pressure CO_2. After incubation, the number of *Streptococcus mutans* was determined by colony counting, and the values were expressed as colony forming units (CFU) per milligram of wet biofilm.[39]

Chemical Analyses

Water-insoluble polysaccharide analysis

A 1 M sodium hydroxide solution was added to the biofilm (0.1 mL/ mg^{-1}). The samples were homogenized and kept under constant agitation for 3 h at room temperature, and then centrifuged for 3 min at 12000 g. The concentration of water-insoluble polysaccharide in the supernatant was determined using the phenol-sulfuric method.[43]

Calcium analysis

Calcium concentration in the samples was determined by spectrophotometry in an automatic microplate reader (Molecular Devices, VersaMax Program, Sunnyvale, California, USA) with a calcium sensitive reagent (Arsenazo III).[44] The reader was previously calibrated with standard solutions (0.02 mM Ca to 0.40 mM Ca). The absorbance was set at 650 nm and the values obtained were expressed as µg Ca/mg dry weight biofilm.

Fluoride analysis

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 that were previously calibrated with various standard solutions (0.025 to 2.00 µg F/mL). The standard solutions were prepared in TISAB II at pH 5.0 (20 g NaOH/L) and 1 M HCl. The readings were expressed in millivolts (mV) and then transformed to µg F/mL through linear regression of the calibration curve. The results were expressed as µg F/mg of dry weight biofilm.

Inorganic phosphorus analysis

The inorganic phosphorus (P_i) concentration was determined colorimetrically [45] in an automatic microplate reader (Molecular Devices, VersaMax Program, Sunnyvale, California, USA) that had been calibrated with standard solutions (0.6 to 9.6 µg P/mL). The readings were performed at 660 nm and the results expressed as to µg P_i /mg of dry weight biofilm.

Cross-sectional Microhardness Analysis

At the end of each experimental period, the enamel slabs were longitudinally sectioned through the centre of the orthodontic brackets. One of the halves was embedded

in acrylic resin, and the cut surface was serially polished with an aluminum oxide disk of 400, 600 and 1200 grain, as well as a 1 µm diamond paste (Buehler). Two sequences of indentations were made. The first sequence of indentations was placed 10 µm from one end of the brackets while the second was placed 10 µm away from the other end. Fifteen indentations were then made at depths of 15, 20, 25, 30, 35, 45, 50, 55, 60, 85, 100, 125, 150, 175 and 200 µm from the outer enamel surface in a V-shaped pattern (Figure 1).[16] The distance between each indentation was 100 µm. The hardness profile was determined using a Shimadzu HMV-2 microhardness tester (Tokyo, Japan) and a Knoop diamond indenter under a 25 g load for 5 s. The data were expressed as Knoop hardness number since there are two different relationship between hardness and volume percent mineral in the literature.[46,47]

Statistical Analysis

The data were checked for equal variance and normal error distribution, and then calcium and CFU data were transformed to $\log_{10}$. Statistical analysis was performed using SAS software (SAS Institute Inc., version 8.01, Cary, NC, USA), with the significance level set to $p < 0.05$. For validation of the microbial model, all groups including the control were compared using analysis of variance (ANOVA) followed by Tukey's test. The same tests were then used to assess differences between treatments for microbiological, chemical and cross-sectional microhardness analyses.

RESULTS

The mean Knoop hardness number (KHN) values for the experimental groups were significantly lower than the values for control group without *S mutans* inoculation (305.61 ± 51.12, $p<0.05$). Table 1 shows that groups TL and FL presented the highest KHN values and that these treatments showed a statistically stronger effect than that observed for group T (control group). The lowest KHN value was found for group T; however this value was not significantly different from the value for group F. A significant difference was found between groups FL and F, whereas TL and F presented statistically similar KHN values (Table 1).

Table 2 summarizes the biochemical and microbiological composition of dental plaque samples; no statistically significant differences were found.

DISCUSSION

Inhibition of caries around orthodontic brackets is an important clinical issue since this kind of lesion occurs in 50% of orthodontic patients.[2,5] This study was designed to investigate the effects of enamel irradiation with a CO_2 laser in association with fluoride-releasing bonding materials on the inhibition of enamel demineralization around orthodontic brackets. An *in vitro* microbiological caries model was chosen for use in this study since brackets make the patient dental hygiene more difficult, thereby facilitating biofilm accumulation.[48] Microbiological caries models are more suitable than chemical models due to more clinically relevant biofilm accumulation around orthodontic brackets. Our data demonstrate that the microbiological model used in the present study was cariogenic to bovine dental enamel because the KHN values of the treated slabs were significantly decreased.

Although there is consensus that the use of human teeth is more relevant for demineralization studies, bovine teeth have also been used for *in vitro* microbial models.[39] The use of bovine instead of human teeth is advantageous because bovine teeth are easier to obtain and to manipulate.[49] They also have a relatively more uniform chemical composition, which gives rise to less variation in the experimental response to cariogenic treatments.[49]

The results of the present study demonstrate for the first time that 10.6 μm CO_2 laser irradiation could inhibit enamel demineralization around orthodontic brackets after *in vitro* microbiological cariogenic challenge. However, the efficacy of CO_2 laser irradiation for inhibition of demineralization of the enamel surface has been well-established for *in vitro* [21-28, 30, 32, 33] chemical models and *in situ* [29, 30] caries.

Our results show that CO_2 laser irradiation alone or in combination with a fluoride-releasing bonding material could significantly inhibit demineralization around orthodontic brackets in bovine enamel, since TL and FL groups presented high KHN values that statistically differed from the control group (T). In addition, the significant difference

between groups FL and F (table 1) suggests that the combination of CO_2 laser treatment and a fluoride-releasing bonding material has a greater effect than the fluoride-releasing bonding material alone. Moreover, although F and TL were not significantly different, the irradiated groups tended to perform better than the group receiving the fluoride-releasing material alone (table 1). The favorable results in the lased groups may be have been promoted by changes to the enamel surface that could cause decomposition of carbonated hydroxyapatite, transforming it to a less soluble hydroxyapatite phase that is more resistant to acid dissolution.[30,32,50]

Significant differences in the inhibition of demineralization were not found between T and F or TL and FL, indicating that the effect of fluoride was not as evident as that of the laser. These results were supported by chemical analyses that also showed a lack of significant differences in the dental biofilm fluoride levels between these groups (table 2). This contrasts with several previous studies that compared non-fluoride releasing composite resin with resin-modified glass ionomer cement and observed a significant decrease in enamel mineral loss around orthodontic brackets for fluoride-releasing material.[6,15,16] This difference may be partially explained by the dental sterilization process used in the present study; to avoid any non-experimental microbial contamination, all enamel slabs were immersed in sterile deionized distilled water and submitted to gamma radiation for 24 h. Because deionized distilled water has a pH of around 5.5-6 and contains much less fluoride than resin-modified glass ionomer cement, some fluoride may have leached into the water used for storage.[51] Furthermore, according to Lin *et al.*[52], McNeill *et al.*[53], and Carvalho and Cury [54] most fluoride is released during the first day. To our knowledge, this does not affect the relevance of the obtained results since inhibition of caries for longer than 24 h is expected for any type of preventive approach. Nevertheless, most studies performed using fluoridated products may show greater inhibition of caries by the glass ionomer due recharging of the fluoride by refluoridation.[52,55]

Microbiological analyses showed no significant differences among experimental groups inoculated with *Streptococcus mutans*. This result indicates that the fluoride released from the bonding material was unable to inhibit the growth of *Streptococcus mutans*, which is in line with the findings of van Dijken JW *et al.*[56], who observed no

relationship between fluoride releasing capacity and the inhibition of bacteria. Moreover, studies by Montanaro *et al.*[57] and Chin *et al.*[58] showed that materials used for bonding did not reduce the adherence of bacteria to the teeth.

No significant differences among groups were observed for levels of water-insoluble polysaccharides, calcium or inorganic phosphate. Extracellular polysaccharides, calcium and inorganic phosphorus present in the dental biofilm matrix play an important role in the caries process; polysaccharides facilitate streptococcal adherence [59] to the biofilm and increase its porosity, thereby enhancing acid diffusion to the enamel surface.[60] In the present research, the lack of significant differences in biochemical factors among experimental groups may be explained by the use of identical amounts of sucrose for cariogenic challenge; this disaccharide serves as the specific substrate for insoluble polysaccharide production and it is known that in the presence of carbohydrates, low concentrations of Ca, F and P_i are found in dental biofilm.[61]

Under the conditions of this study and considering the results, we conclude that CO_2 laser irradiation at $\lambda = 10.6$ μm either alone or in combination with the release of fluoride by the bonding material can inhibit demineralization of enamel around orthodontic brackets *in vitro*. However, there was no evidence of an additional effect when the enamel was treated with a combination of CO_2 laser and fluoride-releasing material. Further *in situ* and *in vivo* studies are suggested.

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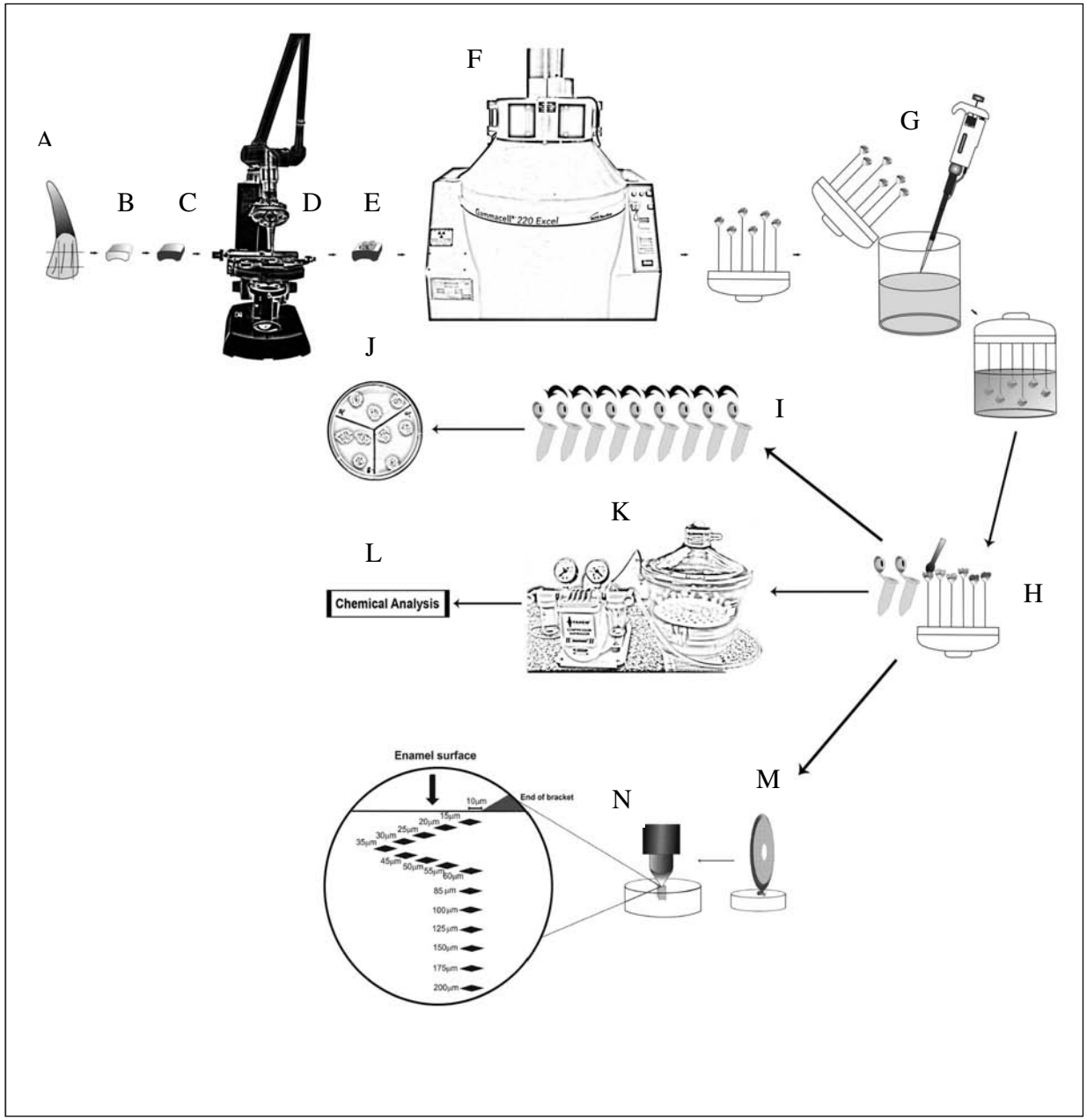

Figure 1. A – Bovine incisor. B – Enamel slabs. C – Enamel slabs coted with acid-resistant varnish. D – Laser irradiation. E – Enamel slabs with brackets. F – Sterilization Procedure. G – Microbiological caries model. H – Collection of biofilm. I – Dilution of biofilm suspension. J – Bacterial plating. K – Biofilm dehydration. L – Chemical analyses. M – Longitudinal section of enamel slabs. N – Schematical representation of microhardness.

Table 1. Mean Knoop microhardness value for each treatment.

Groups	Knoop Hardness Number Means (± SD*)
T (Control)	195.56 ± 87.37 [c]
F	209.86 ± 75.00 [bc]
TL	218.23 ± 113.61 [ab]
FL	229.10 ± 82.73 [a]

Means followed by distinct letters differ statistically [Tukey test ($p<0.05$)]
*SD: Standard deviation

Table 2. Mean values (±SD) of microbiological and biochemical parameters for each group.

Group	Analysis - Means (± SD)				
	10^7CFU/mg	Ca (µg/mg)	P_i (µg/mg)	F (µg/mg)	PI (µg/mg)
T	**2.54±2.58**	**340.5 ±27.01**	**0.162 ± 0.134**	**0.001 ± 0.005**	**213.206 ± 421.746**
F	**2.90±3.08**	**329.5 ± 143.97**	**0.149 ± 0.066**	**0.010 ± 0.021**	**111.208 ± 43.501**
TL	**2.59±3.13**	**412.3 ± 228.80**	**0.170 ± 0.104**	**0.0009 ± 0.002**	**124.626 ± 37.488**
FL	**2.30±4.0**	**411.8 ± 252.59**	**0.148 ± 0.029**	**0.002 ± 0.007**	**138.83 ± 118.893**

SD: Standard deviation
Means were not statistically different by Tukey test ($p>0.05$)

CONCLUSÃO

A irradiação do esmalte com o laser de CO_2 (λ =10,6 µm e 10,0 J/cm^2), sozinho ou combinado ao material de colagem liberador de fluoreto, foi capaz de reduzir a perda mineral do esmalte dentário bovino ao redor de braquetes ortodônticos *in vitro,* quando submetido a situações de alto desafio cariogênico com biofilme de *Streptococcus mutans*.

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* De acordo com a norma da UNICAMP/FOP, baseadas na norma do International Committee of Medical Journal Editors – Grupo de Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

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ANEXO 1

INFORMAÇÃO CCPG/OO2/06[6]

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato e a impressão das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG n° 1985/96, que trata da possibilidade do formato alternativo ao já estabelecido, a CCPG resolve:

Artigo 1º - O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverão obrigatoriamente conter:

I. Capa com formato único ou em formato alternativo que deverá conter informações relativas ao nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, sendo o projeto gráfico das capas definido pela PRPG.
II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.
III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.
IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).
V. Sumário.
VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.
VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.
VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.
IX. Todas as páginas com numeração "impar" serão impressas como "frente" e todas as páginas com numeração "par" serão impressas como "verso".

§ 1º - A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; glossário; apêndice; anexos.

§ 2º - A dissertação ou tese deverá ser apresentada na língua portuguesa, com exceção da possibilidade permitida no artigo 2° desta Informação.

§ 3º - As dissertações e teses cujo conteúdo versar sobre pesquisa envolvendo seres humanos, animais ou biossegurança, deverão apresentar anexos os respectivos documentos de aprovação.

Artigo 2º - A critério do orientador e com aprovação da CPG da Unidade, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

§ único - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

Artigo 3º - Dependendo da área do conhecimento, a critério do orientador e com aprovação da CPG da Unidade, a dissertação ou tese poderá ser apresentada em formato alternativo, desde que observados os incisos I, II, III IV, V e VII do artigo 1º.

Artigo 4º - Para impressão, na gráfica da Unicamp, dos exemplares definitivos de dissertações e teses defendidas, deverão ser adotados os seguintes procedimentos:

§ 1º - A solicitação para impressão dos exemplares de dissertações e teses poderá ser encaminhada à gráfica da Unicamp pelas Unidades, que se responsabilizarão pelo pagamento correspondente.

§ 2º - Um original da dissertação ou tese, em versão definitiva, impresso em folha tamanho carta, em uma só face, deve ser encaminhado à gráfica da Unicamp acompanhado do formulário "Requisição de Serviços Gráficos", onde conste o número de exemplares solicitados.

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão encaminhados à Unidade em, no máximo, cinco dias úteis.

§ 4º - No formulário "Requisição de Serviços Gráficos" deverão estar indicadas as páginas cuja reprodução deva ser feita no padrão "cores" ou "foto", ficando entendido que as demais páginas devam ser reproduzidas no padrão preto/branco comum.

§ 5º - As dissertações e teses serão reproduzidas no padrão frente e verso, exceção feita às páginas iniciais e divisões de capítulos; dissertações e teses com até 100 páginas serão reproduzidas no padrão apenas frente, exceção feita à página que contém a ficha catalográfica.

§ 6º - As páginas fornecidas para inserção deverão ser impressas em sua forma definitiva, ou seja, apenas frente ou frente/verso.

§ 7º - O custo, em reais, de cada exemplar produzido pela gráfica será definido pela Administração Superior da Universidade.

Artigo 5º - É obrigatória a entrega de dois exemplares para homologação.

Artigo 6º - Esta Informação entrará em vigor na data de sua publicação, ficando revogadas as disposições em contrário, principalmente as Informações CCPG 001 e 002/98 e CCPG/001/00.

Campinas, 13 de setembro de 2006

Profa. Dra. Teresa Dib Zambon Atvars
Presidente
Comissão Central de Pós-Graduação

ANEXO 2

Figura 1. Laser de CO_2.

Figura 2. Laser de CO_2 acoplado ao microscópio para varredura da área de esmalte.

ANEXO 3

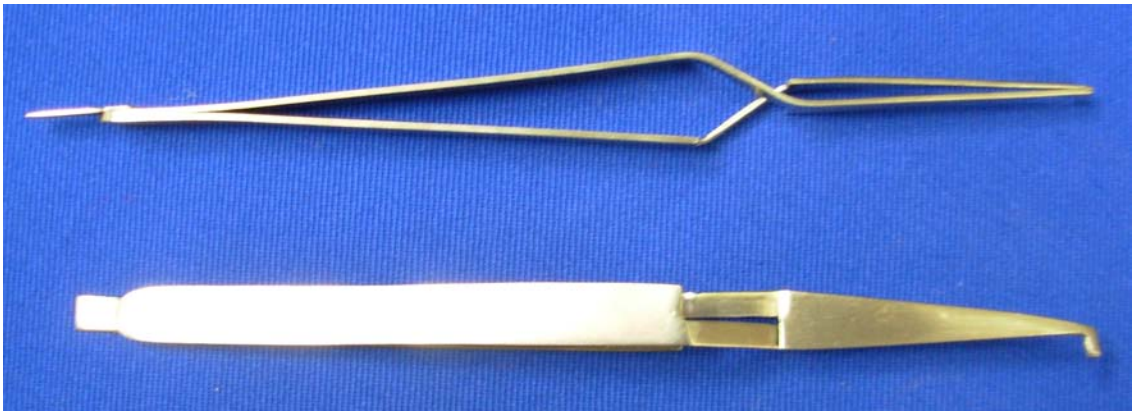

Figura 1. Pinça Ortodôntica utilizada para colagem dos braquetes.

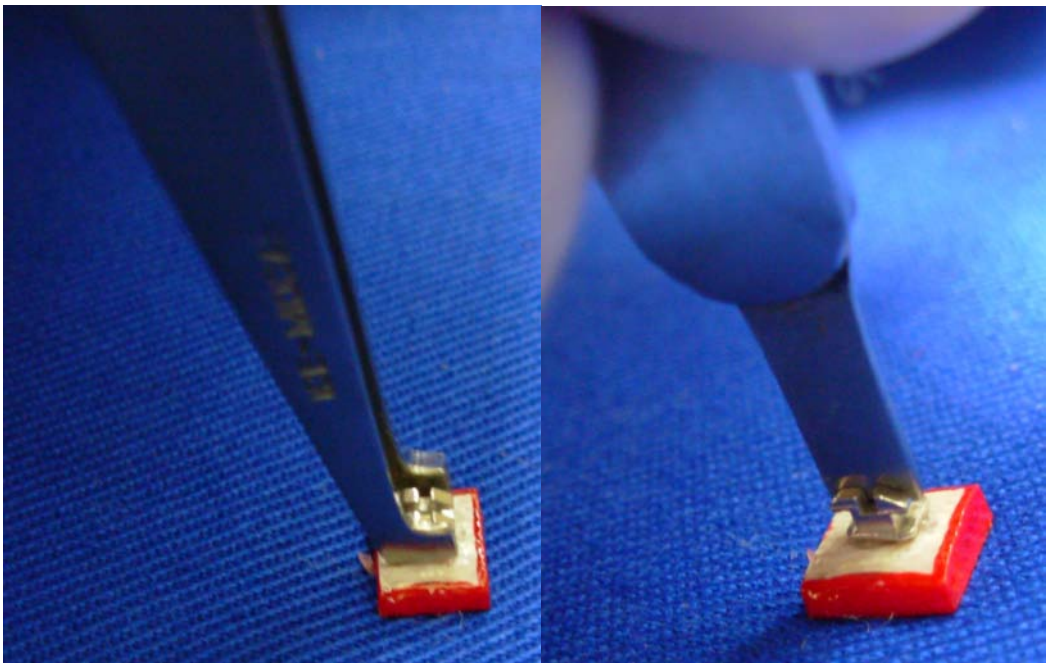

Figura 2. Posicionando o braquete no espécime de esmalte.

ANEXO 4

Figura 1. Modelo microbiológico – Capela – 1- grupos no meio de cultura, 2- solução salina 0,9% esterilizada, 3- novo meio de cultura.

ANEXO 5

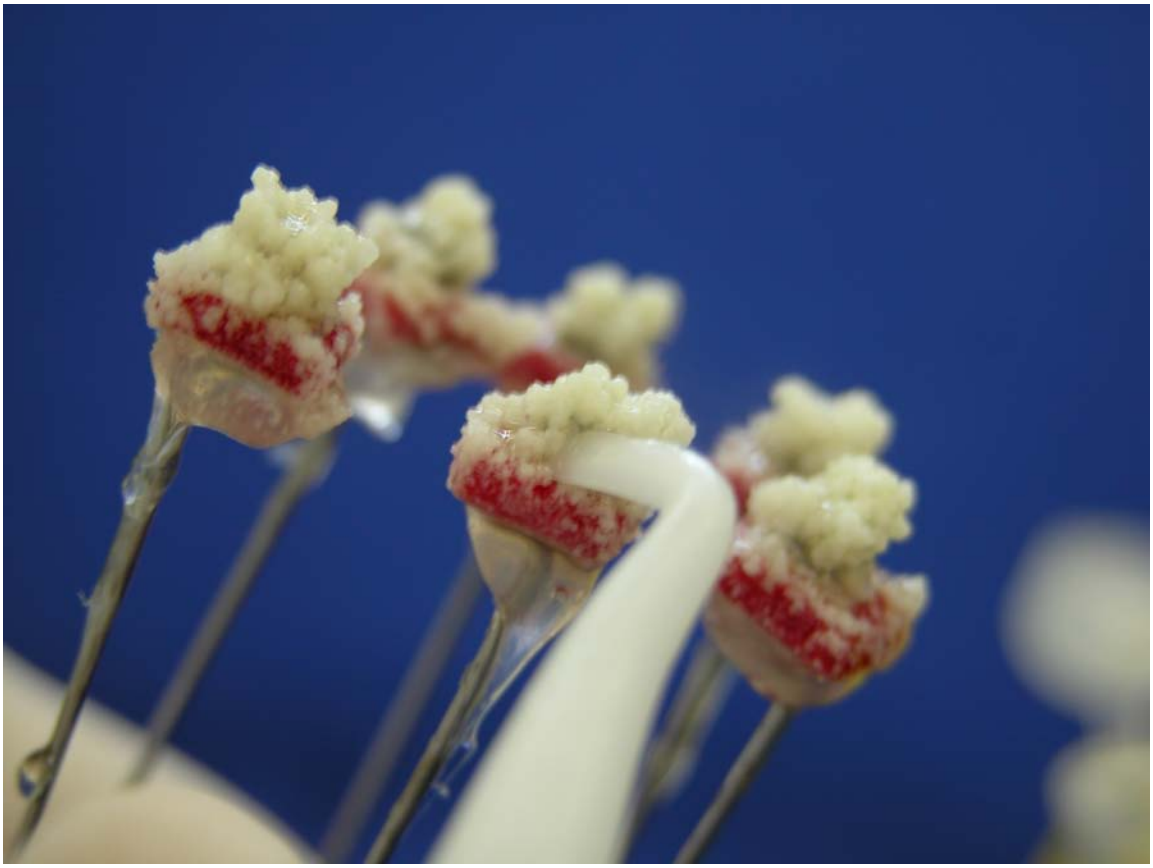

Figura 1. Coleta do biofilme dentário.

ANEXO 6

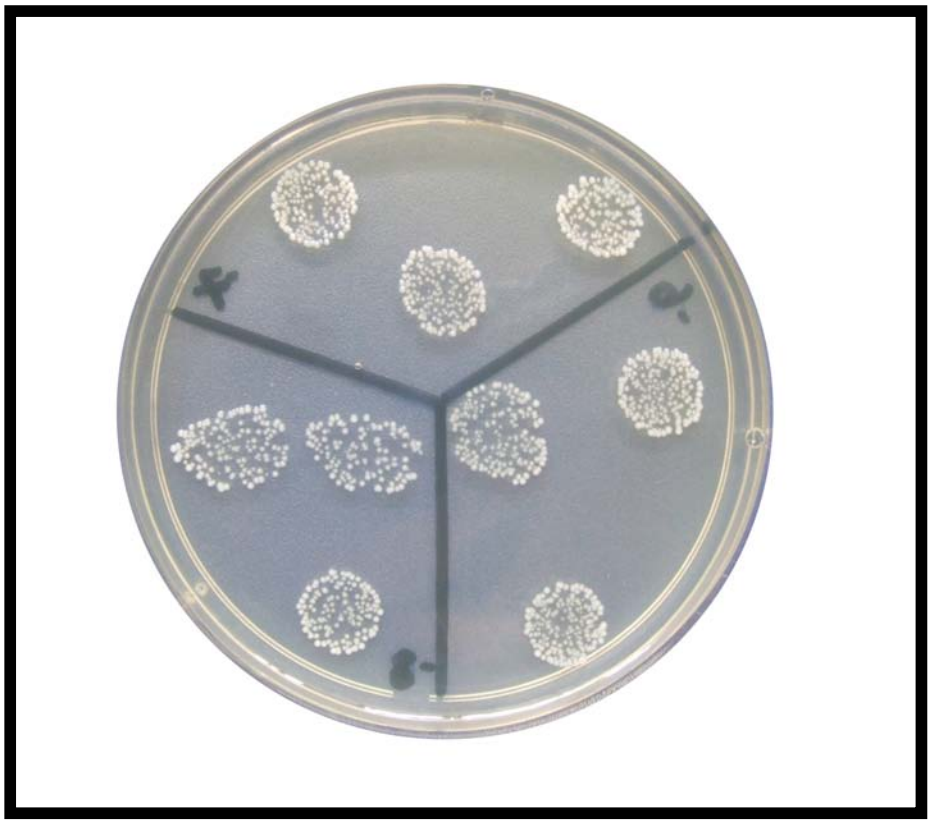

Figura 1. Colônias de estreptococos do grupo mutans. Biofilme após a diluição em série decimal no meio de cultura.

ANEXO 7

Figura 1. Microdurômetro HMV-2 tester Shimadzu.

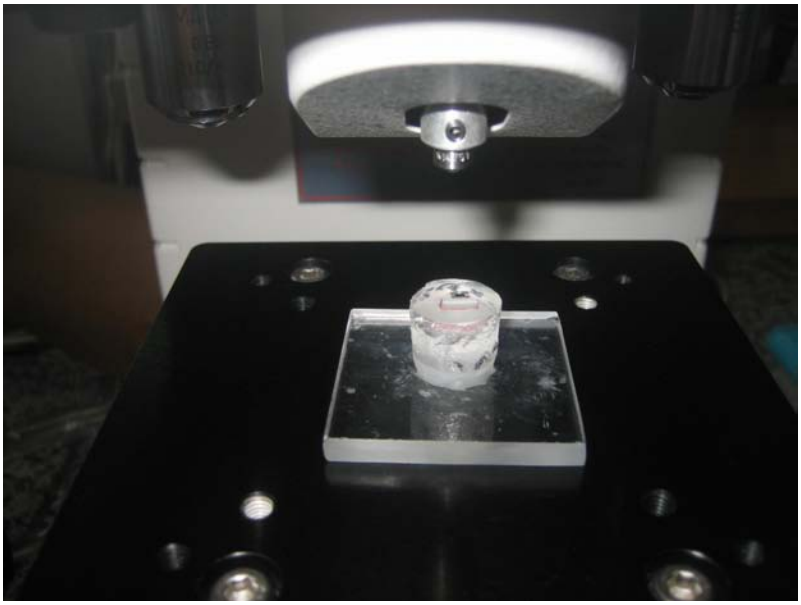

Figura 2. Microdurômetro com o espécime embutido em posição para início da indentação.

ANEXO 8

Figura 1. Eletrodo íon-seletivo Orion 96-09.

ANEXO 9

Figura 1. Leitor automático de microplacas utilizado para determinação da concentração de cálcio e fósforo.

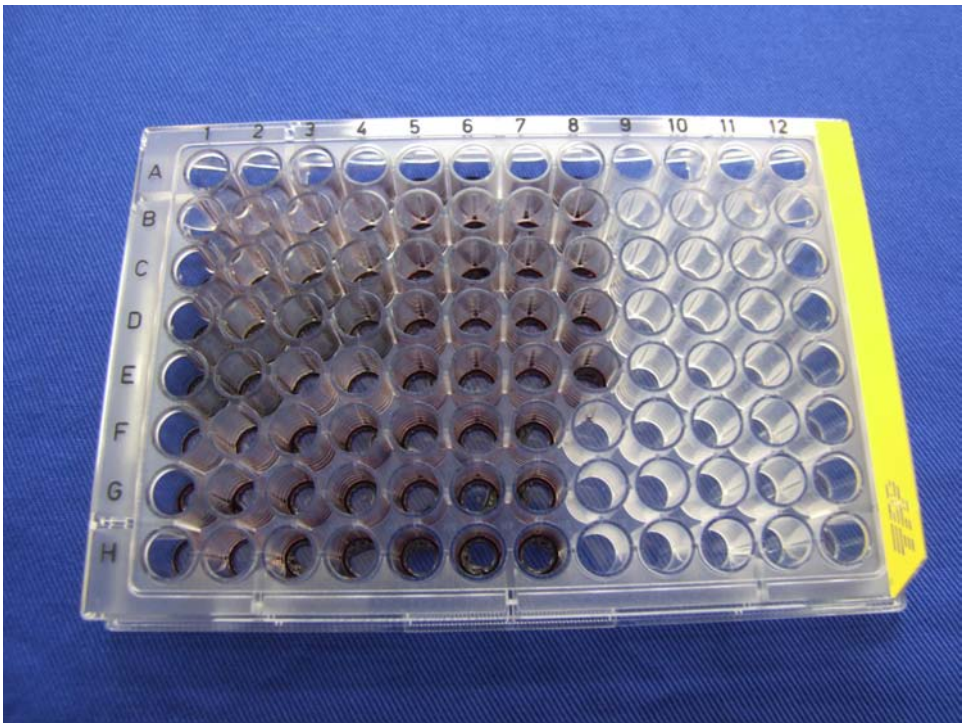

Figura 2. Microplaca utilizada para determinação da concentração de cálcio e fósforo.