ALINE ROGÉRIA FREIRE DE CASTILHO

PROPRIEDADES BIOLÓGICAS E MECÂNICAS DE UM CIMENTO DE IONÔMERO DE VIDRO ASSOCIADO À CLOREXIDINA OU À DOXICICLINA

Tese apresentada à Faculdade de Odontologia de Píracicaba da Universidade Estadual de Campinas, para a obtenção do Título de Doutor em Odontologia – Área de Odontopediatria.

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"A vida me ensinou...
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(Charles Chaplin)

RESUMO

Procedimentos de remoção parcial de cárie, indicados para os dentes com lesão de cárie profunda, utilizam-se do preceito de retirar a dentina mais amolecida e infectada, deixando-se uma fina camada de dentina afetada por cárie, sobre a câmara pulpar, evitando-se assim, exposição pulpar mecânica. Contudo, algumas bactérias cariogênicas podem permanecer na dentina por indeterminados períodos de tempo, podendo ocasionar progressão da lesão. Assim, estudos conduzidos com o propósito de melhorar a propriedade antibacteriana de materiais restauradores, sem, contudo produzir efeitos citotóxicos em células odontoblastóides e capaz de manter as propriedades básicas do material pode ser uma alternativa no tratamento de lesões de cárie profundas, uma vez que não existe um produto comercialmente disponível que associe todas estas características. No intuito de facilitar a apresentação desta Tese, a mesma foi dividida em dois capítulos, como descrito nas proposições seguintes. **Capítulo 1:** avaliar *in vitro* as propriedades biológicas (ação antibacteriana contra *Streptococcus mutans, Lactobacillus acidophilus, Lactobacillus casei* e *Actinomyces viscosus,* e citotoxicidade em células de linhagem odontoblástica MDPC-23) e mecânicas (resistência à compressão e à tração diametral) de um cimento de ionômero de vidro modificado por resina (Fuji Lining LC), contendo digluconato de clorexidina em diferentes concentrações (0,2%, 0,5%, 1,25% e 2,5%) e assim determinar a concentração terapêutica para utilização desta substância em tratamentos restauradores. **Capítulo 2**: verificar *in vitro* o comportamento de um cimento de ionômero de vidro modificado por resina (Fuji Lining LC) contendo hiclato de doxiciclina nas concentrações 1,5%, 3% e 4,5% frente a diferentes patógenos cariogênicos (*Streptococcus mutans, Lactobacillus acidophilus, Lactobacillus casei* e *Actinomyces viscosus*), células odontoblástóides MDPC-23 e quando submetidos aos ensaios mecânicos de resistência à compressão e à tração diametral. Os resultados observados em ambos os estudos mostraram que a incorporação de antimicrobianos ao cimento de ionômero de vidro, é capaz de melhorar significativamente o efeito inibitório do cimento contra microrganismos cariogênicos. A adição de digluconato de clorexidina a 2,5% promoveu ligeira alteração no metabolismo e morfologia das células

MDPC-23. O hiclato de doxiciclina, quando incorporado ao ionômero de vidro mesmo em maiores concentrações, não causou efeitos tóxicos em cultura de células odontoblásticas MDPC-23. Além disso, o cimento de ionômero de vidro contendo digluconato de clorexidina a 2,5% apresentou resistência à compressão reduzida, sem alteração da propriedade de resistência a tração diametral do cimento. A adição de hiclato de doxiciclina não alterou as propriedades mecânicas do cimento. Assim, a adição de hiclato de doxiciclina e de digluconato de clorexidina nas concentrações estudadas produziram aumento na atividade antimicrobiana sem efeitos citotóxicos e alteração nas propriedades mecânicas da mistura, exceto para o digluconato de clorexidina a 2,5%, que apresentou o menor resistência a compressão e alterações no metabolismo e morfologia de células pulpares.

PALAVRAS-CHAVE: agentes antibacterianos, cultura de células, cimento de ionômero de vidro, resistência à compressão, resistência à tração.

ABSTRACT

Partial caries removal approaches are indicated to the management of deep caries. It consists of the incomplete removal of softened carious dentine during cavity preparation, leaving a soft dentine layer over the pulp, so that it is not mechanically exposed. However, some viable cariogenic bacteria have been found in the remaining affected dentine, which may promote caries lesion progression. Therefore, studies aimed to improve inhibitory effect of the restorative materials, without cytotoxic effects on odontoblast cells and able to keep properties of material could be an alternative to the treatment of deep caries lesions. Nevertheless, the disposal-marketed products do not associate all those features. In order to facilitate the accomplishment of this Thesis, it was divided into two chapters, as described on the following descriptions. **Chapter 1:** to evaluate *in vitro* biological properties (antibacterial effect against *Streptococcus mutans, Lactobacillus acidophilus, Lactobacillus casei* and *Actinomyces viscosus,* and cytotoxicity at odontoblast cell line MDPC-23) and mechanical properties (compressive strength and diametral tensile strength) of a resin-modified glass ionomer cement (Fuji Lining LC) containing different concentrations of chlorhexidine digluconate (0.2%, 0.5%, 1.5% and 2.5%) and, thus to determine the therapeutic concentration of it for using in restorative dental treatment. **Chapter 2**: to verify *in vitro* the performance of a resin-modified glass ionomer cement (Fuji Lining LC) containing the antibiotic doxycycline hyclate at 1.5%, 3% and 4.5% against some cariogenic pathogens (*Streptococcus mutans, Lactobacillus acidophilus, Lactobacillus casei and Actinomyces viscosus*); to analyze it indirect contact with odontoblast-like MDPC-23 cells; and, to reveal the mechanical properties under compressive strength and diametral tensile strength. Results of both studies proved that the incorporation of antimicrobials at adequate proportions into the glass ionomer cement have the ability to become better inhibitory effects of the cement against cariogenic bacteria. The 2.5% chlorhexidine digluconate added to glass ionomer cement promoted slight alteration on the mebolism and morfology of MDPC-23 cells. The incorporation of doxycycline hyclate to glass ionomer cement, even in the highest concentration, did not cause toxic effects on culture of odontoblast-like MDPC-23 cells. In addition, 2.5% chlorhexidine

digluconate-containing glass ionomer cement decreased the compressive strength of cement, although there was no difference to diametral tensile strength. The adding of doxycycline hyclate did not alter mechanical properties of cement. It was concluded that doxycycline hyclate and chlorhexidine digluconate in the studied concentrations produced increased antimicrobial activity without cytotoxic effects and changes in mechanical properties of the mixture, except for the 1.25% chlorhexidine digluconate that presented the lowest compressive strength and alterations on metabolism and morphology of pulp cells.

KEY WORDS: antibacterial agents, cell culture, glass ionomer, compressive strength, diametral tensile strength.

SUMÁRIO

Por muito tempo, a remoção completa do tecido cariado foi considerada a estratégia ideal no tratamento de lesões de cárie dentária (Fusayama, 1979). Todavia, atualmente, a remoção parcial de cárie, tem sido considerada terapia de escolha no tratamento de lesões profundas, com o intuito de manter a integridade do tecido pulpar. Técnica similar ao tratamento pulpar indireto, a remoção parcial de cárie, tem sido utilizada para obtenção de resultados mais previsíveis, uma vez que, a dentina mais amolecida e infectada é removida, deixando-se uma fina camada de dentina afetada por cárie, porção mais profunda e endurecida da lesão, sob a restauração provisória (Ricketts, 2001).

Estudos têm demonstrado a eficiência clínica da remoção incompleta de dentina afetada por cárie em dentes com lesões de cárie profunda, baseada na ausência de sintomatologia e de sinais radiográficos de alterações patológicas apicais e periapicais, além da redução da microbiota da dentina remanescente, após 3 a 12 meses do procedimento clínico (Bjørndal et al, 1997; Maltz et al., 2002; Oliveira et al., 2006; Franzon et al., 2007; Maltz et al., 2007). Entretanto, alguns patógenos cariogênicos como *S. mutans*, são capazes de permanecer na dentina por longos períodos de tempo, mesmo com adequada restauração do elemento dentário, o que pode favorecer progressão da lesão de cárie ou mesmo comprometimento da restauração (Lula et al., 2009).

O efeito inibitório dos cimentos de ionômero de vidro (CIV) sobre o crescimento de microrganismos cariogênicos tem aplicação reconhecida nos procedimentos de remoção incompleta de cárie (Duque et al., 2009; Gruythuysen et al., 2010). Além da considerável atividade antibacteriana, os CIV apresentam propriedades mecânicas, físicas e biológicas desejáveis como material forrador/base. Dentre as propriedades mais importantes destacam-se a adesão à estrutura dentinária, adequados coeficiente de expansão térmica, módulo de elasticidade e resistência à compressão (Sidhu, 2010), além de ser biologicamente aceitável quando aplicados em cavidades profundas (Costa et al., 2003). Estudos com cimentos de ionômero de vidro modificados por resina (CIVMR), entre eles, o Fuji Lining LC têm apresentado destaque devido à sua baixa citotoxicidade quando aplicado em contato com células odontoblastóides (Aranha et al., 2006). Em contrapartida,

provavelmente, devido sua reduzida capacidade de liberar flúor (Loyola-Rodriguez et al., 1994), quando comparado a outros CIVMR, o Fuji Lining LC não elimina completamente a microbiota cariogênica, em lesões cariosas profundas (Duque et al., 2009).

Na tentativa de eliminar as bactérias residuais em dentes restaurados após a remoção parcial de cárie, pesquisadores incorporaram antimicrobianos, como clorexidina, em diferentes concentrações, ao cimento de ionômero de vidro convencional ou modificado por resina e obtiveram considerável ação antimicrobiana (Jedrychowski et al., 1983; Sanders et al., 2002; Takahashi et al., 2006; Frencken et al., 2007; Türkün et al., 2008). A clorexidina é um dos agentes antimicrobianos mais seguros e efetivos contra estreptococos orais (Pucher e Daniel 1992; Jenkins et al 1993; Hildebrandt, 1996), devido a atividade bactericida de amplo espectro apresentada, capaz de afetar, principalmente, estreptococos do grupo mutans, além de atuar contra outras espécies gram positivas, gram negativas, fungos e leveduras, aeróbias facultativas e anaeróbias (Emilson, 1977). Também com a finalidade de melhorar o efeito inibitório contra patógenos cariogênicos, outras substâncias antimicrobianas, como antibióticos, foram incorporadas aos CIV para aumentar a atividade antibacteriana do cimento. Em estudo *in vivo*, Pinheiro et al. (2005) observaram redução de mais de 98% da contagem de bactérias totais isoladas de dentina infectada após remoção parcial de cárie e restauração com CIV associado à metronidazol 1%, ciprofloxacina 1% e cefaclor 1% em dentes decíduos de crianças de 4 a 10 anos de idade.

As tetraciclinas formam um grupo de antibióticos usados tanto sistemicamente quanto localmente no tratamento de diversas infecções bacterianas (Golub *et al.,* 1991). Derivado semi-sintético biodegradável da tetraciclina, o hiclato de doxiciclina é um antibiótico de amplo espectro, com reconhecida ação bacteriostática (Bogren et al., 2008). Considerado um dos antimicrobianos de maior eficácia local dentre os frequentemente utilizados, este antibiótico é amplamente utilizado contra patógenos periodontais (Paquette et al., 2008; Sela et al., 2009), além de ser capaz de inibir a degradação de matriz extracelular, por meio da inativação de colagenase e metaloproteinase-9 (MMP-9) (Gu et al., 2010). Assim, antibióticos de liberação local e controlada como o hiclato de doxiciclina, podem ser uma alternativa segura e eficaz no tratamento de patologias orais, entre elas, a cárie dentária. Entretanto, nada se conhece a respeito do comportamento desta substância

antimicrobiana frente a microrganismos cariogênicos ou em contato com células odontoblásticas.

Embora a remoção parcial de cárie em dentes decíduos com lesões extensas em dentina tenha ampla divulgação na atualidade e, consequentemente, seja habitualmente incorporada à rotina do tratamento odontológico de crianças jovens, ainda não se conhece um material que apresente capacidade de atuar sobre a progressão do processo carioso, por meio de seu efeito antimicrobiano, e ainda ser considerado biologicamente aceitável quando em contato com células pulpares, sem contanto, afetar as propriedades mecânicas do cimento, necessárias para a manutenção do dente decíduo na cavidade bucal até a erupção do dente permanente.

Com base nos pressupostos descritos, este estudo teve por objetivos específicos:

1) Avaliar o efeito antibacteriano *in vitro* de um cimento de ionômero de vidro contendo os antimicrobianos digluconato de clorexidina e hiclato de doxiciclina em diferentes concentrações.

2) Avaliar o efeito citotóxico *in vitro* do cimento de ionômero de vidro contendo digluconato de clorexidina ou hiclato de doxiciclina, em diferentes concentrações, sobre células odontoblásticas.

3) Avaliar as propriedades mecânicas (resistência à compressão e à tração diametral) do cimento de ionômero de vidro associado ao digluconato de clorexidina ou ao hiclato de doxiciclina.

Os objetivos do presente estudo[1] foram:

Capítulo 1

Antimicrobial-RMGIC association - New combination is a promising candidate for treatment of caries lesions

Capítulo 2

Mechanical and biological characterization of resin-modified glass-ionomer cement containing doxycycline hyclate[2]

1 Esta tese de doutorado foi realizada no formato alternativo, com base na resolução da CCPG/002/06, a qual dispõe a respeito do formato das teses de mestrado e doutorado aprovados pela UNICAMP.

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Antimicrobial-RMGIC association - New combination is a promising candidate for treatment of caries lesions

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ABSTRACT

An effective antimicrobial agent that is also acceptable to tooth tissues would inhibit cariogenic microorganism, allowing caries process be arrested. This study was focused on the effect of 0.2, 0.5, 1.25 and 2.5% chlorhexidine digluconate added to a resin-modified glass ionomer cement (RMGIC) on antibacterial activity, toxicity on MDPC-23 odontoblast-like cells and mechanical properties of the cement. Antibacterial activity of material was evaluated against *Streptococcus mutans, Lactobacillus acidophilus, Lactobacillus casei and Actinomyces viscosus* using agar diffusion test. For the cytotoxicity tests, cell metabolism and morphology were investigated by MTT assay and SEM, respectively. In addition, the compressive and diametral tensile strengths were measured. Data from antibacterial activity and cell culture were submitted to Kruskal Wallis and Mann-Whitney tests and mechanical tests to One-way ANOVA and Tukey tests ($p<0.05$). RMGIC containing 2.5% chlorhexidine digluconate showed the highest growth inhibitions of strains but it affected the metabolism and morphology of MDPC-23 cells, and also decreased the compressive strength of cement. The use of 1.25% chlorhexidine digluconate was shown to improve considerably the inhibitory effects of RMGIC against cariogenic bacteria, without cytotoxic effects and no disturbing of mechanical properties of cement and opens a new perspective for treatment of dental caries.

Keywords: chlorhexidine digluconate; cell culture; antibacterial activity; mechanical properties; glass ionomer cement.

1. Introduction

Partial caries removal approaches for dental caries managing have gained great importance in the last decade since scientific literature have suggested that only the softened (infected) dentin, structurally disarranged should be removed from carious tissue [1]. The remineralization capacity of hardest (affected) dentin has also led to acceptance that partial caries removal is a practice to avoid excessive excavation and the risk of pulp exposure in deep cavities [2]. This way, this procedure could induce dentin repair, arrest of the carious process and maintain pulp vitality [3,4].

Even after removal of the infected layer and adequate sealing, viable bacteria have been consistently found in the remaining affected dentine after different period of evaluation, irrespective of the material applied on the residual carious dentine [3-7]. Therapeutic benefit was gained when antimicrobial substances have been used in association with glass ionomer cement to contribute to residual infection elimination, and thus, minimizes the risk of recurrent caries and damage to the pulp [8].

Among the different antimicrobial agents used to control dental microorganisms reduction, chlorhexidine has been considered one of the most effective and safe substance. It presents wide spectrum against Gram positive bacteria specially mutans streptococci, Gram negative, aerobic and facultative anaerobic bacteria, yeasts and fungi [9]**.** Therefore, chlorhexidine might to be the promise substance in the caries treatment since its characteristics agree with the properties to establish of health and function of tooth. This way, studies have suggested the incorporation of this agent to glass ionomer cements to improve their inhibitory action on residual microorganisms contributing to reduction of secondary caries [10-14].

Although the addition of chlorhexidine to glass ionomer cement must increase the antimicrobial activity of a dental material, the presence of that substance might produce toxicity to pulp cells, when applied in deep cavities, modify physical characteristics of the cement or both. Studies have demonstrated that high concentrations of chlorhexidine cause damage on odontoblastic lineage [15] or jeopardize basic properties of the materials [13,14]. For a secure and adequate dental treatment, concentration of this antimicrobial agent to be used in association with dental materials into cavities must be defined before its application. In vitro study demonstrated a slight caries-inhibiting effect of chlorhexidine-containing glass ionomer cement without compromising its physical characteristics [8]; however, none demonstrated if their combination can affect odontoblast cells, essential property to preserve pulp health. Furthermore, glass ionomer cements are used as liners on affected dentin during partial caries removal procedures, and the association of those liners materials and chlorhexidine digluconate was not studied yet. This way, this study intend to determine the therapeutic concentration of chlorhexidine digluconate that is necessary to produce anticariogenic action without cause toxic effects on odontoblast-like cells and no interferes on the mechanical properties when incorporated to a liner resin-modified glass ionomer cement (RMGIC).

2. Materials and methods

2.1. Dental materials

The liner RMGIC chosen for this study was GC Fuji Lining LC (Lot 0710021, GC Corporation, Tokyo, Japan) that was modified by the addition of chlorhexidine digluconate (C9394 Sigma-Aldrich, Steinheim, Germany). Chlorhexidine digluconate (20%) was added

at 0.2%; 0.5%; 1.25% and 2.5% concentrations to the liquid of the GC Fuji Lining LC keeping the original ratio of power/liquid (1.4 g: 1.0 g). The control group was GC Fuji Lining LC with no antimicrobial agent. Those concentrations were determined previously (pilot study) using minimal inhibitory concentration and minimal bactericidal concentration assays. The composition of the RGMIC is presented at Table 1.

2.2 Microbial strains and growth media

Stock cultures of *Streptococcus mutans* (UA159), *Lactobacillus acidophilus* (ATCC#IAL-523), *Lactobacillus casei* (ATCC #193) and *Actinomyces viscosus* (T14V # IAL.5) from Microbiology and Immunology Laboratory of Piracicaba Dental School - University of Campinas, Piracicaba, São Paulo, Brazil were used in this study. For each experiment, cells were cultured freshly from frozen stock on brain–heart infusion broth (BHI; DIFCO Laboratories, Detroit, MI, USA) for 24 hours at 37°C in 10% CO2 incubator. After confirming viability and absence of contamination by plating in specific medium and Gram techniques, cultures were again grown in BHI for 18-24h at 37^{o}C and adjusted to a concentration of 1 x 10^{8}cells/mL to obtain an inoculum for subsequent tests.

2.3 Agar diffusion test

In each sterilized Petri dish (20x100 mm), a base layer containing 15 mL of BHI agar mixed with 300 μL of each inoculum was prepared. After solidification of the culture medium, six wells measuring 5 mm in diameter were made in each plate and completely filled up with one of experimental control material (RMGIC with chlorhexidine digluconate

0.2, 0.5, 1.25 and 2.5%) or control group (RMGIC). All materials were handled under aseptic conditions according to the manufacturer's instructions and inserted into wells using a syringe (Centrix Inc., Shelton, USA). The cements were light activated for 30 seconds using a halogen curing unit (Curing Light XL3000, 3MESPE). The light intensity (410mW/cm2) was monitored by a radiometer (Optilux 500, Demetron Kerr, Danbury, CT, USA). Ten microliters of aqueous 0.2% chlorhexidine digluconate was applied on sterile filter paper discs (n=6), also 5 mm in diameter, which acted as a control of the experiment. The plates were kept for 2 h at room temperature for the diffusion of the materials and then were incubated at 37^{o}C for 24 h. After this period, inhibition zones around the materials were measured with a digital caliper.

2.4. Culture of MDPC-23 cells

Immortalized cells of the odontoblast-like cell line (MDPC-23) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), with 100 IU/mL penicillin, 100 μg/mL streptomycin and 2 mmol/L glutamine (Gibco) in an humidified incubator with 5% CO_2 and 95% air at 37oC (Isotemp; Fisher Scientific, Pittsburgh, PA, USA). MDPC-23 cells were sub-cultured every 3 days until an adequate number of cells were obtained for the study. The cells were then seeded (30.000 cells/cm^2) in sterile 24-well plates (Costar Corp., Cambridge, MA, USA), which were maintained in the humidified incubator with 5% CO_2 and 95% air at 37oC for 48 h.

2.3.2. *Analysis of Cell Metabolism by MTT assay*

The RMGIC containing or not 0.2, 0.5, 1.25 and 2.5% of chlorhexidine digluconate were hand-mixed and applied into stainless-steel molds with cylindrical apertures. Vitrebond (3MESPE) was considered positive control for this experiment, because have high cytotoxic effect on odontoblast cells [16]. Ten round-shaped samples of each group (2mm thick and 4 mm diameter) were prepared, light-cured for 30 seconds and maintained for 1 h at 37o C - 100% humidity. Then, specimens were inserted separately in sterile 24-well plates containing DMEM medium for 24h. After this period, 800 µL of extract of each well were applied to previously culture MDPC-23 cells for 24h. Eight out of 10 specimens were used for analysis of cell metabolism by the cytochemical demonstration of succinic dehydrogenase (SDH) activity, which is a measure of the mitochondrial respiration of the cells, employing the methyl tetrazolium (MTT) assay. For the MTT assay, the extracts were aspirated and replaced by 900 µL of DMEM plus 100 µL of MTT solution (5 mg/mL sterile PBS; Sigma Chemical Co., St. Louis, MO, USA). Thereafter, the culture medium with the MTT solution were aspirated and replaced by 600 µL of acidified isopropanol solution (0.04 N HCl) in each well to dissolve the formazan crystals resulting from the cleavage of the MTT salt ring by the SDH enzyme present in the mitochondria of viable cells. Three 100 µL aliquots of each well were transferred to 96-well plates (Costar Corp., Cambridge, MA, USA). Cell viability was evaluated by spectrophotometry as being proportional to the absorbance measured at 570 nm wavelength with an ELISA microplate reader (model 3550-UV, Bio-Rad Laboratories, Hercules, CA, USA).

The values obtained from the 3 aliquots were averaged to provide a single value for each well. The means were calculated for the groups and transformed into percentages,

which represented the inhibitory effect of the mitochondrial activity of the cells by the extracts. The negative control (DMEM) was defined as having 100% of cell metabolism.

2.6 Analysis of cell morphology by scanning electron microscopy

Cell morphology was examined by scanning electron microscopy (SEM) using two representative wells of each group. For such purpose, sterile 12-mm-diameter cover glasses (Isotemp; Fisher Scientific) were placed on the bottom of the wells of sterile 24-well plates immediately before seeding of the MDPC-23 cells. Then, the extracts were applied on the cells and the plates were incubated for 24 h, in the same way as described before. Following this period, the extracts were aspirated and the viable cells that remained adhered to the glass substrate were fixed in 1 mL of buffered 2.5% glutaraldehyde for 60 min. The cells were the subjected to three 5-min rinses with 1 mL PBS, post-fixed in 1% osmium tetroxide for 60 min and processed for examination with a scanning electron microscope (JEOL-JMS-T33A; JEOL, Tokyo, Japan).

2.7 Measurement of Mechanical Properties

Four experimental groups (RMGIC-containing 0.2%; 0.5%; 1.25% and 2.5% chlorhexidine digluconate) and one control group (RMGIC) were established as described above for each mechanical assay, compressive strength (n= 50) and diametral tensile strength (n=50). Briefly, GC Fuji Lining LC was mixed by agglutination of powder to liquid associated or not to chlorhexidine at 0.2%; 0.5%; 1.25% and 2.5% and then the mixture was placed with Centrix syringe (Centrix Inc., Shelton, USA) into cylindrical molds (4 mm high x 2 mm diameter). After that, they were exposed to a light source

(Curing Light XL3000, 3MESPE), with 410 mW/cm^2 of light intensity for 30 seconds. Afterwards, the specimens were stored in distilled water for 24 hours at 37ºC. Specimens were submitted to compressive strength in an Instron universal test machine (4411, Instron Co., Canton, Mass, USA) at a crosshead speed of 1.0 mm/min until failure occurred. Compressive strength values (kgf/cm^2) were calculated by dividing the load (F) by the cross-sectional area and converted in MPa. Diametral tensile strength was carried out in an Instron universal test machine (4411, Instron Co., Canton, Mass, USA) at 0.5 mm/min crosshead speed. Diametral tensile strength values (kgf/cm^2) were calculated using the equation: DTS = 2F/3.14DT, where F is the failure load, D the diameter, and T the height of the specimen. DTS values were converted into MPa.

2.8 Statistical Analysis

Data from antibacterial and cytotoxic effects were submitted to Kruskal Wallis and Mann-Whitney tests, and those from mechanical properties were submitted to one-way ANOVA and Tukey tests for ($p<0.05$).

3. Results

3.1 Antibacterial activity

The mean values of the inhibition zones for each material are shown in **Figure 1**. The concentrations of 0.2 and 0.5% chlorhexidine digluconate did not have effect on the antibacterial activity of RMGIC. The incorporation of 1.25 and 2.5% chlorhexidine digluconate improved significantly the inhibitory activity of cement on all bacteria tested, except for 1.25% chlorhexidine digluconate against *L. acidophilus*. When compared these

two groups, there was statistically difference between them for *S. mutans* and *L. acidophilus.*

3.2. Toxicity on odontoblast-like cells

Figure 2 shows the cell metabolism (SDH activity) following application of the culture medium treated or not with the experimental materials. The 2.5% chlorhexidine digluconate in association with RMGIC caused significant reduction in the metabolism of MDPC-23 cells, when compared to controls (RMGIC and DMEM). Vitrebond showed the highest cytotoxic effects, decreasing the metabolic activity in 93%. RMGIC associated to 0.2 and 0.5 CHX increased significantly the SDH activity (14.03% and 12.6%, respectively) and statistically differed from control group (DMEM), showing that low concentrations of chorhexidine digluconate could stimulate cell metabolism. There was no difference between 1.25% chlorhexidine digluconate and RMGIC and DMEM groups.

Images of SEM indicated that chlorhexidine digluconate concentrations up to 1.25% when incorporated to RMGIC do not affect cell morphology. However, RMGIC containing chlorhexidine digluconate at 2.5% concentration altered slightly the morphology of MDPC-23 cells (**Figure 3).**

3.3 Measurements of Mechanical tests

The means and standard deviations of the values obtained for mechanical tests are showed in **Figure 4 and 5**. The 2.5% chlorhexidine digluconate showed significantly lower compressive strength when compared to the control without antimicrobial. No significant differences were observed among groups for diametral tensile test ($p<0.05$), demonstrating

that the incorporation of chlorhexidine digluconate into RMGIC up to 2.5% concentration did not modify this mechanical property of the liner material.

4. Discussion

Chlorhexidine substance has been proved its efficacy against oral pathogens [9]. In this study, the addition of 1.25% and 2.5% chlorhexidine digluconate to liner resin-modified glass ionomer cement increased substantially its inhibitory activity against the tested oral bacteria when compared with RMGIC alone. However, it is interesting to note that *L. acidophilus* was the most resistant microorganism to the inhibitory effects of RMGIC containing chlorhexidine digluconate. According to Botelho [17], the addition of chlorhexidine to glass ionomer cement is less effective against that cariogenic bacteria than the addition of cetylpyridinium chloride and benzalkonium chloride. Notwithstanding, chlorhexidine substance added to glass ionomer cement has a significant residual release effect for some weeks [13] then it could inhibit remain microorganisms, including *L. acidophilus*.

Some studies have evaluated the release of chlorhexidine substance from glass ionomer cements using high performance liquid chromatography (HPLC) analysis and demonstrated conflicting results about antibacterial effects. Some of them related that inhibitory activity against pathogens was dependent upon the concentration of that antimicrobial [11,17] and others showed no dose-response effects [8,18]. In this study, antibacterial effect seems to be concentration-dependent since 1.25% and 2.5% concentrations produced the better results as obtained by Türkün et al. [14]. These findings are also according to Ribeiro and Ericson [11] that evaluated antibacterial effect *in vitro*

against mutans streptococci that lasted up to 80 days when combined clorhexidine gluconate with glass ionomer cement. Characteristics such as viscosity and hardness of glass ionomer cement could determine amounts of antimicrobial released [8].

Even maintaining the original ratio of power/liquid, the adding of any substance could affect important characteristics of glass ionomer cement. Antimicrobials could enhance the cytotoxic effect of dental material or interfere in their mechanical properties. Although chlorhexidine digluconate is a potential antimicrobial with many desirable biological characteristics such as inhibition of dentin metalloproteinases [19], it may causes immediate hypersensitivity and other unwanted responses including inhibit protein synthesis and mitochondrial activity [20,21]. For those reasons, both quantity and oral administration of the chlorhexidine digluconate must be controlled. The liner RMGIC Fuji Lining LC was chosen for this study because its low toxicity on odontoblast-like cells. Aranha et al. [22] evaluated SDH activity after exposition to some RMGICs and verified that Fuji Lining LC provided minimal reduction in the cellular metabolism (9.3%) compared to Vitrebond (80.7%) that is highly cytotoxic. The toxicity of Vitrebond is caused by the percentage of HEMA (20-30%) that is higher than Fuji Lining LC (8-10%). In relation to toxicity of chlorhexidine substance on culture cells, studies in the literature evaluated only the cytotoxicity of that antimicrobial agent applied directly on cells, not associated with some dental material. Lessa et al. [23] evaluated 0.06, 0.12, 0.2, 1 and 2% chlorhexidine digluconate on odontoblast-like cells for 60s to 24h and observed that the antimicrobial had a dose-time dependent toxic effect on MDPC-cells. The higher concentration of chlorhexidine digluconate and longer its contact time with odontoblast cells, the more intense the cytotoxic effect of that chemical agent. In the present study,

extracts obtained after incubation of RMGIC specimens associated with 0.2 to 1.25% of chlorhexidine digluconate for 24h did not increase the toxicity on odontoblastic lineage cells. However, the 2.5% chlorhexidine digluconate concentration reduced significantly cell metabolism and changed it morphology.

The idea of incorporating chlorhexidine digluconate into dental materials used for filling or lining such as glass ionomer cement is based on the improvement of their antimicrobial activity. However, the addition of that antimicrobial substance to glass ionomer cement can affect mechanical properties of cement [8,13,24]. Therefore, the particular antimicrobial agent and its quantity are important aspects to determine if the characteristics of the dental material could be affected. In this study, the inhibitory action of RMGIC against all tested strains was improved by the presence of chlorhexidine digluconate, as well the mechanical properties of the cement was kept, except the 2.5% chlorhexidine digluconate concentration for compressive strength test. Our results are according to Takahashi et al. [8] that observed that 2% chlorhexidine diacetate or greater significantly decreased the compressive strength and the bond strength to dentin of conventional glass ionomer cement. Those authors suggested that the decrease in mechanical properties could be attributed to slight modifications in powder/liquid ratios by adding antimicrobial. In the present study, 2.5% chlorhexidine digluconate affected two important properties of glass ionomer cement: cytotoxicity on odontoblast-like cells and compressive strength of cement. Then, chlorhexidine digluconate up to 1.25% could be the ideal and safety concentration to take in RMGIC used as liner in deep cavities.

Findings of this study demonstrated that the using of chlorhexidine digluconate in combination with RMGIC maximizes the antimicrobial activity of cement. Usually there is

no antimicrobial added to dental materials but possibly the combination of antimicrobial to glass ionomer cement could be better protection against cariogenic bacteria and should avoid caries progression. Therefore, we propose that chlorhexidine digluconate must be a potential candidate as a therapeutic agent in caries management, especially in partial caries removal procedures, and could be further developed as a constituent for dental materials.

5. Conclusion

Chlorhexidine digluconate at 1.25% added to resin-modified glass ionomer cement had marked antimicrobial activity against cariogenic bacteria, neither caused damage to odontoblast-like cells nor affected mechanical properties of the cement showing that that mixture provides an alternative approach for treatment of caries lesions after partial caries removal procedures.

6. Acknowledgements

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7. References

1. Fusayama T. Two layers of carious dentin: diagnosis and treatment. Oper Dent 1979;4:63-70.

2. Hayashi M, Fujitani M, Yamaki C, Momoi Y. Ways of enhancing pulp preservation by stepwise excavation-A systematic review. J Dent. 2010;21.

3. Björndal L, Larsen T, Thylstrup A. A clinical and microbiological study of deep carious lesion during stepwise excavation using long treatment intervals. Caries Res 1997;31:411-412.

4. Björndal L, Larsen T. Changes in the cultivable flora in deep carious lesions following a stepwise excavation procedure. Caries Res 2000;34:502-508.

5. Weerheijm KL, Kreulen CM, de Soet JJ, Groen HJ, van Amerongen WE. Bacterial counts in carious dentine under restorations: 2-year in vivo effects. Caries Res 1999; 33:130-134.

6. Pinto AS, Araújo FB, Franzon R, Figueiredo MC, Henz S, Garcia-Godoy F. Clinical and microbiological effect of calcium hydroxide protection in indirect pulp capping in primary teeth. Am J Dent 2006;19:382-387.

7. Duque C; Negrini TC, Sacono NT, Spolidorio DMP, Costa CAS, Hebling J. Clinical and microbiological performance of resin-modified glass-ionomer liners after incomplete dentine caries removal. Clin Oral Invest 2009; 13:465-471.

8. Takahashi Y, Imazato S, Kaneshiro AV, Ebisu S, Frencken JE, Tay FR. Antibacterial effects and physical properties of glass-ionomer cements containing chlorhexidine for the ART approach. Dent Mater 2006; 22:647-652.

9. Emilson CG. Susceptibility of various microorganisms to chlorhexidine. Scand J Dent Res 1977; 85:255-265.

10. Frencken JE, Imazato S, Toi C, Mulder J, Mickenautsch S, Takahashi Y, Ebisu S. Antibacterial effect of chlorhexidine- containing glass ionomer cement in vivo: a pilot study. Caries Res. 2007;41:102-7.

11. Ribeiro J, Ericson D. In vitro antibacterial effect of chlorhexidine added to glass-ionomer cements. Scand J Dent Res 1991;99:533-540.

12. Hoszek A, Ericson D. In vitro fluoride release and the antibacterial effect of glass ionomers containing chlorhexidine gluconate. Oper Dent 2008;33:696-670.

13. Sanders BJ, Gregory RL, Moore K, Avery DR. Antibacterial and physical properties of resin modified glass-ionomers combined with chlorhexidine. J Oral Rehabil 2002;29:553-558.

14. Türkün LS, Türkün M, Ertuğrul F, Ateş M, Brugger S. Long-term antibacterial effects and physical properties of a chlorhexidine-containing glass ionomer cement. J Esthet Restor Dent 2008;20:29-44.

15. Souza LB, Aquino SG, Souza PPC, Hebling J, Costa CAS. Cytotoxic effects of different concentrations of chlorhexidine to the odontoblast cell line MDPC-23. Am J Dent. 2007;20:400-404.

16. Souza PP, Aranha AM, Hebling J, Giro EM, Costa CA. In vitro cytotoxicity and in vivo biocompatibility of contemporary resin-modified glass-ionomer cements. Dent Mater 2006;22:838-844.

17. Botelho MG. Inhibitory effects on selected oral bacteria of antibacterial agents incorporated in a glass ionomer cement. Caries Res 2003;37:108-114.

18. Jedrychowski JR, Caputo AA, Kerper S. Antibacterial and mechanical properties of restorative materials combined with chlorhexidines. J Oral Rehabil 1983;10:373-381.

19. Tjäderhane L, Larjava H, Sorsa T, Uitto VJ, Larmas M, Salo T. The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions. J Dent Res. 1998;77:1622-1629.

20. Chang YC, Huang FM, Tai KW, Chou MY. The effect of sodium hypochlorite and chlorhexidine on cultured human periodontal ligament cells. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001;92:446-450.

21. Beaudouin E, Kanny G, Morisset M, Renaudin JM, Mertes M, Laxenaire MC, Mouton C et al. Immediate hypersensitivity to chlorhexidine: literature review. Eur Ann Allergy Clin Immunol 2004;36:123-126.

22. Aranha AM, Giro EM, Souza PP, Hebling J, de Souza Costa. Effect of curing regime on the cytotoxicity of resin-modified glass-ionomer lining cements applied to an odontoblast-cell line. Dent Mater 2006;22:864-869.

23. Lessa FC, Aranha AM, Nogueira I, Giro EM, Hebling J, Costa CA. Toxicity of chlorhexidine on odontoblast-like cells. J Appl Oral Sci 2010;18:50-58.

24. Palmer G, Jones FH, Billington RW, Pearson GJ. Chlorhexidine release from an experimental glass ionomer cement. Biomaterials 2004;25:5423-5431.

Table 1 – Composition, batch number of GC Fuji Lining LC (GC Corporation) and antimicrobial used in the study.

Material	Composition	%	Manufacturer	Batch number
Fuji Lining LC* Powder	Alumino-silicate glass	100	GC Corp., Tokyo, Japan	0710021
Fuji Lining LC* Liquid	Polyacrylic acid 2-Hydroxyethyl methacrylate Proprietary Ingredient	65 – 75 8 – 10 5 – 15	GC Corp., Tokyo, Japan	0710021
Chlorhexidine digluconate 20%	Chlorhexidine digluconate Solubility – H_2O	20	Sigma-Aldrich, Steinheim, Germany	C9394

*Material Safety Data Sheet information. **Sigma Aldrich (www.sigma-aldrich.com)

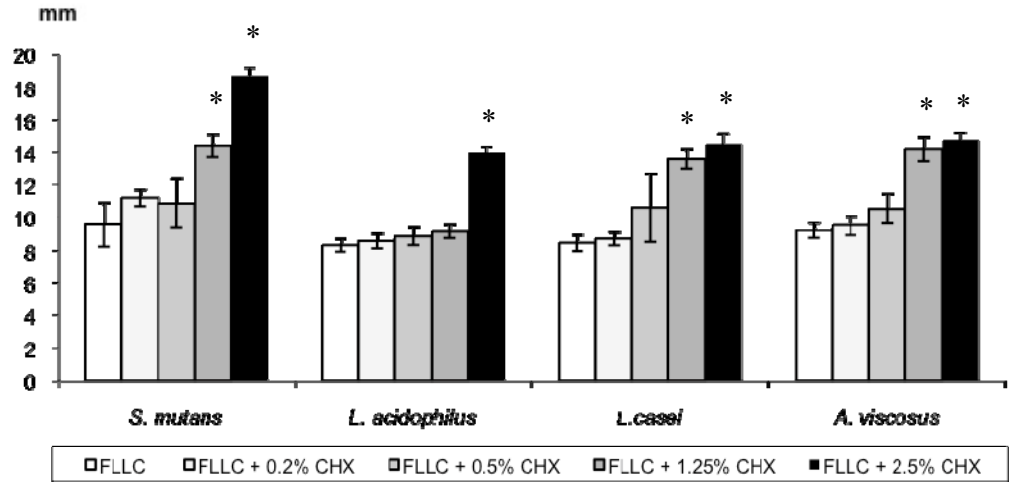

Figure 1. Means and standard deviations of inhibition zones for Fuji Lining LC (FLLC) associated or not to different concentrations of chlorhexidine digluconate (CHX) against *S. mutans, L. acidophilus, L. casei,* and *A. viscosus.*

* Values obtained for these test groups differ statistically from the control group (FLLC), according to Kruskal Wallis and Mann-Whitney tests ($p \leq 0.05$).

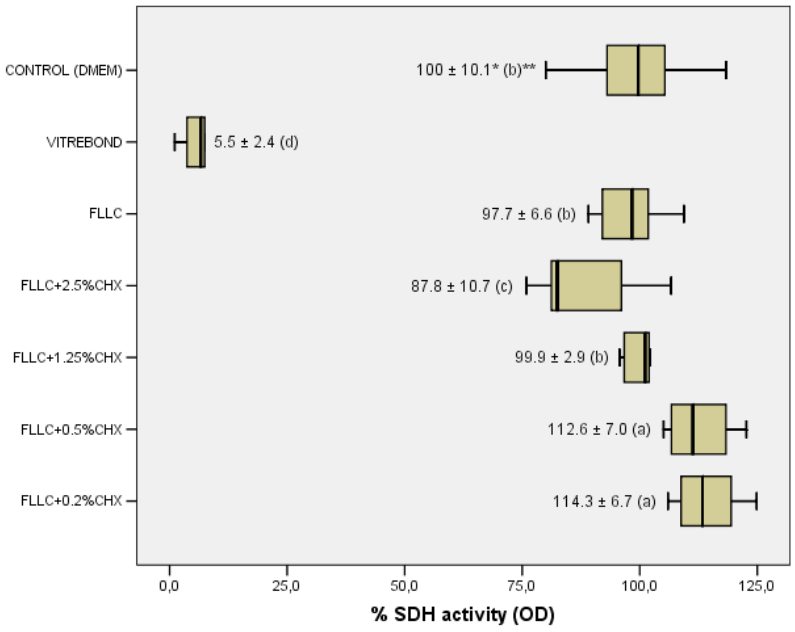

Figure 2. Box-whisker plot (minimum [lower quartile–median–upper quartile] maximum) of the cell metabolism (MTT assay) results for each group. * Mean±standard deviation. The vertical line in the box is the median. ** Groups identified with the same letter do not differ statistically (Mann-Whitney; $p>0.05$).

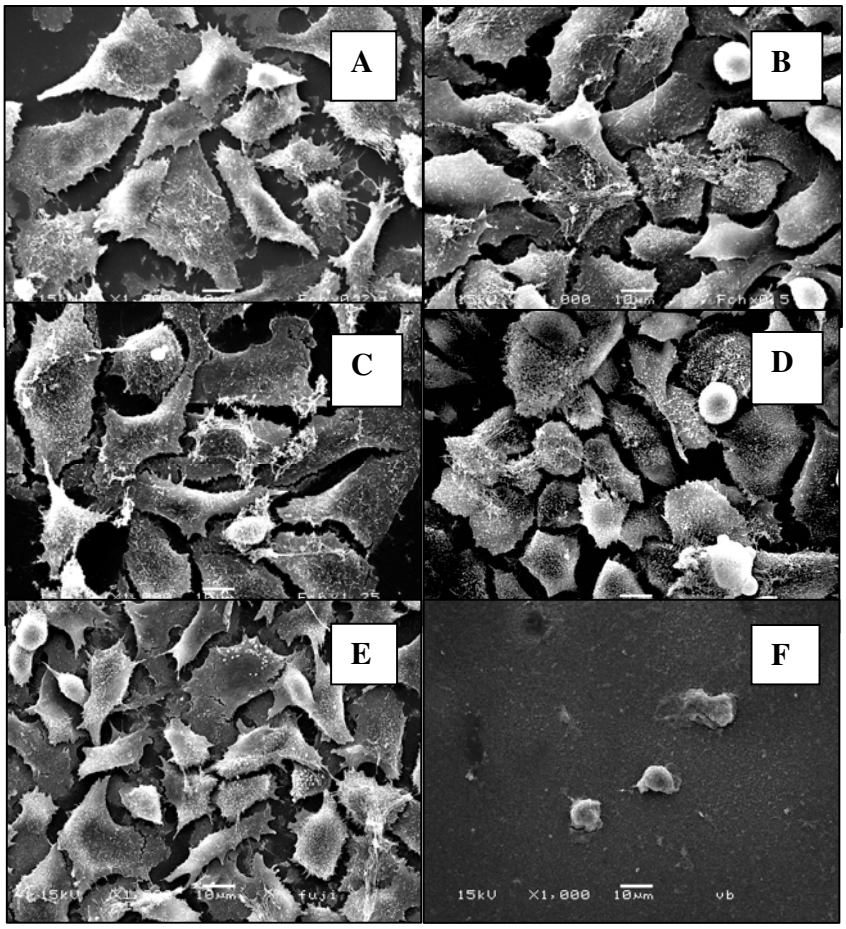

Figure 3. MDPC-23 cells adhered to the glass substrate after exposure to extracts of (A) Fuji Lining LC (FLLC) containing 0.2% chlorhexidine digluconate; (B) FLLC containing 0.5% chlorhexidine digluconate; (C) FLLC containing 1.25% chlorhexidine digluconate; (D) FLLC containing 2.5% chlorhexidine digluconate; (E) Control group – FLLC without chlorhexidine digluconate; and (F) Vitrebond. Normal cell morphology was observed for extracts of FLLC containing chlorhexidine digluconate concentrations up to 1.25%: numerous MDPC-23 cells, near confluence, remained adhered to the glass substrate and exhibited an elongated morphology with several thin cytoplasmatic prolongations originating from their membrane. Cells treated with FLLC containing 2.5% chlorhexidine digluconate exhibited slightly morphological alteration. A small number of cells were observed for Vitrebond (SEM original magnification x1000).

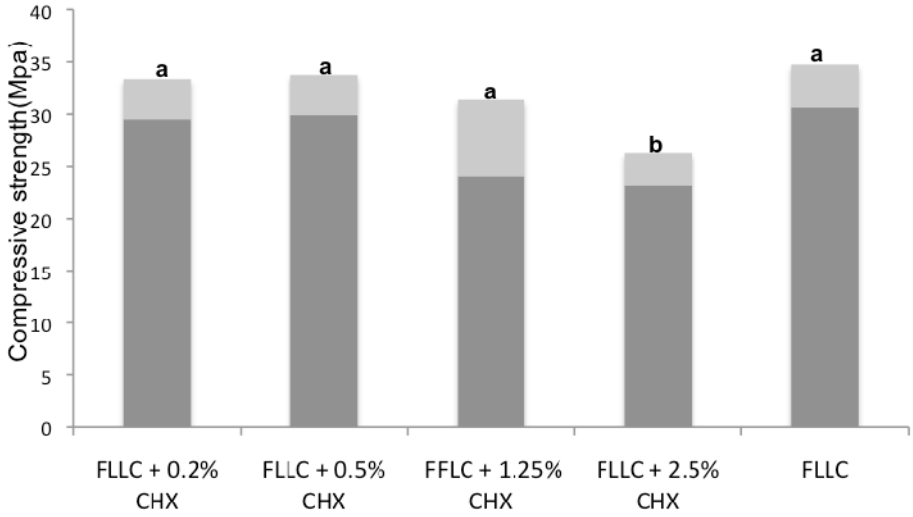

Figure 4. Mean (dark grey) and standard deviation (grey) of compressive strength values obtained for the different groups. Different letters indicate statistically different groups (ANOVA; $p<0.05$).

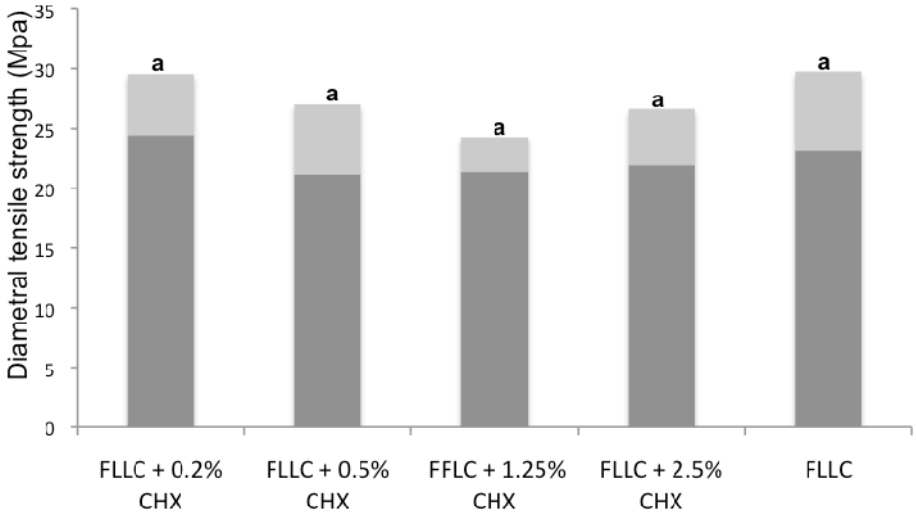

Figure 5. Mean (dark grey) and standard deviation (grey) of diametral tensile strength values obtained for the different groups. Groups identified with the same letter do not differ statistically (ANOVA; $p>0.05$).

Mechanical and biological characterization of resin-modified glass-ionomer cement containing doxycycline hyclate

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Abstract

Objectives

To characterize the mechanical and biological properties of a resin-modified glass ionomer cement (RMGIC) containing doxycycline hyclate.

Methods

The antibacterial effect of RMGIC containing 1.5, 3.0 and 4.5% doxycycline hyclate was assessed using agar diffusion test against *Streptococcus mutans, Lactobacillus acidophilus, Lactobacillus casei* and *Actinomyces viscosus*. Base layers of BHI agar and 300 μL of each inoculum were prepared in Petri dishes with 6 wells (5 mm) that were completely filled up with materials. After 24h incubation, zones of bacterial growth inhibition were measured using digital caliper. Cytotoxicity tests used 50 specimens made in sterilized metal molds, including Vitrebond as positive control. Extracts from every specimen were applied on the MDPC-23 cells for 24h. The MTT assay and SEM evaluated cell metabolism and morphology, respectively. 80 cylindrical specimens (4 mm high x 2 mm diameter) were made from the previously cited groups, and were submitted to universal testing machine (Instron 4411) at a crosshead speed of 1.0 mm/min for compressive and 0.5 mm/min for diametral tensile strength, respectively. Data from antibacterial and cytotoxic effects, and mechanical properties were submitted to appropriated statistical tests ($p<0.05$).

Results

All tested groups showed growth inhibitions of all strains ($p<0.05$), without cause toxic effect to the MDPC-23 cells as well as mechanical properties alterations.

Significance

The incorporation of doxycycline hyclate up to 4.5% into RMGIC inhibit important oral

microorganisms, without modify important biological and mechanical characteristics of the dental material, suggesting a new alternative for treatment of dental caries.

Keywords: Antibiotic; Glass-ionomer cement; Antibacterial activity; Cell culture; Mechanical properties

1. Introduction

Stepwise excavation procedures have been suggested for the management of deep dentinal lesions for both primary and permanent dentitions in order to induce the remineralization of affected dentin and maintain pulp vitality avoiding endodontic treatment [1]. However, after partial caries removal many microorganisms can remain alive in dentin substrate even in the presence of a standard sealing [1], [2], [3] and [4].

Different approaches have been described in the literature adding antimicrobial agents to dental materials, contributing to residual infection control [5]. In fact, antibacterial treatment of the dentine can suppresses the growth of remained bacteria under existing restorations and thus minimizes the risk of recurrent caries and damage to the pulp [5]. Besides, some studies have demonstrated that chlorhexidine associated with glass ionomer cement, although improve the antibacterial effect, can affect the mechanical properties of the mixing [5], [6] and [7].

This way, even the presence of antibiotic into the dental material can be effective against oral pathogens, other important biological and mechanical properties must be evaluated before clinical application. One of them is the pulp response to dental materials, when they are used in deep cavities. Odontoblasts are specialized cells that play a key role in the pulpal healing process and formation of the mineralized tissue barrier [8]. A chemical injury to the primary odontoblasts would impair the repair capacity of the pulpo-dentinal complex by inducing apoptosis or death of these cells due to a cytotoxic effect [9]. Therefore, an ideal antimicrobial agent should also present low or preferably no toxic effects to pulp cells, especially odontoblasts [10].

Tetracyclines have been used both systemically and locally in the treatment of various infectious diseases. It is now recognized that the tetracycline family of antibiotics also can inhibit the catalytic activities of human collagenases and gelatinases especially metalloproteinases - MMPs [11]. The choice for doxycycline hyclate in this study is based in this property, because caries progression is not only dependent on the bacterial activity but is also related to release of MMPs from dentine that may cause acceleration of dentin destruction [12].

Although application of antibiotics for prevention and treatment of dental caries is not frequently recommended since there is a speculation about the risk of development of resistant bacterial strains [13], the lack of agents with marked antimicrobial activity, low cellular toxicity and that to be capable to not modify original mechanical properties have stimulated the search for new alternatives therapies. However, it is important to elucidate that only the indiscriminate use of these drugs would induce microorganism resistance. This study aimed to determine the therapeutic concentration of doxycycline hyclate to be incorporated to resin-modified glass ionomer cement necessary to produce at the same time anticariogenic action without toxic effect on odontoblast-like cells and damage to mechanical properties of the dental material. Three hypotheses were tested when added 1.5%, 3.0% and 4.5% doxycycline hyclate to resin-modified glass ionomer cement (RMGIC): 1. It will improve the antibacterial effect of RMGIC mix; 2. It does not affect odontoblast-like cells – no citotoxity effect; and 3. Its mechanical properties (compression and diametral tensile strength) will no be disturbed.

2. Materials and methods

2.1. Materials

The GC Fuji Lining LC (Lot 0710021, GC Corporation, Tokyo, Japan) liner, a resin-modified glass ionomer cement (RMGIC) was used in this study. It was modified by adding 1.5%; 3.0% and 4.5% doxycycline hyclate (D9891 Sigma-Aldrich, Steinheim, Germany) w/w to the liquid of the GC Fuji Lining LC keeping original ratio of powder/liquid proportion (1.4 g: 1.0 g) [14]. The control group was GC Fuji Lining LC with no antimicrobial agent. Those concentrations were determined previously using minimal inhibitory concentration and minimal bactericidal concentration assays. The composition of the RGMIC is presented at **Table 1**.

2.2. Microbial strains and growth media

Stock cultures of *Streptococcus mutans* (UA159), *Lactobacillus acidophilus* (ATCC#IAL-523), *Lactobacillus casei* (ATCC #193) and *Actinomyces viscosus* (T14V # IAL.5) from Microbiology and Immunology Laboratory of Piracicaba Dental School - University of Campinas, Piracicaba, São Paulo, Brazil were used in this study. For each experiment, cells were cultured freshly from frozen stock on brain–heart infusion broth (BHI; DIFCO Laboratories, Detroit, MI, USA) for 24 hours at 37°C in 10% CO_2 incubator. After confirming viability and absence of contamination by plating in specific medium and Gram techniques, cultures were again grown in BHI for 18-24h at 37^{o}C and adjusted to a concentration of 1 x 10^{8}cells/mL to obtain an inoculum for subsequent tests.

2.3 Agar diffusion test

In each sterilized Petri dish (15 x 90 mm), a base layer containing 15 mL of BHI agar mixed with 300 μL of each inoculum was prepared. After solidification of the culture medium, six wells measuring 5 mm in diameter were made in each plate and completely filled up with one of experimental (RMGIC with doxycycline hyclate 1.5, 3.0 and 4.5%) or control group (RMGIC). All materials were handled under aseptic conditions according to the manufacturer's instructions and inserted into wells using a syringe (Centrix Inc., Shelton, USA). The cements were light activated for 30 seconds using a halogen curing unit (Curing Light XL3000, 3MESPE St Paul, MN, US). The light intensity (410 mW/cm^2) was monitored by a radiometer (Optilux 500, Demetron Kerr, Danbury, CT, US). Ten microliters of 5 mg/mL doxycycline hyclate solution was applied on sterile filter paper discs (n=6), also 5 mm in diameter, which acted as a control of the experiment. The plates were kept for 2 h at room temperature for the diffusion of the materials and then were incubated at 37^{o}C for 24 h. After this period, inhibition zones around the materials were measured with a digital caliper.

2.4 Culture of MDPC-23 Cells

Immortalized cells of the odontoblast-like cell line (MDPC-23) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), with 100 IU/mL penicillin, 100 μg/mL streptomycin and 2 mmol/L glutamine (Gibco) in an humidified incubator with 5% CO^2 and 95% air at 37^{o}C (Isotemp; Fisher Scientific,

Pittsburgh, PA, USA). MDPC-23 cells were sub-cultured every 3 days until an adequate number of cells were obtained for the study. The cells were then seeded (30.000 cells/cm^2/well) in sterile 24-well plates (Costar Corp., Cambridge, MA, USA), which were maintained in the humidified incubator with 5% CO^2 and 95% air at 37^oC for 48 h.

2.5 Analysis of Cell Metabolism by MTT Assay

The RMGIC containing or not 1.5, 3.0 and 4.5% of doxycycline hyclate were hand-mixed and applied into stainless-steel molds with cylindrical apertures. Vitrebond (3MESPE) was considered positive control for this experiment, because have high cytotoxic effect on odontoblastic cells [10]. Ten round-shaped samples of each group (2 mm thick and 4 mm diameter) were prepared, light-cured for 30 seconds and maintained for 1 h at 37^o C - 100% humidity. Then, specimens were inserted separately in sterile 24-well plates containing DMEM medium for 24h. After this period, 800 μL of extract of each well were applied to previously culture MDPC-23 cells for 24h. Eight out of 10 specimens were used for analysis of cell metabolism by the cytochemical demonstration of succinic dehydrogenase (SDH) activity, which is a measure of the mitochondrial respiration of the cells, employing the methyl tetrazolium (MTT) assay. For the MTT assay, the extracts were aspirated and replaced by 900 μL of DMEM plus 100 μL of MTT solution (5 mg/mL sterile PBS; Sigma Chemical Co., St. Louis, MO, USA). Thereafter, the culture medium with the MTT solution was aspirated and replaced by 600 μL of acidified isopropanol solution (0.04 N HCl) in each well to dissolve the formazan crystals, resulting from the cleavage of the MTT salt ring by the SDH enzyme present in the mitochondria of viable cells. Three 100 μL aliquots of each well were transferred to 96-well plates (Costar Corp., Cambridge, MA,

USA). Cell viability was evaluated by spectrophotometry as being proportional to the absorbance measured at 570 nm wavelength with an ELISA microplate reader (model 3550-UV, Bio-Rad Laboratories, Hercules, CA, USA).

The values obtained from the 3 aliquots were averaged to provide a single value for each well. The means were calculated for the groups and transformed into percentages, which represented the inhibitory effect of the mitochondrial activity of the cells by the extracts. The negative control (DMEM) was defined as having 100% of cell metabolism.

2.6 Analysis of Cell Morphology by Scanning Electron Microscopy

Cell morphology was examined by scanning electron microscopy (SEM) using two representative wells of each group. For such purpose, sterile 12-mm-diameter cover glasses (Fisher Scientific) were placed on the bottom of the wells of sterile 24-well plates immediately before seeding of the MDPC-23 cells. Then, the extracts were applied on the cells and the plates were incubated for 24 h, in the same way as described before. Following this period, the extracts were aspirated and the viable cells that remained adhered to the glass substrate were fixed in 1 mL of buffered 2.5% glutaraldehyde for 60 min. The cells were subjected to three 5-min rinses with 1 mL PBS, post-fixed in 1% osmium tetroxide for 60 min and processed for examination with a scanning electron microscope (JEOL-JMS-T33A; JEOL, Tokyo, Japan).

2.7 Measurements of Mechanical Properties

Three experimental groups (RMGIC-containing 1.5, 3 and 4.5% doxycycline hyclate) and one control group (RMGIC) were established as described above for each mechanical

assay, compressive strength (n= 40) and diametral tensile strength (n=40). Briefly, GC Fuji Lining LC was mixed by agglutination of powder to liquid associated or not to doxycycline hyclate at 1.5, 3.0 and 4.5% and then the mixture was placed with Centrix syringe (Centrix Inc., Shelton, USA) into cylindrical molds (4 mm high x 2 mm diameter). After that, they were exposed to a light source (Curing Light XL3000, 3MESPE), with 410 mW/cm^2 of light intensity for 30 seconds. Afterwards, the specimens were stored in distilled water for 24 hours at 37ºC. Specimens were submitted to compressive strength in an Instron universal test machine (4411, Instron Co., Canton, Mass, USA), in vertical position with load at a crosshead speed of 1.0 mm/min until failure occurred. Compressive strength values (kgf/cm^2) were calculated by dividing the load (F) by the cross-sectional area and converted in MPa. Diametral tensile strength was carried out with an Instron universal test machine (4411, Instron Co., Canton, Mass, USA) in horizontal position at 0.5 mm/min crosshead speed. Diametral tensile strength values (kgf/cm^2) were calculated using the equation: DTS = 2F/3.14DT, where F is the failure load, D the diameter, and T the height of the specimen. DTS values were converted into MPa.

2.8 Statistical Analysis

Data from antibacterial and cytotoxic effects were submitted to Kruskal Wallis and Mann-Whitney tests, and those from mechanical properties were submitted to one-way ANOVA and Tukey tests for ($p<0.05$).

3. Results

3.1 Antibacterial activity

The mean values of the inhibition zones for each tested material are shown in **Table 2**. All concentrations added to glass RMGIC produced inhibitory zones against tested cariogenic bacteria. Antibacterial activity of RMGIC containing 3.0% and 4.5% doxycycline hyclate was statistically higher than 1.5% concentration, except to *A. viscosus*. RMGIC control group showed the lowest antibacterial effect, with the lowest inhibition zone.

3.2 Cell Metabolism

The results of cell metabolism response by MTT tests obtained after exposure of the MDPC-23 cells to extracts of RMGICs associated or not with doxycycline hyclate are presented in **Figure 1**. There was no statistically significant difference ($p>0.05$) among the control (DMEM) and experimental groups. All concentrations of doxycycline hyclate did not cause toxic effects to the MDPC-23 cells and were not significantly different from each other ($p>0.05$). The positive control (Vitrebond) was the most cytotoxic to the cultured MDPC-23 cells with decreasing cell metabolism by 95%. Overall, 1.5% and 4.5% doxycycline hyclate concentrations reduced cell metabolism (6% and 3%, respectively) and 3.0% doxycycline hyclate increased cell metabolism in 1%. None of these results was statistically different from that obtained for the control group (DMEM).

3.3 Cell Morphology

In the negative control group (DMEM), the MDPC-23 cells showed normal morphology. They were on confluence and organized as epithelioid nodules (**Figure 2A**). For the experimental groups, cells with similar morphology to those seen in the negative control group were observed. A larger number of cells remained adhered to the glass substrate

when extracts of RMGIC with 1.5, 3.0 and 4.5% doxycycline hyclate was applied to the cells (**Figure 2B, 2C, 2D**), similar to control group (**Figure 2E**). In the positive control group (Vitrebond), the small number of MDPC-23 cells that remained adhered to the glass substrate presented a round shape as well as total loss or maintenance of few cellular processes on the cytoplasmic membrane demonstrating high cell toxicity (**Figure 2F**).

3.4 Measurements of Mechanical tests

The means and standard deviations of the values obtained for mechanical tests are showed in **Figure 3** and **4**. No significant differences were observed among groups for both mechanical tests ($p<0.05$), showing that all tested doxycycline hyclate concentrations did not modify original properties of the liner material.

4. Discussion

Clinical studies have demonstrated that residual bacteria can resist under restoration after partial caries removal procedures for months or until years [1], [2], [3] and [4]. For increasing the success rate of these procedures, many studies have demonstrated the antimicrobial benefits of incorporating chlorhexidine or antibiotics into glass ionomer cement (GIC) [5], [6], [7], [14], [15], [16], [17] and [18]. However, some of these studies have showed that addition of chlorhexidine salts decreased the mechanical properties of GIC [5], [15] and [17]. Besides, high doses of chlorhexidine could have some undesirable responses including inhibit protein synthesis and mitochondrial activity [19] if it is in contact with pulp cells is considered toxic, with a dose-dependent effect [20].

A few studies evaluated the incorporation of antibiotics in GIC and its effect on cariogenic bacteria and on the mechanical properties [14] and [18]. In this study, the addition of doxycycline hyclate to Fuji Lining LC increased antibacterial activity against some important cariogenic bacteria when compared with RMGIC alone (control group). The same way, Yesilyurt et al. [14] observed an increase of inhibitory activity of glass ionomer cement containing ciprofloxacin, metronidazole and minocycline against *S. mutans* and *L. casei*. In this context, Pinheiro et al. [18] observed a reduction of more than 98% of counting of bacteria isolated from infected dentin after partial caries removal of deciduous teeth children and sealing with glass ionomer cement associated with 1% metronidazole, 1% ciprofloxacin and 1% cefaclor.

In the current study, the addition of doxycycline hyclate to the RMGIC did not modify the mechanical properties of the material. No changes were noted in the compressive strength and diametral tensile strength values even with the highest antibiotic concentration added (4.5%). Our results were different that to obtained by Yesilyurt et al. [14]. These authors observed that the associated antibiotics at 3.0 and 4.5% reduced compressive resistance and bond strength to dentin when compared to control group without antimicrobial agents. A low quantity of these antibiotics (1.5%) had a substantial antimicrobial effect without cause significant alterations on mechanical properties. However, they used a three antibiotic mixture, ciprofloxacin, metronidazole and minocycline, added to powdered GIC (Fuji IX) to obtain concentration ratios of 1.5, 3.0 and 4.5% w/w. It has been considered that different kinds of materials mixed with different antibiotics can perform in a different way.

Findings in this study revealed that the addition of doxycycline hyclate to the Fuji Lining LC is better choice than other liner cements [21]. This affirmation is based on antibacterial characteristics of this antibiotic and mainly because this combination did not cause toxicity to pulp cells. Although, the RMGIC Vitrebond provided the greatest inhibition zones against *Streptoccocus mutans, Streptoccocus sobrinus, Lactobacillus acidophilus* and *Actinomyces viscosus*, overcoming the conventional glass ionomers cements Ketac Molar (3M ESPE) and Fuji IX (GC America) [22], it is extremely toxic to odontoblastic-like cells due to the presence of high concentration of resin monomers such as HEMA (more than 80%) in its chemical composition [10]. In addition, according to Hebling et al. [21] the remaining cells from Vitrebond presented intense morphological alterations, confirmed in this study. No morphological alterations of MDPC-23 were verified in this study by adding doxycycline hyclate at 1.5, 3.0 and 4.5% to the Fuji Lining LC. In agreement to our findings, cytotoxic effects of doxycycline hyclate were also not observed in experiments with this antibiotic on seeded fibroblasts, even over weeks of qualitative determination of cell viability at the highest doxycycline hyclate concentrations [23].

In summary, the findings of the current study demonstrated that the incorporation of doxycycline hyclate up to 4.5% concentration into Fuji Lining LC maximizes the antimicrobial activity against oral pathogens, without cause toxic effects to pulp cells or influence on the mechanical properties of the cement. Thus, doxycycline hyclate may be promising candidate for the treatment of dentin after partial caries removal procedures. Usually there is no substance added to dental materials but the combination of antimicrobial agents to restorative materials may be a better protection against cariogenic

bacteria and caries progression. Based on this study, additional *in vivo* studies are recommended in order to demonstrate the antibacterial effect on growth and viability of remaining bacteria in deep cavities when incomplete caries excavation is used.

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REFERENCES

[1] Bjorndal L and Larsen T. Changes in the cultivable flora in deep carious lesions following a stepwise excavation procedure. Caries Res, 2000; 34:502-508.

[2] Weerheijm KL, Kreulen CM, de Soet JJ, Groen HJ and van Amerongen WE. Bacterial counts in carious dentine under restorations: 2-year in vivo effects. Caries Res, 1999; 33:130-134.

[3] Pinto AS, Araújo FB, Franzon R, Figueiredo MC, Henz S and Garcia-Godoy F. Clinical and microbiological effect of calcium hydroxide protection in indirect pulp capping in primary teeth. Am J Dent, 2006; 19:382-387.

[4] Duque C, Negrini Tde C, Sacono NT, Spolidorio DM, de Souza Costa CA and Hebling J. Clinical and microbiological performance of resin-modified glass-ionomer liners after incomplete dentine caries removal. Clin Oral Investig, 2009; 13:465-471.

[5] Takahashi Y, Imazato S, Kaneshiro AV, Ebisu S, Frencken JE and Tay FR. Antibacterial effects and physical properties of glass-ionomer cements containing chlorhexidine for the ART approach. Dent Mater, 2006; 22:647-652.

[6] Sanders BJ, Gregory RL, Moore K and Avery DR. Antibacterial and physical properties of resin modified glass-ionomers combined with chlorhexidine. J Oral Rehabil, 2002; 29:553-558.

[7] Türkün LS, Türkün M, Ertuğrul F, Ateş M and Brugger S. Long-term antibacterial effects and physical properties of a chlorhexidine-containing glass ionomer cement. J Esthet Restor Dent, 2008; 20:29-44.

[8] Arana-Chavez VE and Massa LF. Odontoblasts: the cells forming and maintaining dentine. Int J Biochem Cell Biol, 2004; 36:1367-1373.

[9] Costa CA, Edwards CA and Hanks CT. Cytotoxic effects of cleasing solutions recomended for chemical lavage of pulp exposures. Am J Dent, 2001; 14:25-30.

[10] Souza PP, Aranha AM, Hebling J, Giro EM and Costa CA. In vitro cytotoxicity and in vivo biocompatibility of contemporary resin-modified glass-ionomer cements. Dent Mater, 2006; 22:838-844.

[11] Golub LM, Ramamurthy NS, McNamara TF, Greenwald RA and Rifkin BR. Tetracyclines inhibit connective tissue breakdown: new therapeutic implications for an old family of drugs. Crit Rev Oral Biol Med, 1991; 2:297-322.

[12] Tjäderhane L, Larjava H, Sorsa T, Uitto VJ, Larmas M and Salo T. The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions. J Dent Res, 1998; 77:1622-1629.

[13] Ostela I, Karhuvaara L and Tenovuo J. Comparative antibacterial effects of chlorhexidine and stannous fluoride-amine fluoride containing dental gels against salivary mutans streptococci. Scand J Dent Res, 1991; 99:378-383.

[14] Yesilyurt C, Er K, Tasdemir T, Buruk K and Celik D. Antibacterial activity and physical properties of glass-ionomer cements containing antibiotics. Oper Dent, 2009; 34:18-23.

[15] Jedrychowski JR, Caputo AA and Kerper S. Antibacterial and mechanical properties of restorative materials combined with chlorhexidines. J Oral Rehabil, 1983; 10:373-381.

[16] Botelho MG. Inhibitory effects on selected oral bacteria of antibacterial agents incorporated in a glass ionomer cement. Caries Res, 2003; 37:108-114.

[17] Palmer G, Jones FH, Billington RW and Pearson GJ. Chlorhexidine release from an experimental glass ionomer cement. Biomaterials, 2004; 25:5423-5431.

[18] Pinheiro SL, Simionato MR, Imparato JC and Oda M. Antibacterial activity of glass-ionomer cement containing antibiotics on caries lesion microorganisms. Am J Dent, 2005; 18:261- 266.

[19] Chang YC, Huang FM, Tai KW and Chou MY. The effect of sodium hypochlorite and chlorhexidine on cultured human periodontal ligament cells. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2001; 92:446-450.

[20] Lessa FC, Aranha AM, Nogueira I, Giro EM, Hebling J and Costa CA. Toxicity of chlorhexidine on odontoblast-like cells. J Appl Oral Sci, 2010; 18:50-58.

[21] Hebling J, Lessa FC, Nogueira I, Carvalho RM and Costa CA. Cytotoxicity of resin-based light-cured liners. Am J Dent, 2009; 22:137-142.

[22] Duque C, Negrini Tde C, Hebling J and Spolidorio DM. Inhibitory activity of glass-ionomer cements on cariogenic bacteria. Oper Dent, 2005; 30:636-640.

[23] Myers SA and Wolowacz RG. Tetracycline-based MMP inhibitors can prevent fibroblast-mediated collagen gel contraction in vitro. Adv Dent Res, 1998; 12:86-93.

Table 1 – Composition, batch number of GC Fuji Lining LC (GC America) and antimicrobials used in the study.

Material	Composition	%	Manufacturer	Batch number
Fuji Lining LC* Powder	Alumino-silicate glass	100	GC Corp., Tokyo, Japan	0710021
Fuji Lining LC* Liquid	Polyacrylic acid 2-Hydroxyethyl methacrylate Proprietary Ingredient	65 – 75 8 – 10 5 – 15	GC Corp., Tokyo, Japan	0710021
Doxycycline** Hyclate	Doxycycline hydrochloride hemiethanolate hemihydrate Solubility – H_2O (50 mg/mL)	≥ 98.0% (TLC) ≤ 5% water ≤ 7% ethanol	Sigma-Aldrich, Steinheim, Germany	D9891

*Material Safety Data Sheet information. **Sigma Aldrich (www.sigma-aldrich.com)

Table 2. Mean (Standard Deviation) of inhibition zones obtained for experimental and control group (FLLC without DOX).

Microorganism	EXPERIMENTAL GROUPS			
	FLLC* + 1.5% DOX	**FLLC + 3% DOX**	**FLLC + 4.5% DOX**	**FLLC**
Streptococcus mutans	15.96 (0.70)[a**]	19.69 (0.86)[b]	20.72 (1.28)[b]	9.55 (1.34)[c]
Lactobacillus acidophilus	24.35 (0.39)[a]	29.91(1.57)[b]	28.85 (0.46)[b]	8.3 (0.39)[c]
Lactobacillus casei	13.69 (0.98)[a]	20.20 (1.19)[b]	24.00 (1.72)[c]	8.44 (0.5)[d]
Actinomyces viscosus	14.85 (1.34)[a]	13.45 (1.15)[a]	12.61 (0.40)[a]	9.2 (0.45)[c]

*FLLC – Fuji Lining LC; DOX – doxycycline hyclate

**Means followed by different small letters indicate statistical difference between groups considering each microorganism separately ($p \leq 0.05$).

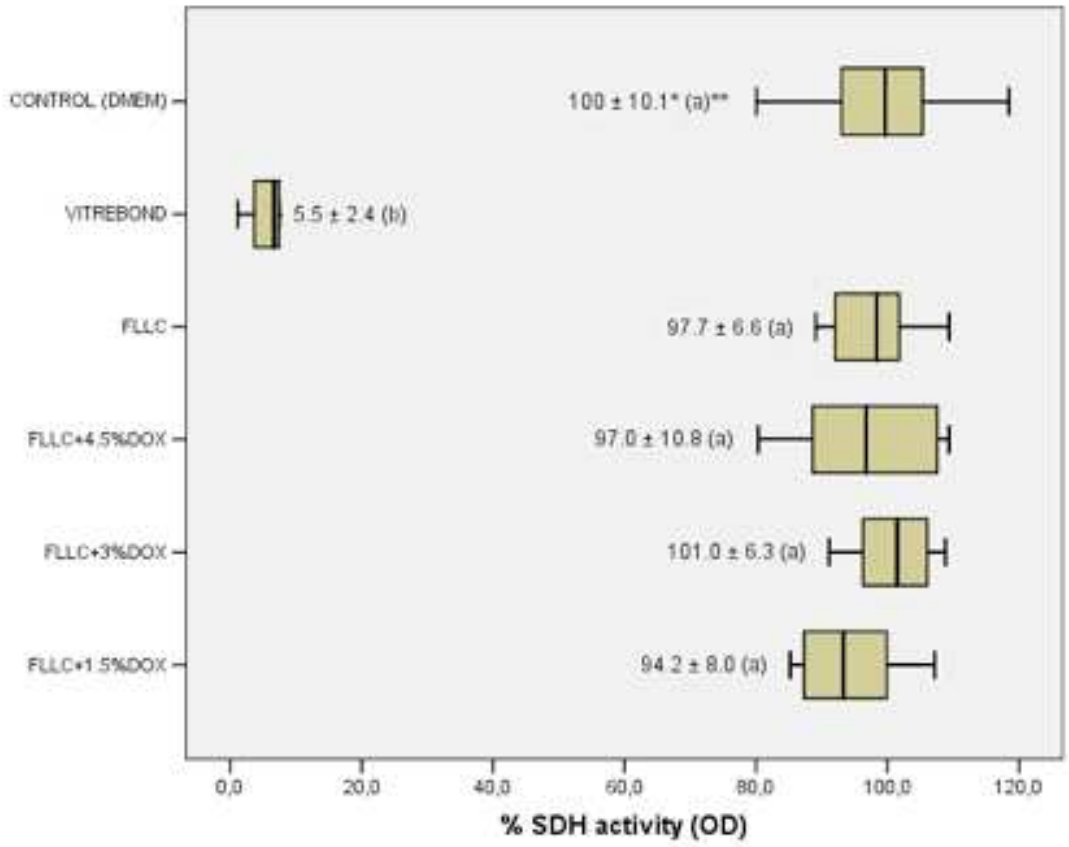


Figure 1. Box-whisker plot (minimum [lower quartile–median–upper quartile] maximum) of the cell metabolism (MTT assay) results for each group. * Mean±standard deviation. The vertical line in the box is the median. ** Groups identified with the same letter do not differ statistically (Mann-Whitney; $p>0.05$).

FLLC – Fuji Lining LC; DOX – doxycycline hyclate

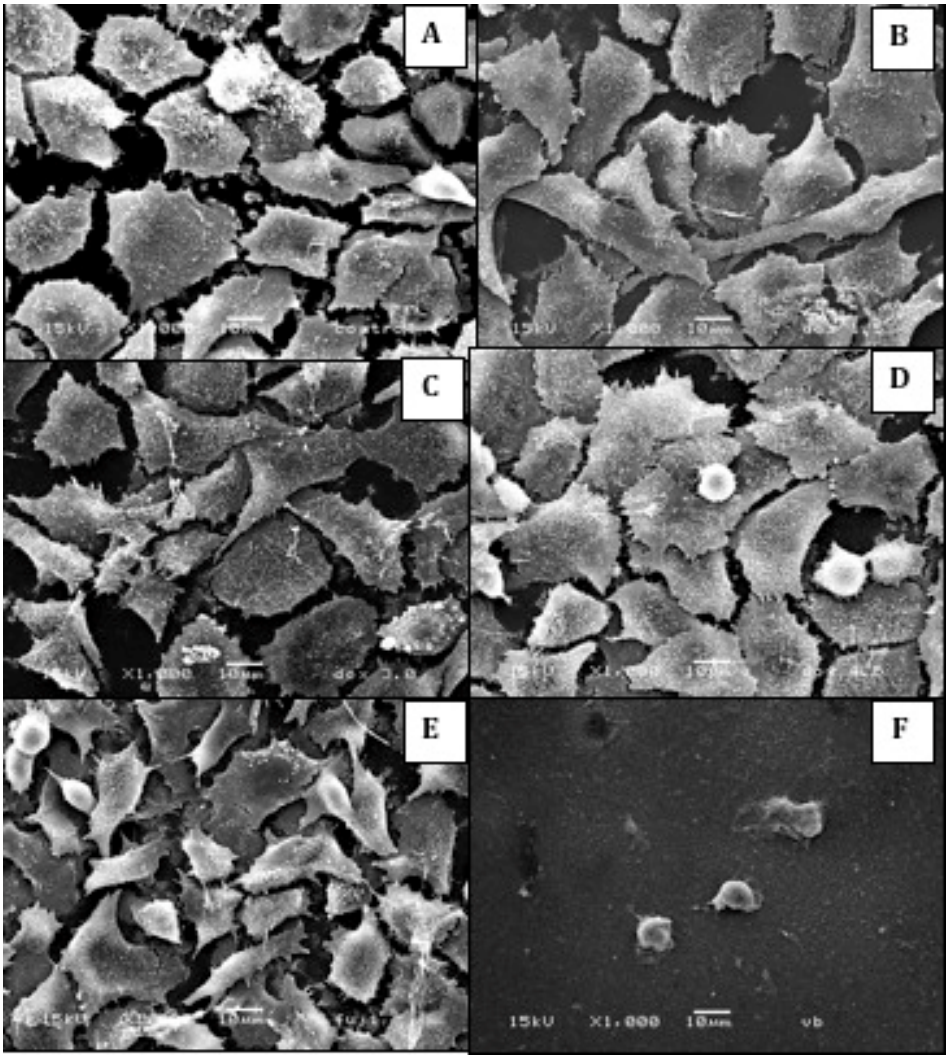


Figure 2. MDPC-23 cells adhered to the glass substrate. Negative control (DMEM) (A). After exposure to extracts of (B) Fuji Lining LC (FLLC) containing 1.5% DOX; (C) FLLC containing 3.0% DOX; (D) FLLC containing 4.5% DOX; (E) Control group – FLLC without DOX; and (F) Vitrebond. Except for Vitrebond, normal cell morphology was observed for all groups: numerous MDPC-23 cells, near confluence, remained adhered to the glass substrate and exhibited an elongated morphology with several thin cytoplasmatic prolongations originating from their membrane (SEM original magnification x1000).

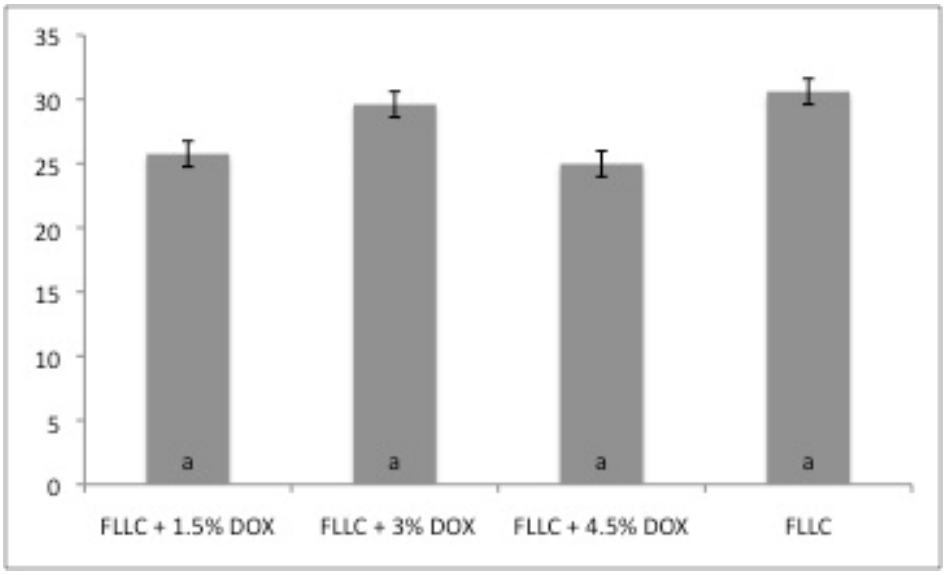


Figure 3. Mean and standard deviation (vertical lines) of compressive strength values obtained for the different groups. Groups identified with the same letter do not differ statistically (ANOVA; $p>0.05$).

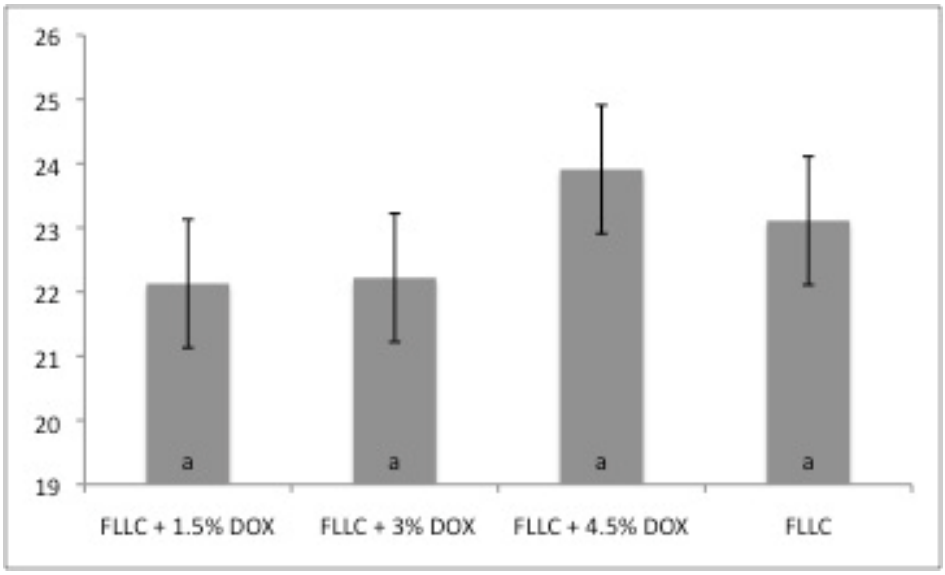


Figure 4. Mean and standard deviation (vertical lines) of diametral tensile strength values obtained for the different groups. Groups identified with the same letter do not differ statistically (ANOVA; $p>0.05$).

Com os procedimentos de mínima intervenção no manejo da cárie dentária em voga, a remoção parcial de cárie em cavidades profundas têm sido amplamente utilizada na tentativa de induzir a reparação dentinária e paralisar o processo carioso, mantendo assim, a vitalidade pulpar (Bjørndal and Larsen, 2000). Sabe-se, no entanto, que após a realização destes procedimentos menos invasivos, microrganismos ainda permanecem viáveis no substrato dentinário, mesmo na presença de adequado selamento da cavidade (Lula et al., 2009).

O cimento de ionômero de vidro foi desenvolvido por Wilson & Kent em 1972 e, desde então, tem variada aplicabilidade na Odontologia, especialmente em Odontopediatria, devido as características desejáveis encontradas neste material (translucidez, estética aceitável, adesão aos tecidos duros dentários, coeficiente de expansão térmica semelhante ao da dentina e liberação de flúor) (Sidhu, 2010). O cimento escolhido para este estudo (Fuji Lining LC) apresenta baixa atividade antibacteriana (Loyola-Rodriguez et al., 1994). Quando associado aos antimicrobianos digluconato de clorexidina ou ao hiclato de doxiciclina, o cimento de ionômero de vidro exibiu propriedade antibacteriana potencializada pela ação das substâncias adicionadas *in vitro,* com efeito inibitório de diferentes microrganismos cariogênicos. Contudo, para que um material tenha relevância clínica, outras propriedades, além de efeito inibitório de bactérias patogênicas, devem também ser consideradas para que a indicação e utilização deste sejam adequadamente empregadas na prática odontológica.

A manutenção da vitalidade do complexo dentino-pulpar é fundamental para a vida funcional do dente, devendo ser considerada prioridade na escolha das estratégias de tratamento odontológico. Tal importância é explicada pelo fato de que as células pulpares, além de manterem a homeostase tecidual após o desenvolvimento dentário, também controlam as reações de defesa em resposta a determinada injúria tecidual e os eventos de reparo que levam à regeneração dentinária (Smith, 2003). Além disso, a presença do fluido

que percorre os túbulos dentinários é fundamental para a manutenção da sensibilidade (Brännström e Aström, 1972) e propriedades mecânicas da dentina (Marshall et al., 1997).

Para que o emprego de substâncias antimicrobianas em materiais odontológicos restauradores seja seguro, concentrações adequadas da solução devem ser utilizadas, levando-se em conta a toxicidade do produto. O digluconato de clorexidina utilizado até a concentração de 1,25% e o hiclato de doxiciclina mesmo em suas maiores concentrações (3% e 4,5%), quando adicionados ao cimento de ionômero de vidro mostraram comportamento biologicamente aceitável, semelhante ao grupo controle negativo, sem provocar alterações na morfologia ou no metabolismo de células pulpares odontoblásticas, comportamento este que justifica sua aplicabilidade na prática clínica.

Em adição às propriedades inibitória e de biocompatibilidade tecidual, a incorporação de aditivos também deve ser capaz de manter as propriedades básicas do material restaurador de forma que haja mínima ou nenhuma modificação na estrutura deste (Turkun et al., 2003). Os ensaios mecânicos de resistência à compressão e à tração diametral são testes comumente utilizados para determinar as propriedades mecânicas de cimentos de ionômero de vidro (Williams et al., 1991; Cattani-Lorente et al., 1994; Gladys et al., 1997; Xie et al., 2000; Pereira et al., 2002; Yap et al., 2003). O ensaio de resistência à compressão, por simular o processo de mastigação, permite uma significativa representação da integridade mecânica do material testado e está indicado para ensaios com materiais friáveis, como os cimentos de ionômero de vidro (Naasan & Watson, 1998). Já o ensaio de resistência à tração diametral, adaptação do teste de resistência à tração para utilização em materiais friáveis, como o concreto e os cimentos de ionômeros de vidro, traduz a propagação de tensão ao longo do corpo do material. Em suma, a determinação da resistência à tração, entre outras propriedades, é de fundamental importância; pois, o estresse mastigatório é o principal responsável por insucessos clínicos de materiais restauradores (McKinney et al., 1987). A adição de digluconato de clorexidina até 1,25% e de hiclato de doxiciclina até 4,5% ao ionômero de vidro estudado não modificou as propriedades mecânicas do cimento.

Embora a incorporação de digluconato de clorexidina e de hiclato de doxiciclina ao cimento de ionômero de vidro aumente a atividade antibacteriana do cimento

contra patógenos cariogênicos, sem afetar células odontoblásticas ou propriedades mecânicas do cimento, estudos adicionais, incluindo avaliação *in vivo*, são recomendados para melhor estabelecer as características e aplicação da associação antimicrobiano-cimento de ionômero de vidro.

Diante dos objetivos e da metodologia empregada no presente estudo, concluiu-se que:

1. A incorporação de substâncias antimicrobianas como digluconato de clorexidina e hiclato de doxiciclina ao cimento de ionômero de vidro potencializa consideravelmente a ação inibitória do cimento contra *Streptococcus mutans, Lactobacillus acidophilus, Lactobacillus casei* e *Actinomyces viscosus*.

2. A adição de digluconato de clorexidina até a concentração de 1,25% e de hiclato de doxiciclina a 4,5% ao cimento de ionômero de vidro é biologicamente aceitável, pois não agride células odontoblásticas MDPC-23.

3. O cimento de ionômero de vidro contendo digluconato de clorexidina a 1,25% ou hiclato de doxiciclina a 4,5% não mostrou alterações em suas propriedades mecânicas.

4. A adição de hiclato de doxiciclina e de digluconato de clorexidina nas concentrações estudadas produziram aumento na atividade antimicrobiana, sem efeitos citotóxicos e alteração nas propriedades mecânicas da mistura, exceto para o digluconato de clorexidina a 2,5%, que apresentou menor resistência a compressão e alteração no metabolismo e morfologia celular.

1. Aranha AM, Giro EM, Souza PP, Hebling J, de Souza Costa CA. Effect of curing regime on the cytotoxicity of resin-modified glass-ionomer lining cements applied to an odontoblast-cell line. Dent Mater. 2006;22(9):864-9.
2. Bjørndal L, Larsen T, Thylstrup A. A clinical and microbiological study of deep carious lesion during stepwise excavation using long treatment intervals. Caries Res. 1997;31(6):411-2.
3. Bjørndal L, Larsen T. Changes in the cultivable flora in deep carious lesions following a stepwise excavation procedure. Caries Res. 2000; 34(6):502-8.
4. Bogren A, Teles RP, Torresyap G, Haffajee AD, Socransky SS, Wennström JL. Locally delivered doxycycline during supportive periodontal therapy: a 3-year study. J Periodontol. 2008;79(5):827-35.
5. Brännström M, Aström A. The hydrodynamics of the dentine; its possible relationship to dentinal pain. Int Dent J. 1972;22(2):219-27.
6. Costa CA, Giro EM, do Nascimento AB, Teixeira HM, Hebling J. Short-term evaluation of the pulpo-dentin complex response to a resin-modified glass-ionomer cement and a bonding agent applied in deep cavities. Dent Mater. 2003;19(8):739-46.
7. Duque C, Negrini T de C, Hebling J, Spolidorio DM. Inhibitory activity of glass-ionomer cements on cariogenic bacteria. Oper Dent. 2005; 30(5):636-40.
8. Emilson CG. Susceptibility of various microorganisms to chlorhexidine. Scand J Dent Res. 1977;85(4):255-65.
9. Falster CA, Araujo FB, Straffon LH, Nör JE. Indirect pulp treatment: in vivo outcomes of an adhesive resin system vs calcium hydroxide for protection of the dentin-pulp complex. Pediatr Dent. 2002;24(3):241-8.

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

10. Franzon R, Casagrande L, Pinto AS, García-Godoy F, Maltz M, de Araujo FB. Clinical and radiographic evaluation of indirect pulp treatment in primary molars: 36 months follow-up. Am J Dent. 2007 Jun;20(3):189-92.
11. Fusayama T. Two layers of carious dentin: diagnosis and treatment. Oper Dent. 1979;4(2):63-70.
12. Gladys S, Van Meerbeek B, Braem M, Lambrechts P, Vanherle G. Comparative physico-mechanical characterization of new hybrid restorative materials with conventional glass-ionomer and resin composite restorative materials. J Dent Res 1997;76:883-94.
13. Golub LM, Ramamurthy N, McNamara T, Greenwald R, Rifkin B Tetracyclines inhibit connective tissue breakdown: new therapeutic implications for an old family of drugs. Crit Rev Oral Biol Med 1991;2(3):297-322.
14. Gruythuysen R, van Strijp G, Wu MK. Long-term survival of indirect pulp treatment performed in primary and permanent teeth with clinically diagnosed deep carious lesions. J Endod. 2010;36(9):1490-3.
15. Gu Y, Lee HM, Sorsa T, Simon SR, Golub LM. Doxycyline inhibits mononuclear cell-mediated connective tissue breakdown. FEMS Immunol Med Microbiol. 2010;58(2):218-25.
16. Hildebrandt GH. Effect of repeated treatment with sustained-release chlorhexidine mouth guards on salivary levels of mutans streptococci. Caries Res. 1996;30(6):445-53.
17. Jenkins S, Addy M, Newcombe R. The effects of a chlorhexidine toothpaste on the development of plaque, gingivitis and tooth staining.J Clin Periodontol. 1993;20(1):59-62.
18. Loyola-Rodriguez JP, Garcia-Godoy F, Lindquist R. Growth inhibition of glass ionomer cements on mutans streptococci. Pediatr Dent. 1994;16(5):346-9.
19. Lula EC, Monteiro-Neto V, Alves CM, Ribeiro CC. Microbiological analysis after complete or partial removal of carious dentin in primary teeth: a randomized clinical trial. Caries Res. 2009;43(5):354-8.
20. Maltz M, de Oliveira EF, Fontanella V, Bianchi R. A clinical, microbiologic, and radiographic study of deep caries lesions after incomplete caries removal. Quintessence

Int. 2002 Feb;33(2):151-9.

21. Maltz M, Oliveira EF, Fontanella V, Carminatti G. Deep caries lesions after incomplete dentine caries removal: 40-month follow-up study. Caries Res. 2007;41(6):493-6. Epub 2007 Oct 5.
22. Marshall GW Jr, Inai N, Wu-Magidi IC, Balooch M, Kinney JH, Tagami J, Marshall SJ. Dentin demineralization: effects of dentin depth, pH and different acids. Dent Mater. 1997;13(6):338-43.
23. McKinney JE, Antonucci JM, Rupp NW. Wear and microhardness of glass-ionomer cements. J Dent Res. 1987 Jun;66(6):1134-9.
24. Naasan MA, Watson TF. Conventional glass ionomers as posterior restorations: a status report for the American Journal of Dentistry. Am J Dent 1998; 11(1): 36-45, 1998.
25. Oliveira EF, Carminatti G, Fontanella V, Maltz M. The monitoring of deep caries lesions after incomplete dentine caries removal: results after 14-18 months. Clin Oral Investig. 2006 Jun;10(2):134-9
26. Paquette DW, Ryan ME, Wilder RS. Locally delivered antimicrobials: clinical evidence and relevance. J Dent Hyg. 2008;82 Suppl 3:10-5
27. Pereira LC, Nunes MC, Dibb RG, Powers JM, Roulet JF, Navarro MF. Mechanical properties and bond strength of glass-ionomer cements. J Adhes Dent 2002;4:73-80.
28. Pinheiro SL, Simionato MR, Imparato JC, Oda M. Antibacterial activity of glass-ionomer cement containing antibiotics on caries lesion microorganisms. Am J Dent. 2005;18(4):261-6.
29. Pucher JJ, Daniel JC. The effects of chlorhexidine digluconate on human fibroblasts in vitro. J Periodontol. 1992;63(6):526-32.
30. Ricketts D. Management of the deep carious lesion and the vital pulp dentine complex. Br Dent J. 2001 Dec 8;191(11):606-10.
31. Sanders BJ, Gregory RL, Moore K, Avery DR. Antibacterial and physical properties of resin modified glass-ionomers combined with chlorhexidine. J Oral Rehabil. 2002;29(6):553-8.
32. Sela MN, Babitski E, Steinberg D, Kohavi D, Rosen G. Degradation of collagen-guided

tissue regeneration membranes by proteolytic enzymes of Porphyromonas gingivalis and its inhibition by antibacterial agents. Clin Oral Implants Res. 2009;20(5):496-502.

33. Sidhu SK. Clinical evaluations of resin-modified glass-ionomer restorations. Dent Mater 2010;26:7-12.
34. Smith AJ.Vitality of the dentin-pulp complex in health and disease: growth factors as key mediators. J Dent Educ. 2003;67(6):678-89.
35. Takahashi Y, Imazato S, Kaneshiro AV, Ebisu S, Frencken JE, Tay FR. Antibacterial effects and physical properties of glass-ionomer cements containing chlorhexidine for the ART approach. Dent Mater. 2006;22(7):647-52.
36. Türkün LS, Türkün M, Ertugrul F, Ates M, Brugger S. Long-term antibacterial effects and physical properties of a chlorexidine-containing glass ionomer cement. J Esthet Restor Dent 2008;20(1):29-44.
37. Williams JA, Billington RW. Changes in compressive strength of glass ionomer restorative materials with respect to time periods of 24 h to 4 months. J Oral Rehabil 1991;18:163-8.
38. Wilson AD, Kent BE. A new translucent cement for dentistry - the glass ionomer cement. Br Dent J 1972;132:133-5.
39. Xie D, Brantley WA, Culbertson BM, Wang G. Mechanical properties and microstructures of glass-ionomer cements. Dent Mater 2000;16:129-38.
40. Yap AUJ, Pek YS, Cheang P. Physico-mechanical properties of a fast-set highly viscous GIC restorative. J Oral Rehabil 2003;30:1-8.

APÊNDICE 1. Ilustrações da metodologia empregada nos capítulos 1 e 2.

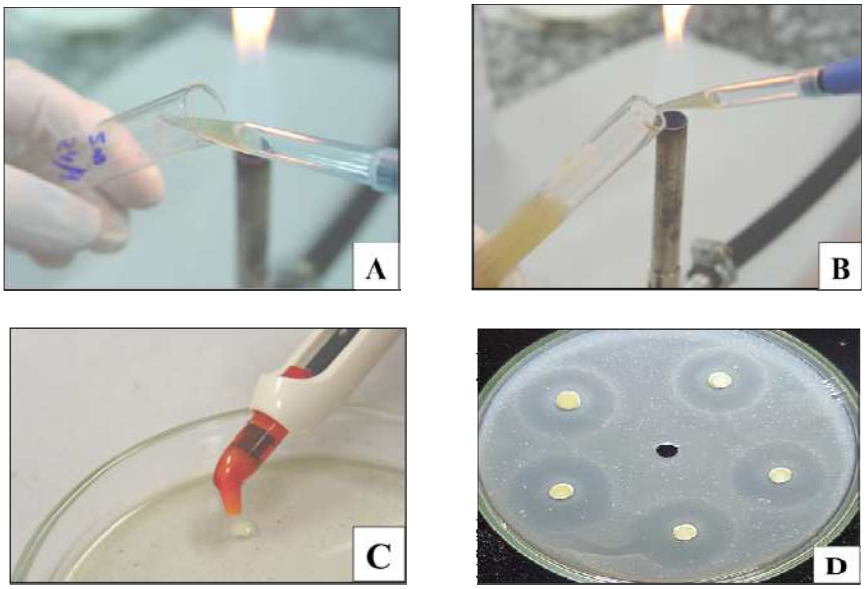
Sequência do método de difusão em ágar.

A - Preparo dos inóculos de *Streptococcus mutans, Lactobacillus acidophilus, Lactobacillus casei* e *Actinomyces viscosus* em meio de cultura Brain-Heart-Infusion (BHI; DIFCO Laboratories, Detroit, MI, USA).

B - Ajuste da concentração de células bacterianas em 1 x 10^8mo/mL para utilização no preparo das placas base contendo 15 mL de BHI e 300 µL de inóculo.

C - Preenchimento dos poços com um dos materiais estudados, com auxílio de uma seringa (Centrix Inc., Shelton, USA).

D – Halos de inibição produzidos após 24h de incubação a 37^{o}C.



APÊNDICE 2. Ilustrações da metodologia empregada nos capítulos 1 e 2.

Sequência do ensaio de citotoxicidade pelo método MTT.

A - Incorporação dos antimicrobianos ao cimento de ionômero de vidro.

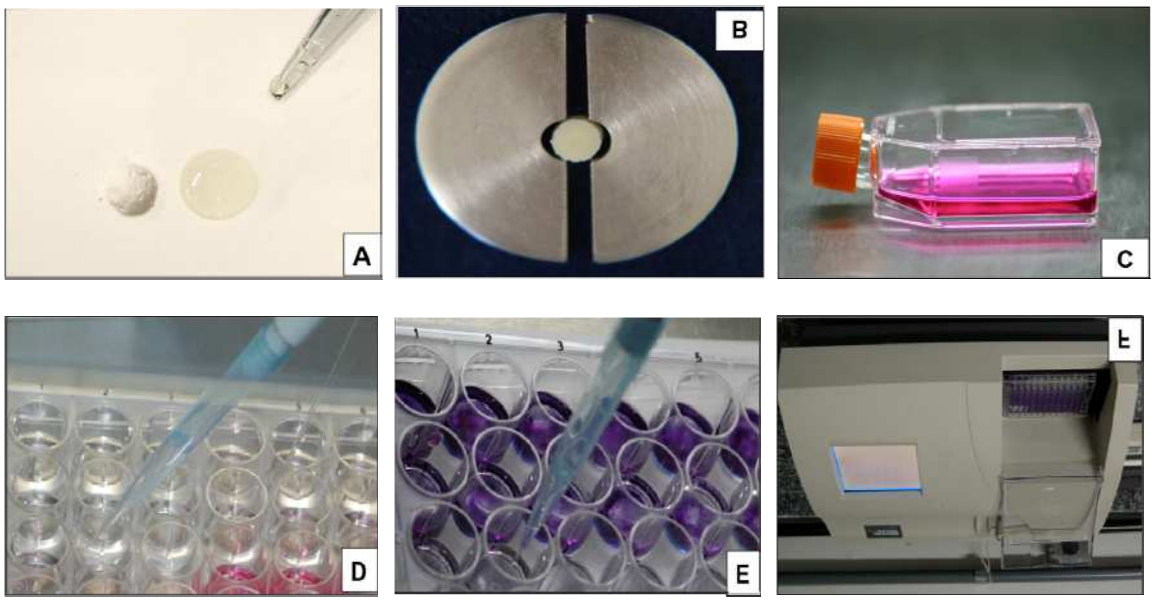
B - Preparo dos corpos-de-prova (4mm X 2mm) utilizando-se matrizes metálicas.

C - Cultura de células de linhagem odontoblástica MDPC-23.

D - Aspiração dos extratos produzidos pelos corpos-de-prova em contato com células MDPC-23 em meio de cultura DMEM, seguida da adição de solução de MTT (5 mg/mL PBS; Sigma Chemical Co., St. Louis, MO, USA).

E - Aplicação de solução acidificada de isopropanol (0.04 N HCl).

F - Análise da viabilidade celular por espectofotometria em leitor de Elisa (modelo 3550-UV, Bio-Rad Laboratories, Hercules, CA, USA).

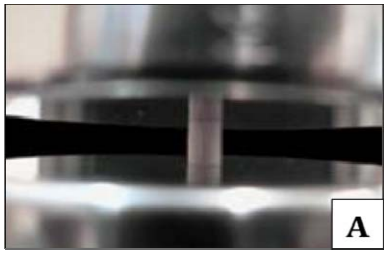


APÊNDICE 3. Ilustrações da metodologia empregada nos capítulos 1 e 2.

Figuras dos ensaios mecânicos.

A - Resistência à compressão.

B - Resistência à tração diametral.



ANEXO 1 - Resolução CCPG/002/06 a qual dispõe a respeito do formato das teses de mestrado e doutorado aprovados pela UNICAMP (Parte I).

INFORMAÇÃO CCPG/OO2/066

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.

6 Disponível em: http://www.prpg.unicamp.br/ccpg_inf002_06.pdf

ANEXO 1 - Resolução CCPG/002/06 a qual dispõe a respeito do formato das teses de mestrado e doutorado aprovados pela UNICAMP (Parte II).

§ 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 - Certificado do Comitê de Ética em Pesquisa

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 das propriedades biológicas e físico-mecânicas de cimentos de ionômero de vidro associados à clorexidina ou à doxiciina como materiais forradores em procedimentos de remoção parcial de cárie**", protocolo nº **031/2008**, dos pesquisadores **ALINE ROGÉRIA FREIRE DE CASTILHO, ANDRÉIA BOLZAN DE PAULA, CRISTIANE DUQUE e REGINA MARIA PUPPIN RONTANI**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 26/05/2008.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Evaluation of biological and physico-mechanical properties of glass ionomer cements associated to chlorhexidine or doxycycline as liner material in procedures of partial caries removal**", register number **031/2008**, of **ALINE ROGÉRIA FREIRE DE CASTILHO, ANDRÉIA BOLZAN DE PAULA, CRISTIANE DUQUE and REGINA MARIA PUPPIN RONTANI**, 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 26/05/2008.

Prof. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
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.