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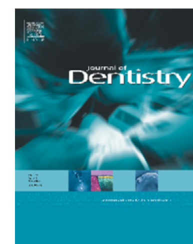

In vitro and in vivo investigation of the biological and mechanical behaviour of resin-modified glass-ionomer cement containing chlorhexidine

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ABSTRACT

Objectives: To evaluate: (1) the in vitro antibacterial, cytotoxic and mechanical properties of a resin-modified glass ionomer cement (RMGIC) containing different concentrations of chlorhexidine (CHX) and (2) the in vivo microbiologic action of the best concentration of CHX associated with the RMGIC applied on remaining dentine after indirect pulp treatment (IPT).

Methods: For the in vitro studies, RMGIC was associated with 0.2, 0.5, 1.25 and 2.5% CHX. Microbiologic evaluation consisted of an agar diffusion test on cariogenic bacteria for 24 h. Odontoblast-like cell metabolism and morphology analyses measured the cytotoxic effects of the RMGIC groups after 24 h. The same groups were submitted to compressive and diametral tensile strength. The in vivo treatment consisted of IPT using an RMGIC associated with the best CHX concentration. Clinical and microbiologic evaluations were performed before and after 3 months.

Results: The use of 1.25% CHX significantly improved the antibacterial effects of the evaluated RMGIC, without causing any detrimental effects to the odontoblast-like cells and on the mechanical properties. This RMGIC and CHX combination completely eliminated mutans streptococci after 3 months of IPT.

Conclusion: The RMGIC and 1.25% CHX mixture showed great biological and mechanical behaviour and could be a good treatment against caries progression.

Clinical significance: The association of CHX with a liner RMGIC opens a new perspective for arresting residual caries after IPT.

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1. Introduction

Partial caries removal approaches for dental caries management have gained great importance in the last decade, since scientific literature has suggested that only the softened (infected) dentine should be removed from carious tissue.[1] The remineralization capacity of affected dentine has also led to the acceptance of partial caries removal as an effective practice to avoid excessive excavation and the risk of pulp exposure in deep cavities.[2] This way, this procedure could induce dentine 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 periods of evaluation, irrespective of the material applied on the residual carious dentine.[3–7] A therapeutic benefit was gained when antimicrobial substances were used in association with a glass ionomer cement to contribute to residual infection elimination, and thus, minimizing the risk of recurrent caries and damage to the pulp.[8]

Among the different antimicrobial agents used to control dental microorganisms, chlorhexidine has been considered as one of the most effective and safe substances. It presents a wide spectrum of activity against Gram positive bacteria, especially mutans streptococci, Gram negative, aerobic and facultative anaerobic bacteria, yeasts and fungi.[9] Therefore, chlorhexidine might be a promising substance in the treatment of caries since its characteristics agree with of the establishment of health and function of teeth. Studies have suggested the incorporation of this agent into glass ionomer cements to improve their inhibitory action on residual microorganisms, contributing to the reduction of secondary caries.[10–14] Although the addition of chlorhexidine into a glass ionomer cement must increase the antimicrobial activity of the dental material, the presence of that substance might produce toxicity to pulp cells when applied in deep cavities, modify the physical characteristics of the cement or both. Studies have demonstrated that high concentrations of chlorhexidine cause damage to odontoblastic cells,[15] or jeopardize the basic properties of materials.[13,14] For a secure and adequate dental treatment, the concentration of the antimicrobial agent to be used in association with dental materials must be defined before its application. An in vitro study demonstrated a slight caries-inhibiting effect of chlorhexidine-containing glass ionomer cement without compromising its physical characteristics[8]; however, no studies have demonstrated if their combination can affect odontoblast cells, an essential property to preserve pulpal health. Furthermore, glass ionomer cements are used as liners on affected dentine during partial caries removal procedures, and the association of those liners materials and chlorhexidine digluconate was not yet been studied. Therefore, this present study determined the therapeutic concentration of chlorhexidine digluconate that is necessary to produce anticariogenic action without causing toxic effects on odontoblast-like cells while also causing no negative effects on the mechanical properties of the restorative material when incorporated into a resin-modified glass ionomer cement. The best chlorhexidine/glass ionomer cement combination was used on residual carious dentine after in vivo indirect pulp treatment for a subsequent microbiological and clinical evaluation. Four hypotheses were tested when adding chlorhexidine digluconate to a resin-modified glass ionomer cement (RMGIC): (1) the in vitro antibacterial effect of the RMGIC/CHX mix is improved; (2) there is no cytotoxic effect on the odontoblast-like cells; (3) the mechanical properties of the RMGIC are not modified; and (4) the RMGIC mix reduces or eliminates any residual bacteria from cavities after indirect pulpal treatment.

2. Materials and methods

2.1. *Dental materials*

The liner resin modified glass ionomer cement (RMGIC) chosen for the current study was GC Fuji Lining LC (FLLC – Lot 0710021, GC Corporation, Tokyo, Japan). The composition of this RGMIC is presented in Table 1. This material was modified by adding 0.2%, 0.5%, 1.25%, and 2.5% chlorhexidine digluconate (CHX – C9394 Sigma–Aldrich, Steinheim, Germany) (w/w) to the liquid of the RMGIC while keeping original powder/liquid ratio (1.4:1.0 g). Those concentrations and the material preparation were determined based on the results obtained from Türkün et al.[14]

2.2. *In vitro evaluations*

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) from the Microbiology and

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

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

[a] Material safety data sheet information.
[b] Sigma–Aldrich (www.sigma-aldrich.com).

Immunology Laboratory of Piracicaba Dental School – University of Campinas, Piracicaba, São Paulo, Brazil were used in this present study. For each experiment, cells were fresh cultured from frozen stock on brain–heart infusion broth (BHI; DIFCO Laboratories, Detroit, MI, USA) for 24 h at 37 °C in 10% CO_2 in an incubator. After confirming the viability and absence of contamination by plating in a specific medium and Gram techniques, cultures were again grown in BHI for 18–24 h at 37 °C and adjusted to a concentration of 1×10^8 cells/mL to obtain an inoculum for subsequent testing.

2.2.2. *Agar diffusion test*[15]

In each sterilized Petri dish (20 mm × 100 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 with one of the experimental control materials (RMGIC with chlorhexidine digluconate 0.2, 0.5, 1.25 and 2.5%) or the control material (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 s using a halogen curing unit (Curing Light XL3000, 3MESPE). The light intensity (410 mW/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 °C for 24 h. After this period, inhibition zones around the materials were measured using a digital calliper.

2.2.3. *Culture of MDPC-23 cells*[16]

Immortalized cells of an 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), with 100 IU/mL penicillin, 100 μg/mL streptomycin and 2 mmol/L glutamine (Gibco) in a humidified incubator with 5% CO_2 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 present 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 37 °C for 48 h.

2.2.4. *Analysis of cell metabolism by MTT assay*[16]

The RMGIC, without or with 0.2, 0.5, 1.25 and 2.5% of chlorhexidine digluconate, was hand-mixed and applied into stainless-steel moulds with cylindrical apertures. Vitrebond (3MESPE) was used as a positive control, because it has a high cytotoxic effect on odontoblast cells.[16] 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 – 100% humidity. The specimens were then inserted separately into sterile 24-well plates containing DMEM medium for 24 h. After this period, 800 μL of extract of 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 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). The culture media with the MTT solution were then 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 the 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% cell metabolism.

2.2.5. *Analysis of cell morphology by scanning electron microscopy*[16]

Cell morphology was examined by scanning electron microscopy (SEM) using two representative wells of each group. For this 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, as described before. Following this incubation, 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 then 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).

2.2.6. *Measurement of mechanical properties – compressive and diametral tensile strength*[17,18]

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, both compressive and diametral tensile strength ($n = 50$, 10 for each material group). Briefly, GC Fuji Lining LC was mixed by agglutination of powder to liquid with or without chlorhexidine and then the mixture was placed with Centrix syringe (Centrix Inc., Shelton, USA) into cylindrical moulds (4 mm high × 2 mm diameter). The specimens were then exposed to a light source (Curing Light XL3000, 3MESPE), with 410 mW/cm^2 light intensity for 30 s. Afterwards, the specimens were stored in distilled water for 24 h at 37 °C. The specimens were submitted to compressive strength testing 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 into 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. The strength values (kgf/cm^2) were calculated using the equation: DTS = 2*F*/3.14*DT*, where *F* was the failure load, *D* the diameter, and *T* the height of the specimen. DTS values were converted into MPa.

2.3. *In vivo study*

After approval by the Ethics Committee of the Piracicaba Dental School – State University of Campinas (FOP-UNICAMP, protocol 031/2008), sixteen primary molars were selected from 10 children of both genders, aged 4–9 years. A signed informed consent was obtained from the legal guardians of the children. Criteria for inclusion in the study were: (a) an active deep carious lesion at the internal half of the dentine thickness of a primary molar that had not been previously restored and that involved the occlusal or occluso-proximal surface; (b) an absence of signs of irreversible damage to the pulp, such as spontaneous pain, a fistula, or tooth mobility; (c) the absence of radiolucencies at the interradicular or periapical region or thickening of the periodontal spaces, absence of internal and external root resorption, absence of calcification of the pulp tissue, as determined by radiographic examination; (d) children presenting no systemic disease or those not using medications.

2.3.1. *Clinical procedures and dentine sampling*[7]

Indirect pulp treatment was performed in two sessions by the same investigator (ARCF), to standardize the clinical procedures and the dentine collection procedures. At the first session, after taking a bitewing radiograph using a standardized positioner, anaesthesia was delivered and a rubber dam applied to isolate the tooth. Pumice-slurry dental prophylaxis and anti-sepsis of the operative area using 0.2% chlorexidine digluconate was performed. Access to the infected dentine was achieved using a high speed sterile carbide bur (#245 – KG Sorensen, Barueri, São Paulo, Brazil) to remove the undermined enamel, when necessary. After removal of the superficial necrotic dentine using a spoon excavator, a sterile round steel bur, compatible with the cavity size, was used at low speed to clean all carious tissue from the enamel-dentine junction and lateral walls, leaving a layer of soft dentine on the cavity floor to avoid pulp exposure. After washing and air-drying the cavities to remove debris, an initial collection (baseline) of carious dentine was sampled from the mesial portion of the cavity floor and inserted into 5 mL of 0.9% NaCl. In order to obtain similar amounts of carious tissue from different collections, a standardized cavity was prepared in the active extremity of an amalgam plugger using a spherical bur at high speed. This cavity was completely filled with the dentine samples (approximately 0.6 mg) removed from each tooth using a sterile spoon excavator. Subsequently, the pulpal wall was entirely covered with one of the randomly selected liner materials: (1) liner RMGIC ($n = 8$) (FL; Lot 0710021, GC Corporation, Tokyo, Japan) containing 1.25% chlorhexidine digluconate (CHX; C9394 Sigma–Aldrich, Steinheim, Germany) or (2) Fuji Lining LC as a control group ($n = 8$). The experimental liner RMGIC was modified by adding CHX to the liquid of the GC Fuji Lining LC while keeping the original powder/liquid ratio (1.4:1.0 g), as described in Section 2.1. The CHX concentration was chosen based in the previous results obtained in this study. The materials were handled according to the manufactures' instructions. The liner RMGIC was light activated for 30 s using a halogen curing unit (Curing Light XL3000, 3MESPE, St. Paul, MN, USA) and the light intensity (410 mW/cm2) was monitored using a radiometer (Optilux 500, Demetron Kerr, Danbury, CT, USA). The cavities were then temporarily restored using a conventional GIC (Ketac Molar, 3 M ESPE, Seefeld, Bavaria, GE). Within 3 months after the initial treatment, the teeth were submitted to clinical and radiographic examination to determine signs and symptoms of pulp vitality. Next, under the same initial conditions of anaesthesia and rubber dam placement, the teeth were reopened. The restorative and liner materials were carefully and completely removed and a new dentine sample was collected, as previously described.

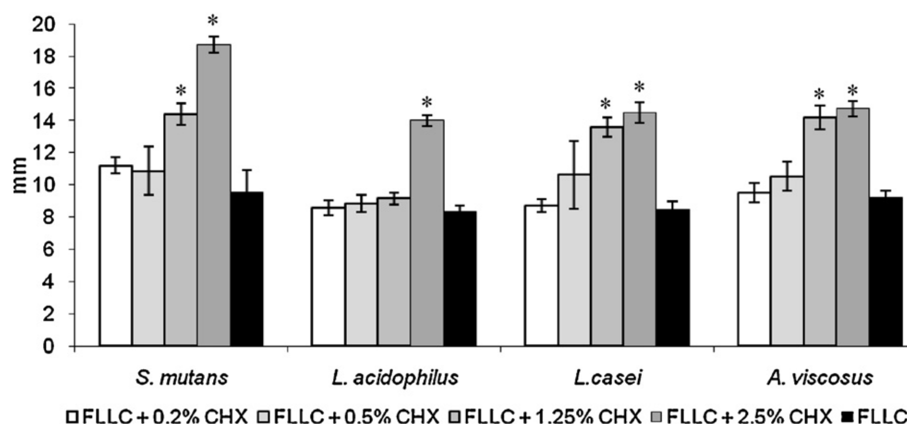

Fig. 1 – Means and standard deviations of inhibition zones for Fuji Lining LC (FLLC) associated or not to different concentrations of chlorhexidine digluconate (CHX) against *S. mutans*, *L. acidophilus*, *L. casei*, and *A. viscosus*. *Values obtained for these test groups differ statistically from the control group (FLLC), according to Kruskal Wallis and Mann–Whitney tests ($p \leq 0.05$).

Finally, when necessary, softened remaining carious dentine was removed, and the teeth were then restored with a light-cured composite resin (Opallis, FGM, Joinvile, SC, Brazil) using a bonding system, Scotchbond Multi-Purpose (3M ESPE, St. Paul, MN, USA), after a new placement of the initial liner material.

2.3.2. *Clinical evaluation criteria*

Before all dentine collections, the dental cavities were copiously washed and carefully air dried, and the colour, consistency and humidity of the carious dentine were blindly evaluated by a second investigator, based on the following criteria[7]: dentine consistency: 0 = hard (similar to normal dentine); 1 = leathery (dentine spoon removes carious tissue when forced); 2 = soft (tissue easily removed by a dentine spoon); dentine colour: 0 = yellow; 1 = light brown; 2 = dark brown; dentine humidity: 0 = dry; 1 = humid

2.3.3. *Microbiological procedures*[7]

Dentine samples immersed in 5 mL of 0.9% NaCl were homogenized in a tube agitator (Vortex, Phoenix AT 56, Munising, Mich., USA) for 1 min. Six decimal dilutions (10^{-1}–10^{-6}) were performed after homogenization. Subsequently, 25 μl aliquots of the dilutions were cultivated in triplicate on a surface containing Bacitracin 0.2 UI/mL Mitis Salivarius – MSB agar for isolation of mutans streptococci (MS). All plates were incubated in a microaerophilic environment at 37 °C for 48 h. After incubation, the total number of colony-forming units per millilitre (CFU/mL) was counted from a representative area of each agar plate, yielding 50–300 colonies using a stereoscopic microscope.

2.4. *Statistical analysis*

Data from the antibacterial and cytotoxic effects were submitted to Kruskal Wallis and Mann–Whitney tests, and the data from the evaluation of the mechanical properties were submitted to one-way ANOVA and Tukey tests ($p < 0.05$). The Wilcoxon's non-parametric test was used to compare the differences in consistency, colour, and humidity of the dentine, before and after the indirect pulp treatment. The counts of MS were compared between the samples collected at baseline and after 3 months within each material group using the same test. Medians and ranges of bacterial counts were expressed as (log(CFU + 1)). The constant 1 was added to the CFU count, since many samples showed zero CFUs after the experimental period. Complementary Mann–Whitney tests were applied to identify differences among the materials. All statistical tests were considered at a 5% level of significance.

3. Results

3.1. *In vitro evaluations*

3.1.1. *Antibacterial activity*

The mean values of the inhibition zones for each material are shown in Fig. 1. The concentrations of 0.2 and 0.5% chlorhexidine digluconate did not have an effect on the antibacterial activity of RMGIC. The incorporation of 1.25 and

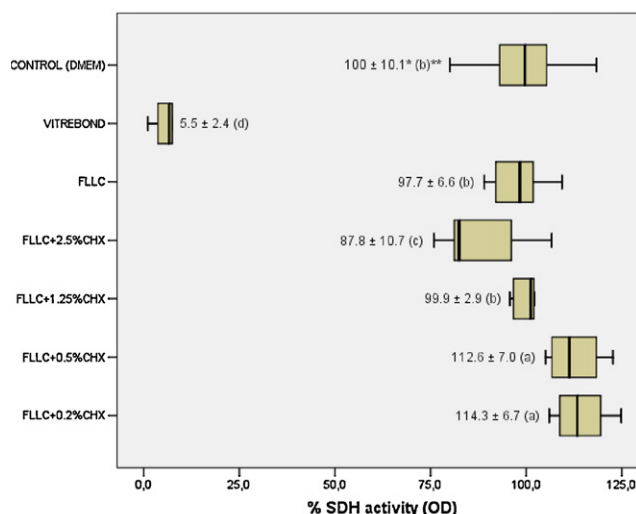

Fig. 2 – Box–Whisker plot (minimum [lower quartile–median–upper quartile] maximum) of the cell metabolism (MTT assay) results for each group. *Mean ± standard deviation. The vertical line in the box is the median. **Groups identified with the same letter do not differ statistically (Mann–Whitney; $p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

2.5% chlorhexidine digluconate significantly improved the inhibitory activity of the cement on all of the bacteria tested, except for 1.25% chlorhexidine digluconate against *L. acidophilus*. When comparing these two groups, there was a statistically significant difference between them for *S. mutans* and *L. acidophilus*.

3.1.2. *Toxicity on odontoblast-like cells*

Fig. 2 shows the cell metabolism (SDH activity) following application of the culture medium with or without 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 the controls (RMGIC and DMEM). Vitrebond showed the highest cytotoxic effects, decreasing the metabolic activity by 93%. RMGIC associated with 0.2 and 0.5 CHX increased significantly the SDH activity (14.03% and 12.6%, respectively) which statistically differed from the control group (DMEM), showing that low concentrations of chlorhexidine digluconate could stimulate cell metabolism. There was no difference between 1.25% chlorhexidine digluconate and the RMGIC and DMEM groups. SEM images indicated that chlorhexidine digluconate concentrations of up to 1.25% when incorporated into RMGIC did not affect cell morphology: 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. However, RMGIC containing chlorhexidine digluconate at a 2.5% concentration slightly altered the morphology of MDPC-23 cells. A small number of cells were observed for Vitrebond (Fig. 3).

3.1.3. *Measurements of mechanical tests*

The means and standard deviations of the values obtained for mechanical testing are shown in Figs. 4 and 5. The 2.5%

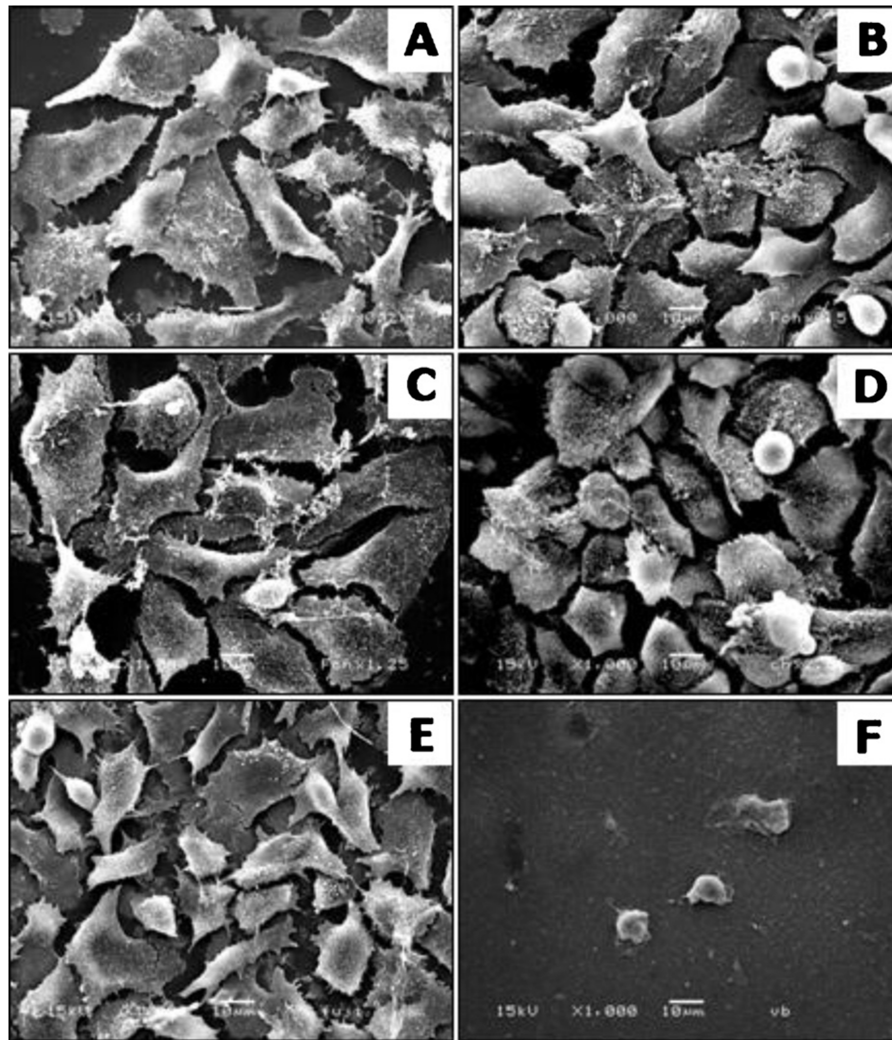

Fig. 3 – MDPC-23 cells adhered to the glass substrate after exposure to extracts of (A) Fuji Lining LC (FLLC) containing 0.2% chlorhexidine digluconate; (B) FLLC containing 0.5% chlorhexidine digluconate; (C) FLLC containing 1.25% chlorhexidine digluconate; (D) FLLC containing 2.5% chlorhexidine digluconate; (E) control group – FLLC without chlorhexidine digluconate; and (F) Vitrebond (SEM original magnification 1000×).

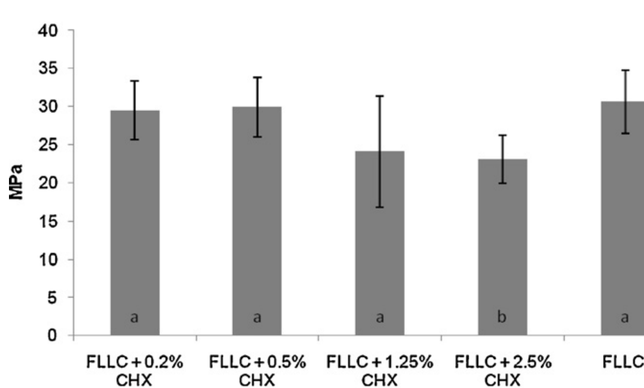

Fig. 4 – Mean (bars) and standard deviation (lines) of compressive strength values obtained for the different groups. Different letters indicate statistically different groups (ANOVA; $p < 0.05$).

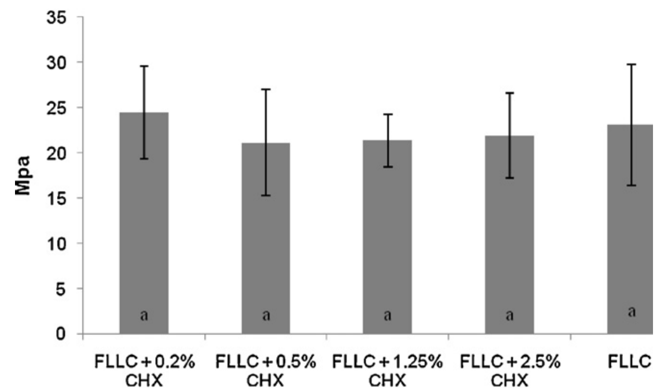

Fig. 5 – Mean (bars) and standard deviation (lines) of diametral tensile strength values obtained for the different groups. Groups identified with the same letter do not differ statistically (ANOVA; $p > 0.05$).

Table 2 – Median and range (minimum–maximum) of clinical scores and SM counts (log(UFC + 1)) according to the collect period and experimental materials.

Groups	Clinical scores	Clinical scores Median (range)		SM Counts Median (range)	
		Baseline	Reentry	Baseline	Reentry
FLLC	Dentine consistency	2 (2–2)[Aa*]	1 (0–2)[Ab]	4.77 (4.53–4.8)[Aa*]	3.75 (3.65–5.86)[Aa]
FLLC + 1.25%CHX		2 (2–2)[Aa]	0 (0–2)[Bb]	4.29 (4.24–4.67)[Aa]	0 (0–0)[Bb]
FLLC	Dentine colour	0 (0–0)[Aa]	1 (0–1)[Ab]		
FLLC + 1.25% CHX		0 (0–0)[Aa]	1 (0–1)[Ab]		
FLLC	Dentine humidity	1 (1–1)[Aa*]	1 (1–1)[Aa]		
FLLC + 1.25% CHX		1 (1–1)[Aa]	1 (1–1)[Aa]		

[*] For each material and collect, median (range) followed by: Same lowercase letters in the rows are not statistically different, according to Wilcoxon and Mann–Whitney test ($p > 0.05$). Same uppercase letters in columns are not statistically different, according to Wilcoxon and Mann–Whitney test ($p > 0.05$).

chlorhexidine digluconate showed significantly lower compressive strength when compared to the control. No significant differences were observed among the groups for diametral tensile test ($p < 0.05$), demonstrating that the incorporation of chlorhexidine digluconate into RMGIC at up to 2.5% concentrations did not modify this mechanical property of the liner material.

3.2. *In vivo investigation*

Three teeth were excluded from this evaluation due to the loss of the restoration (1 from the control group and 2 from the CHX group). The final sample consisted of 13 teeth (control = 7 and CHX = 6) from 10 patients. Table 2 shows the in vivo clinical and microbiological results. None of the patients experienced any sensitivity or discomfort and no clinical or radiographic signs of pulp or periapical alterations were noted during the trial period. None of the teeth presented pulp exposures during the reopening of the cavity. The comparison between the bacterial counts before and after sealing the cavity showed no significant reduction for the control group ($p > 0.05$) and complete elimination of SM (UFC = 0) from the cavities of the experimental group.

4. Discussion

Glass ionomer cements (GIC) are widely used in dentistry for its advantages of non-shrinking setting reaction, combined with adhesion to tooth tissue and fluoride release. However, conventional GIC (CGIC) is quite a brittle material and its mechanical properties are limited. Because of this, resin-modified glass ionomer cements (RMGICs) were introduced to provide a material with improved mechanical properties and the light cure facility.[19] Besides the components of the CGIC, the RMGICs contain usually hydroxyl-ethylmethacrylate (HEMA) and polymerization initiators. The good biocompatibility of the CGIC attributed to minimal setting exothermic, rapid acid neutralization and slow release of beneficial ions was impaired by the incorporation of the monomers, such as HEMA. The liner RMGIC more commonly used by dentists is Vitrebond, but its high percentage of HEMA release has showed to be highly cytotoxic.[20] In this study, the RMGIC Fuji Lining LC was chosen for its good sealing capacity along the cavity wall as well as the reduced cytotoxicity. Aranha et al.[21] evaluated SDH activity after exposition to some RMGICs and verified that Fuji Lining LC provided minimal reduction in the cellular metabolism (9.3%) when compared to Vitrebond (80.7%). Palmer et al.[22] compared the percentage HEMA release from restorative and liner RMGIC. After 24 h of maturation, light-activated Vitrebond specimens released 4.95% of HEMA compared to 0.59% from Fuji Lining LC. When these cements were not light-cured, the percentage of HEMA release was 4.01 for Fuji Lining LC and 59.78 for Vitrebond.

The efficacy of chlorhexidine has been proven against oral pathogens.[9] Therefore, different salts of chlorhexidine, mainly digluconate and diacetate, have been added to GICs for improving their antimicrobial efficacy. The incorporation of this agent in dental materials could reduce or eliminate residual bacteria after caries removal procedures or until prevent secondary caries around the restoration. In this current study, the addition of 1.25% and 2.5% chlorhexidine digluconate to a liner RMGIC substantially increased its inhibitory activity against the tested oral bacteria when compared with RMGIC alone. Similar results were found in other studies, using digluconate[11,12,14] or other chlorhexidine salts.[8,11,13,14,23] Among the microorganisms tested, *L. acidophilus* was the most resistant microorganism to chlorhexidine digluconate-containing RMGI. According to Botelho[23] 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, a chlorhexidine added to a glass ionomer cement has a significant residual release effect for some weeks,[13] which could inhibit remaining microorganisms, including *L. acidophilus*. Türkün et al.[14] evaluated the in vitro long-term antimicrobial effect of the incorporation of chlorhexidine in a CGIC and observed inhibitory halos against both *S. mutans* and *L. acidophilus* after 30–40 days of the initial cement application on agar plates.

Some studies have evaluated the release of chlorhexidine from glass ionomer cements using high performance liquid chromatography (HPLC) analysis and compared the relationship between the percentage of CHX released and its antibacterial effects. Some of the studies related that CHX released from GICs and consequent inhibitory activity against pathogens was dependent upon the concentration of that antimicrobial[11,23] while others showed no dose-response

effects,[8,24] showing conflicting results. Although in this current study was not possible to measure the percentage of CHX released by HPLC analysis, the antibacterial effect was concentration-dependent since 1.25% and 2.5% concentrations produced better results. These present findings are in agreement with Ribeiro and Ericson[11] and Botelho.[23] In both studies, the antibacterial-GIC combination specimens showed significant inhibition which increased with the CHX concentrations. Characteristics such as viscosity and hardness of glass ionomer cements could determine the amounts of antimicrobials released.[8]

When maintaining the original ratio of power/liquid, the addition of any substance could affect important characteristics of glass ionomer cement. Antimicrobials could enhance the cytotoxic effect of a dental material while interfering in its mechanical properties. Although chlorhexidine digluconate is a potential antimicrobial with many desirable biological characteristics, such as inhibition of dentine metalloproteinases,[25] it may cause an immediate hypersensitivity and other unwanted responses, including inhibiting protein synthesis and mitochondrial activity.[25,26] For those reasons, both quantity and oral administration of the chlorhexidine digluconate must be controlled. In relation to the toxicity of the chlorhexidine substance on culture cells, studies in the literature evaluated only the cytotoxicity of that antimicrobial agent applied directly on cells, not in association with dental materials. Lessa et al.[27] evaluated the cytotoxicity of 0.06, 0.12, 0.2, 1 and 2% chlorhexidine digluconate on odontoblast-like cells for 60 s to 24 h and observed that the antimicrobial had a dose-time dependent toxic effect on MDPC-cells. The higher the concentration of chlorhexidine digluconate and the 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 0.2–1.25% of chlorhexidine digluconate for 24 h did not increase the toxicity on odontoblastic lineage cells. However, the 2.5% chlorhexidine digluconate concentration significantly reduced cell metabolism and changed the cell 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 a glass ionomer cement can affect the mechanical properties of that cement.[8,13,28] 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 current study, the inhibitory action of RMGIC against all tested strains was improved by the presence of chlorhexidine digluconate with no negative effects to the mechanical properties of the cement, except the 2.5% chlorhexidine digluconate concentration for compressive strength test. These current results are in agreement with Takahashi et al.[8] who observed that 2% or greater chlorhexidine diacetate significantly decreased the compressive strength and the bond strength to dentine of conventional glass ionomer cement. Those authors suggested that the decrease in mechanical properties could be attributed to slight modifications in the powder/liquid ratios by adding the antimicrobial agent. In the present study, 2.5% chlorhexidine digluconate affected two important properties of glass ionomer cement: the cytotoxicity on odontoblast-like cells and compressive strength of the cement. Alternatively, chlorhexidine digluconate in a concentration of 1.25% could be the ideal and safe concentration in a RMGIC used as a liner in deep cavities. Based on the current in vitro results, an in vivo study was conducted with FLLC and 1.25% CHX. This combination completely eliminated mutans streptococci from dentine samples after 3 months of clinical treatment.

Glass ionomer cements have been successfully used in procedures involving partial caries removal, including indirect pulp treatment, stepwise excavation and atraumatic restorative treatment (ART).[2–7] This is the first study evaluating both in vivo and in vitro properties of a liner RMGIC containing chlorhexidine digluconate as an alternative material for eliminating residual bacteria after indirect pulp treatment. A pilot study conducted by Frencken et al.[10] showed lower microorganism counts in chlorhexidine-containing glass ionomers cements than in conventional GIC for both affected and infected dentine over a 7-day period after ART procedures. In a in situ study, the authors investigated the antibiofilm effects of conventional GIC (CGIC) and RMGIC containing 2% chlorhexidine diacetate. GIC and RMGIC specimens were bonded to buccal surface of the molars of volunteers and left untouched for 4 and 24 h. The bacterial viability was analyzed by confocal laser scanning microscopy that revealed significantly lower microorganism counting to CHX-containing specimens compared to CGIC/RMGIC.[29]

5. Conclusion

The findings of this present study demonstrated that the use of chlorhexidine digluconate in combination with RMGIC maximizes the antimicrobial activity of this cement. There is usually no antimicrobial added to dental materials; however, the combination of an antimicrobial with a glass ionomer cement can provide better protection against cariogenic bacteria and should avoid caries progression. Therefore, the current authors propose that chlorhexidine digluconate is 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.

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