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

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### ARTICLE INFO

#### Article history:

Accepted 18 August 2011

#### Keywords:

Antibiotic  
 Glass-ionomer cement  
 Antibacterial activity  
 Cell culture  
 Mechanical properties

### 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 two experiments – agar diffusion test for 24 h and biofilm assay for 24 h and 7 days – against some cariogenic bacteria. Briefly, base layers of BHI agar and 300  $\mu$ L of each inoculum were prepared in Petri dishes with 6 wells that were completely filled with materials. After 24 h incubation, zones of bacterial growth inhibition were measured using a digital caliper. Biofilm assays were conducted using RMGIC specimens immersed in 24-well plates containing the inoculum in BHI broth. After 24 h and 7 days, each specimen were removed, vortexed and the suspension diluted and inoculated in BHI plates for subsequent bacterial counting. 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 odontoblast-like cells for 24 h. The MTT assay and SEM evaluation determined cell metabolism and morphology, respectively. 80 cylindrical specimens were made from the previously cited groups, and were submitted to testing with a universal testing machine (Instron 4411) using a crosshead speed of 1.0 mm/min for compressive strength 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.

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doi:10.1016/j.archoralbio.2011.08.009

**Results:** All tested groups showed growth inhibition of all tested strains ( $p < 0.05$ ) in 24 h for both microbiological tests, but only 4.5% doxycycline have antibacterial effect after 7 days. None of doxycycline concentrations caused toxic effect to the MDPC-23 cells or presenting alterations to mechanical properties.

**Conclusion:** The incorporation of up to 4.5% doxycycline hyclate into RMGIC inhibits important oral microorganisms, without modifying biological and mechanical characteristics of the dental material, suggesting a new alternative for the treatment of dental caries.

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## 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 dentine and maintain pulp vitality, all whilst avoiding endodontic treatment.<sup>1</sup> However, after partial caries removal, many microorganisms can remain alive in the dentine substrate, even in the presence of a standard sealing.<sup>1–4</sup>

Different approaches have been described in the literature, such as adding antibacterial agents to dental materials, to provide residual infection control.<sup>5</sup> In fact, antibacterial treatment of the dentine can suppress the growth of remaining bacteria under existing restorations, thus minimizing the risk of recurrent caries and damage to the pulp.<sup>5</sup> Studies have demonstrated that chlorhexidine associated with glass ionomer cement, whilst improving the antibacterial effect of the material, can affect its mechanical properties.<sup>5–7</sup>

Whilst the presence of an antibiotic in a dental material can be effective against oral pathogens, other important biological and mechanical properties must be evaluated before clinical application. One evaluation that should be completed before clinical use is the pulp response to dental materials when 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.<sup>8</sup> A chemical injury to the primary odontoblasts would impair the repair capacity of the pulp-dentinal complex by inducing apoptosis, or death, of these cells due to a cytotoxic effect.<sup>9</sup> Therefore, an ideal antibacterial agent should also present low, or preferably no, toxic effects to pulp cells, especially odontoblasts.<sup>10</sup>

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 inhibits the catalytic activities of human collagenases and gelatinases, especially metalloproteinases (MMPs).<sup>11</sup> The choice of doxycycline hyclate in this current study is based in this property, because caries progression is not only dependent on the bacterial activity but also related to the release of MMPs from dentine that may cause acceleration of dentine destruction.<sup>12</sup>

Although the application of antibiotics for the prevention and treatment of dental caries is not frequently recommended, since there is a speculation about the risk of development of resistant bacterial strains,<sup>13</sup> the lack of agents with marked antibacterial activity, low cellular toxicity and that have no modifications to the original mechanical

properties of the restorative materials has stimulated the search for new alternative therapies. However, it is important to note that only the indiscriminate use of these drugs would induce microorganism resistance.

This present study determined the necessary therapeutic concentration of doxycycline hyclate to be incorporated into a resin-modified glass ionomer cement to produce an anti-carious action without either a toxic effect on odontoblast-like cells or reducing the mechanical properties of the dental material. Three hypotheses were tested when adding 1.5%, 3.0% and 4.5% doxycycline hyclate to a resin-modified glass ionomer cement (RMGIC): 1. the antibacterial effect of the RMGIC mix is improved; 2. there is no cytotoxic effect to the odontoblast-like cells; and 3. the mechanical properties (compression and diametral tensile strength) of the RMGIC will not be changed.

## 2. Materials and methods

### 2.1. Dental 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. This material was modified by adding 1.5%; 3.0% or 4.5% doxycycline hyclate (D9891 Sigma–Aldrich, Steinheim, Germany) (w/w) to the liquid of the GC Fuji Lining LC whilst keeping original powder/liquid ratio (1.4 g:1.0 g).<sup>14</sup> The control group was GC Fuji Lining LC with no antibacterial agent. The concentrations used in this current study were determined previously using minimal inhibitory concentration and minimal bactericidal concentration assays. The composition of the RGMIC is presented in Table 1.

### 2.2. Antibacterial tests

#### 2.2.1. 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), obtained from the Microbiology and Immunology Laboratory of Piracicaba Dental School – University of Campinas, Piracicaba, São Paulo, Brazil, were used. For each experiment, cells were cultured freshly from frozen stock on brain-heart infusion broth (BHI; DIFCO Laboratories, Detroit, MI, USA) for 24 h at 37 °C in a 10% CO<sub>2</sub> incubator. After confirming the viability and absence of contamination by plating in a specific medium and using Gram techniques, cultures were again grown in BHI for 18–24 h at 37 °C and adjusted to a concentration of 10<sup>8</sup> cells/mL to obtain an inoculum for subsequent testing.

**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 <sup>a</sup> Powder	Alumino-silicate glass	100	GC Corp., Tokyo, Japan	0710021
Fuji Lining LC <sup>a</sup> Liquid	Polyacrylic acid 2-Hydroxyethyl methacrylate Proprietary ingredient	65–75 8–10 5–15	GC Corp., Tokyo, Japan	0710021
Doxycycline <sup>b</sup> hyclate	Doxycycline hydrochloride hemimethanolate hemihydrate Solubility – H <sub>2</sub> O (50 mg/mL)	≥98.0% (TLC) ≤5% water ≤7% ethanol	Sigma–Aldrich, Steinheim, Germany	D9891

<sup>a</sup> Material safety data sheet information.  
<sup>b</sup> Sigma Aldrich ([www.sigma-aldrich.com](http://www.sigma-aldrich.com)).

### 2.2.2. Agar diffusion test

In each sterilized Petri dish (15 mm × 90 mm), a base layer containing 15 mL of BHI agar, mixed with 300 mL of each inoculums (*S. mutans*, *L. acidophilus*, *L. casei* and *A. viscosus*), was prepared. After solidification of the culture medium, six wells with a 5 mm diameter were made in each plate and completely filled with one of the experimental materials (RMGIC with doxycycline hyclate 1.5, 3.0 and 4.5%) or with the control (RMGIC). All materials were inserted into wells using a syringe (Centrix Inc., Shelton, USA) and were handled under aseptic conditions and according to the manufacturer's instructions. The cements were light activated for 30 s using a halogen curing unit (Curing Light XL3000, 3MESPE, St. Paul, MN, US). The light intensity (410 mW/cm<sup>2</sup>) 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 were then incubated at 37 °C for 24 h in a 10% supplemented CO<sub>2</sub> environment. After incubation, inhibition zones around the materials were measured using a digital calliper.

### 2.2.3. Biofilm assay

The objective was to evaluate the ability of doxycycline incorporated in RMGI to interfere in biofilm formation. To prepare the inoculum, *S. mutans*, *L. acidophilus* and *A. viscosus* were grown as previously described in item 2.2. The RMGIC containing 0, 1.5, 3.0 and 4.5% doxycycline hyclate were hand-mixed and applied into stainless-steel moulds with cylindrical apertures. After polymerization, six specimens of each material (4 mm thick and 2 mm diameter) were inserted in a single well of 24-well polystyrene plates (Costar Corp., Cambridge, MA, USA), with 1 mL of sterile fresh BHI broth. After 1 h, 10 µL of each inoculum adjusted to an optical density (OD) of 0.6 at 550 nm (approximately 10<sup>8</sup> CFU/mL) were inserted in each well. These plates were incubated at 37 °C for 24 h and 7 days in a 10% supplemented CO<sub>2</sub> environment. Culture medium was changed every three days. After these periods, each specimen was washed two times with saline solution (0.9% NaCl) to remove non-adhered cells and then inserted into microtubes containing glass beads in saline solution and vortexed for 1 min. The suspension was diluted in decimal series from 10<sup>-1</sup> to 10<sup>-6</sup> and inoculated in triplicate on BHI agar plates. The colonies were counted and the number of viable bacteria was determined in CFU/mL that corresponded to the cells adhering to the GIC cements after bacterial exposure.

### 2.3. Toxicity on MDPC-23 odontoblastic-like cells

#### 2.3.1. Culture of cells

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% foetal bovine serum (Gibco, Grand Island, NY, USA), 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L glutamine (Gibco) in a humidified incubator at 5% CO<sub>2</sub> and 95% air at 37 °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<sup>2</sup>/well) in sterile 24-well plates (Costar Corp., Cambridge, MA, USA), which were maintained in a humidified incubator at 5% CO<sub>2</sub> and 95% air at 37 °C for 48 h.

#### 2.3.2. Analysis of cell metabolism by MTT assay

The RMGIC containing 0, 1.5, 3.0 and 4.5% doxycycline hyclate were hand-mixed and applied into stainless-steel moulds with cylindrical apertures. Vitrebond (3MESPE) was used as a positive control for this experiment, because it has a high cytotoxic effect on odontoblastic cells.<sup>10</sup> Ten round-shaped samples of each group (2 mm thick and 4 mm diameter) were prepared, light-cured for 30 s and maintained for 1 h at 37 °C in 100% humidity. The specimens were then inserted separately in sterile 24-well plates containing DMEM medium for 24 h. An 800 µL extract from each well was applied to previously cultured MDPC-23 cells for 24 h. Eight out of 10 specimens were used for analysis of cell metabolism using the cytochemical demonstration of succinic dehydrogenase (SDH) activity, which is a measure of the mitochondrial respiration of the cells, using 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 with 600 µL of acidified isopropanol solution (0.04 N HCl) in each well to dissolve the 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

**Table 2 – Mean (standard deviation) of inhibition zones (mm) obtained by agar diffusion test.**

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

FLLC – Fuji Lining LC; DOX – doxycycline hyclate.

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

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% cell metabolism.

### 2.3.3. Analysis of cell morphology by scanning electron microscopy

Cell morphology was examined by scanning electron microscopy (SEM) using two representative wells of each group. Sterile 12-mm-diameter cover glasses (Fisher Scientific) were placed on the bottom of the wells of sterile 24-well plates immediately before seeding the MDPC-23 cells. Then, the extracts were applied on the cells and the plates were incubated for 24 h, as described before. Following incubation, the extracts were aspirated and the viable cells that were adhered to the glass substrate were fixed using 1 mL of buffered 2.5% glutaraldehyde for 60 min. The cells were subjected to three 5-min rinses using 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).

### 2.4. Measurements of mechanical properties

Three experimental groups (RMGIC-containing 1.5, 3 and 4.5% doxycycline hyclate) and one control group (RMGIC without antibiotic) were prepared as described above for each mechanical assay, compressive strength test ( $n = 40$ ) and diametral tensile strength test ( $n = 40$ ). Briefly, GC Fuji Lining LC was mixed by agglutination of powder to liquid associated without or with 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 moulds (4 mm high  $\times$  2 mm diameter). The specimens were then exposed to a light source (Curing Light XL3000, 3MESPE), with 410 mW/cm<sup>2</sup> of light

intensity for 30 s. Afterwards, the specimens were stored in distilled water for 24 h at 37 °C. Specimens were submitted to compressive strength in an Instron universal test machine (4411, Instron Co., Canton, MA, USA), in a vertical position with a load at a crosshead speed of 1.0 mm/min until failure occurred. Compressive strength values (kgf/cm<sup>2</sup>) 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, MA, USA) in a horizontal position and at a 0.5 mm/min crosshead speed. Diametral tensile strength values (kgf/cm<sup>2</sup>) were calculated using the equation:  $DTS = 2F/3.14DT$ , where  $F$  was the failure load,  $D$  was the diameter, and  $T$  was the height of the specimen. DTS values were converted into MPa.

### 2.5. Statistical analysis

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

## 3. Results

### 3.1. Antibacterial activity

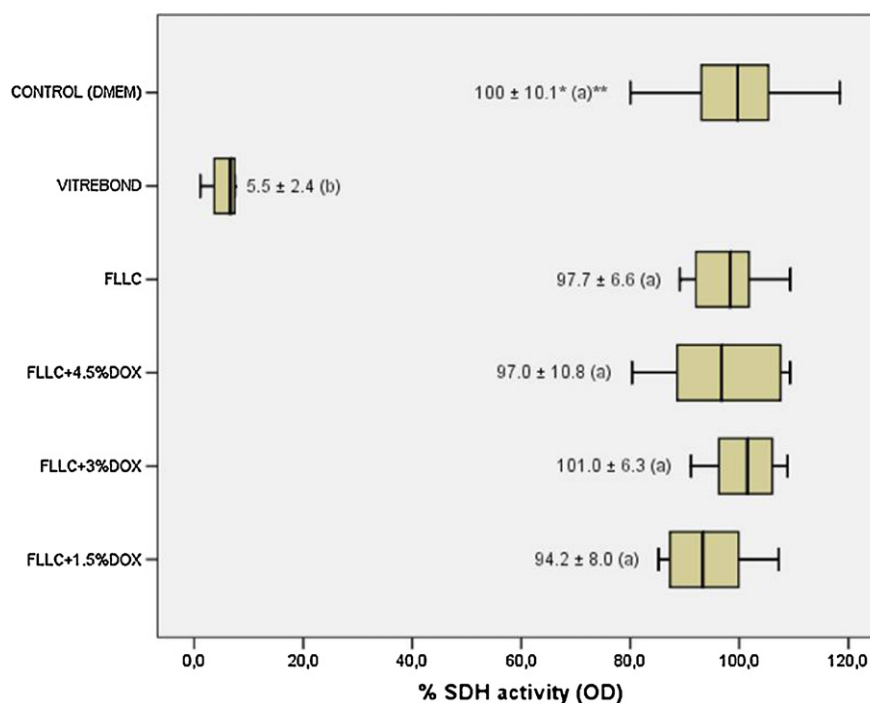
The mean values of the inhibition zones for each tested material are shown in Table 2. All concentrations added to RMGIC produced inhibitory zones against the tested cariogenic bacteria. The antibacterial activity of RMGIC containing 3.0% and 4.5% doxycycline hyclate was statistically higher than the 1.5% concentration, except with *A. viscosus*. The RMGIC control group showed the lowest antibacterial effect, with the smallest inhibition zone. Table 3 shows medians and range

**Table 3 – Median (minimum–maximum) of log(UFC) counts obtained by biofilm test.**

Time	Bacteria	FLLC	FLLC + 1.5% DOX	FLLC + 3.0% DOX	FLLC + 4.5% DOX
24 h	<i>Sm</i>	7.04 (6.85–7.21) <sup>a</sup>	5.68 (5.61–6.08) <sup>b</sup>	5.52 (5.40–5.56) <sup>b</sup>	5.26 (5.09–5.47) <sup>b</sup>
	<i>La</i>	9.35 (9.21–9.36) <sup>a</sup>	5.16 (4.98–6.51) <sup>b</sup>	5.17 (5.02–5.19) <sup>b</sup>	4.56 (4.08–5.21) <sup>b</sup>
	<i>Av</i>	7.18 (7.10–7.27) <sup>a</sup>	5.49 (5.11–6.08) <sup>b</sup>	5.53 (5.42–5.55) <sup>b</sup>	5.23 (5.15–5.39) <sup>b</sup>
7 d	<i>Sm</i>	7.77 (6.45–9.23) <sup>a</sup>	7.28 (7.15–9.43) <sup>a</sup>	8.69(7.41–9.08) <sup>a</sup>	7.20(6.08–8.19) <sup>b</sup>
	<i>La</i>	9.08 (8.81–10.08) <sup>a</sup>	9.08 (9.08–9.08) <sup>a</sup>	9.08 (9.08–9.08) <sup>a</sup>	3.02(2.83–4.08) <sup>b</sup>
	<i>Av</i>	9.30 (6.92–10.08) <sup>a</sup>	8.08 (6.48–8.62) <sup>a</sup>	6.94 (6.08–7.88) <sup>a</sup>	5.20(4.08–5.20) <sup>b</sup>

FLLC – Fuji Lining LC; DOX – doxycycline hyclate; *Sm* – *Streptococcus mutans*; *La* – *Lactobacillus acidophilus*; *Av* – *Actinomyces viscosus*.

Medians followed by different small letters indicate statistical difference between groups of materials considering each microorganism and time separately ( $p \leq 0.05$ ).



**Fig. 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.

(minimum–maximum) of bacterial counts expressed as  $\log(\text{UFC})$  after biofilm assays. In 24 h, all doxycycline concentrations reduced the number of bacteria adhered in the RMGIC. After 7 days, only 4.5% doxycycline had effect on the tested microorganisms.

### 3.2. Odontoblast-like cells metabolism

The results of cell metabolism response by MTT tests obtained after exposure of the MDPC-23 cells to extracts of RMGICs with or without doxycycline hyclate are presented in Fig. 1. There was no statistically significant difference ( $p > 0.05$ ) amongst the control (DMEM) and experimental groups. None of the concentrations of doxycycline hyclate caused 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 by 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 confluent and organized as epithelioid nodules (Fig. 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

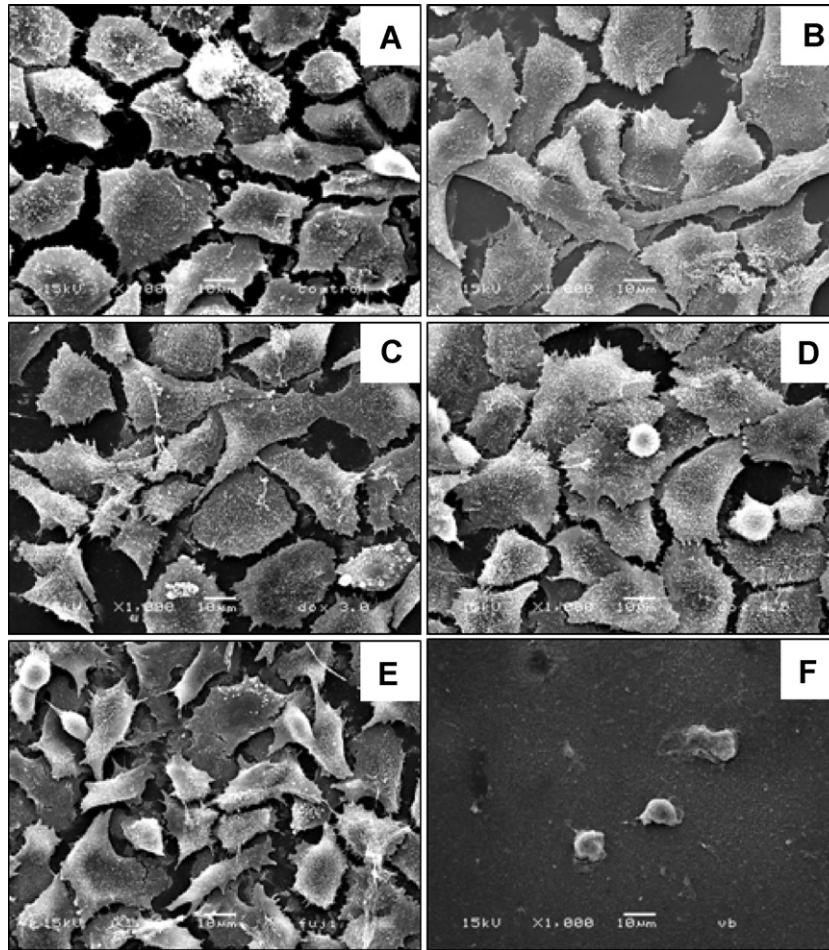
RMGIC with 1.5, 3.0 and 4.5% doxycycline hyclate was applied to the cells (Fig. 2B–D), similar to the control group (Fig. 2E). In the positive control group (Vitrebond), the small number of MDPC-23 cells that remained adhered to the glass substrate presented a round shape as well as a total loss or maintenance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

### 3.4. Measurements of mechanical tests

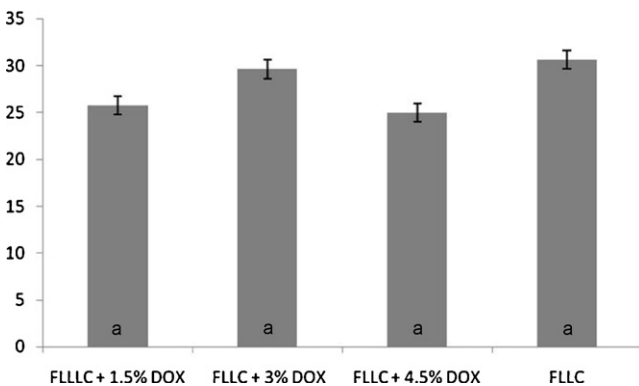
The means and standard deviations of the values obtained for mechanical testing are shown in Figs. 3 and 4. No significant differences were observed amongst groups for both mechanical tests ( $p < 0.05$ ), showing that none of the tested doxycycline hyclate concentrations modified the original properties of the liner material.

## 4. Discussion

Clinical studies have demonstrated that residual bacteria can persist under restorations after partial caries removal procedures for months or years.<sup>1–4</sup> In order to increase the success rate of these restorative procedures, many studies have demonstrated the antibacterial benefits of incorporating antimicrobials into a glass ionomer cement (GIC).<sup>5–7,14–18</sup> However, few studies evaluated the addition of antibiotics into GIC and its effect on cariogenic bacteria.<sup>14,18</sup> Yesilyurt et al.<sup>14</sup> observed an increase of inhibitory activity of a glass ionomer cement containing ciprofloxacin, metronidazole and minocycline against *S. mutans* and *L. casei*. Pinheiro et al.<sup>18</sup>



**Fig. 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 1000×).**

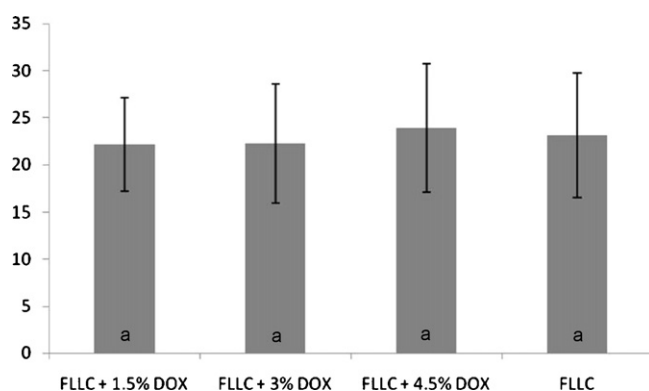


**Fig. 3 – Mean and standard deviation (vertical lines) of compressive strength values obtained for the different groups. Groups identified with the same letter do not differ statistically (ANOVA;  $p > 0.05$ ).**

observed a reduction of more than 98% of bacteria counts isolated from infected dentine after partial caries removal of deciduous teeth in children and sealing the preparations with glass ionomer cement associated with 1% metronidazole, 1% ciprofloxacin and 1% cefaclor.

In this current study, the addition of doxycycline hyclate to Fuji Lining LC increased the antibacterial activity against some important cariogenic bacteria when compared with RMGIC alone (control group). Both antibacterial tests showed significant improvement in the inhibitory action of RMGIC when doxycycline was incorporated. All tested doxycycline concentrations have similar antibacterial effect reducing adherence of microorganisms to glass ionomer cement and consequently interfering in the biofilm formation, after 24 h of incubation. However, only 4.5% doxycycline was effective against tested bacteria after 7 days. *L. acidophilus* was the most sensible bacteria to the antibiotic.

In the present study, doxycycline hyclate was chosen to be incorporated in the RMGIC because of its antibacterial properties and effect on MMP (metalloproteinases) activity. Tetracyclines and their semi-synthetic forms, doxycycline



**Fig. 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;  $p > 0.05$ ).**

and minocycline, have polycyclic structure, amphoteric character and chelating properties binding with bi- and trivalent ions such as iron, aluminium, calcium and magnesium. They are specific inhibitors of prokaryotic ribosome, blocking receptors in 30S subunit that bind to t-RNA, inhibiting the synthesis of protein. Human cells are not affected by tetracycline because eukaryotic ribosomes are structurally different from prokaryotic ones. Because of this, they are considered safe to human cells and effective against gram positive and gram negative, anaerobic and aerobic bacteria.<sup>19</sup> Another property of tetracyclines is to inhibit both activity and secretion of MMPs. Studies have demonstrated their action on MMP-1, MMP-2 and MMP-12, both *in vitro* and *in vivo*.<sup>20,21</sup> Mechanism of caries is thought to be mediated by both bacterial and human proteases. In the caries process, following the dissolution of hydroxyapatite, the collagenous network becomes exposed to enzymatic degradation. However, bacterial collagenases did not resist the pH drop during demineralization, suggesting that host-derived proteolytic enzymes such as MMPs, both in dentine and saliva, have important role in dentine organic matrix degradation.<sup>21</sup> So, the use of tetracyclines and their derivatives could contribute to inactive MMPs and arrest caries progression.

A disadvantage of the antibiotic incorporation into GICs may be increasing the risk of side effects or the development of resistance to the drugs. Yesilyurt et al.<sup>14</sup> evaluated the release of antibiotics using HPLC (high performance liquid chromatography) in 24 h and 7 days and observed that in the tested concentrations (1.5, 3.0 and 4.5% of antibiotic mixture), there was an increase in the antibiotic releasing over time. However, the quantities of antibiotic released were so small that they could not implicate in bacterial resistance.

The incorporation of antimicrobials into GIC may affect their mechanical properties. Some of these studies have shown that the addition of chlorhexidine salts decreased the mechanical properties of GIC, such as compressive strength and bond strength to dentine.<sup>5,15,17</sup> 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%). These present results were different than those obtained by Yesilyurt et al.<sup>14</sup> These authors observed that the

associated antibiotics at 3.0 and 4.5% reduced compressive resistance and bond strength to dentine when compared to a control group without antibacterial agents. A low quantity of these antibiotics (1.5%) had a substantial antibacterial effect without causing significant alterations in the mechanical properties. However, those authors 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).<sup>14</sup> It has been considered that different kinds of materials mixed with different antibiotics can perform differently.

The findings in this current study revealed that the addition of doxycycline hyclate to the GIC is harmless to pulp cells and could be an alternative to other antimicrobials, such as chlorhexidine. High doses of chlorhexidine presented some undesirable biological responses, including inhibiting protein synthesis and mitochondrial activity,<sup>22</sup> which, if in contact with pulp cells is considered toxic, and presented with a dose-dependent effect.<sup>23</sup> Studies showing the effect of chlorhexidine-containing GIC on pulp cells are necessary to compare with our results. The liner cement commonly studied is the RMGIC Vitrebond. This cement provided the greatest inhibition zones against *S. mutans*, *Streptococcus sobrinus*, *L. acidophilus* and *A. viscosus*, with a greater effect than the conventional glass ionomers cements Ketac Molar (3M ESPE) and Fuji IX (GC America).<sup>24</sup> However, Vitrebond is extremely toxic to odontoblastic-like cells due to the presence of a high concentration of resin monomers, such as HEMA (more than 80%), in its chemical composition.<sup>10</sup> In addition, according to Hebling et al.<sup>23</sup> those cells that were not destroyed by Vitrebond presented intense morphological alterations, as confirmed in this present study. No morphological alterations of MDPC-23 were verified in this current study after adding doxycycline hyclate at 1.5, 3.0 and 4.5% to the Fuji Lining LC. In agreement to these current findings, cytotoxic effects of doxycycline hyclate were not observed in experiments on seeded fibroblasts, even over weeks of qualitative determination of cell viability at the highest doxycycline hyclate concentrations.<sup>25</sup>

In summary, the findings of the current study demonstrated that the incorporation of doxycycline hyclate of up to 4.5% concentrations into Fuji Lining LC maximizes the antibacterial activity against oral pathogens, without causing toxic effects to pulp cells or negatively influencing the mechanical

properties of the cement. Thus, doxycycline hyclate may be a promising candidate for the treatment of dentine after partial caries removal procedures. Although there is usually no additive incorporated into dental materials, the combination of antibacterial agents to restorative materials may be a better protection against cariogenic bacteria and caries progression. Based on this current study, additional *in vivo* studies are recommended to demonstrate the antibacterial effect on the growth and viability of remaining bacteria in deep cavities when incomplete caries excavation is used.

## Acknowledgements

This study was based on a thesis submitted to Piracicaba Dental School, University of Campinas (Brazil), in partial fulfillment of the requirements for the PhD degree in Dentistry. The authors thank GC Corporation for supplying Fuji Lining LC.

**Funding:** This investigation was supported by São Paulo State Research Foundation (FAPESP) in part as a PhD scholarship to the first author (Grant Nos. 2008/00359-0; 2008/02606-5).

**Competing interests:** None declared.

**Ethical approval:** Not required.

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